The Genetic Determinants of Resistance to Antifungal Peptides

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List of Abbreviations

-ve	Negative
+ve	Positive
μM	Micromolar
Δ	Gene Knockout
μg	Microgram
μL	Microlitre
%	Percentage
˚C	Degrees Celsius
ABC	ATP-binding Cassette
AFPs	Antifungal Peptides
AMPs	Antimicrobial Peptides
ATP	Adenosine Triphosphate
Ca^{2+}	Calcium ion
CFW	Calcofluor-white
CP29	Chlorophyll a-b binding protein
CWI	Cell Wall Integrity
Bac2a	Bactenecin 2A
BMAP-27	Bovine Myeloid Antimicrobial Peptide-27
BMAP-28	Bovine Myeloid Antimicrobial peptide-28

bp	Base pair
BPTI	Bovine Pancreatic Trypsin Inhibitor (Aprotinin)
DmAMP1	*Dahlia merckii* Defensin-like protein 1
DNA	Deoxyribonucleic Acid
EMS	Ethylmethanesulphonate
ERG	Ergosterol
FIC	Fractional Inhibitory Concentration
GlcCer	Glucosylceramides
GIPC	Glycosyl Inositol Phosphoryl Ceramides
GPI	Glycerophosphatidylinositol
GSLs	Glycosphingolipids
h	Hour
H_2O_2	Hydrogen peroxide
HBD2	Human beta-defensin-2
HCC	Hexamine (III) Cobalt Chloride
hGAPDH	Human glyceraldehyde-3-phosphate dehydrogenase
HNP1	Human neutrophil peptide 1
HOG	High-osmolarity Glycerol
HsAFP1	*Heuchera sanguinea* antifungal peptide 1
INDEL	Insertion or Deletion

kDa	Kilodalton
LL-37	Human cathelicidin 37 amino acids in length
LPS	Lipopolysaccharide
L-PG	Lysyl-phosphatidyl glycerol
$M(IP)_2C$	Mannosyldiinositolphosphorylceramide
MAPK	Mitogen Activated Protein Kinase
MF	Major Facilitator
Mg^{2+}	Magnesium ion
MIC	Minimum Inhibitory Concentration
Min	Minute
mM	Millimolar
mL	Millilitre
MtDef4	*Medicago truncatula* defensin 4
MUC7	Mucin 7
NaCl	Sodium chloride
NaD1	*Nicotiana alata* Defensin 1
NaD2	*Nicotiana alata* Defensin 2
NBDs	Nucleotide-binding Domains
OD	Optical Density
pH	Potential of hydrogen

PIA	Polysaccharide Intercellular Adhesin
PLs	Phospholipids
($PI(4,5)P_2$)	Phosphatidylinositol 4,5-bisphosphate (PIP2)
PA	Phosphatidic acid
PCR	Polymerase Chain Reaction
PDB	Potato Dextrose Broth
PMAP-23	Porcine myeloid antimicrobial peptide 23
PvD1	*Phaseolus vulgaris* Defensin 1
ROS	Reactive Oxygen Species
Rpm	Revolutions Per Minute
Rsafp1	*Raphanus sativus*-antifungal protein 1
SDS	Sodium Dodecyl Sulfate
SMAP-27	Sheep myeloid antimicrobial peptide-27
SMAP-29	Sheep myeloid antimicrobial peptide-29
SNP	Single Nucleotide Polymorphism
SNVs	Single Nucleotide Variant
TPP3	Tomato pistil predominant 3 defensin
UV	Ultraviolet
wt	Wildtype
YPD	Yeast Extract–Peptone–Dextrose

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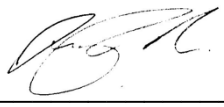
Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis submitted for the award of any other degree or diploma.

No other person's work has been used without due acknowledgement in the main text of the thesis.

The thesis has not been submitted for the award of any degree of diploma in any other tertiary institution.

Amanda McColl

Signed  _____ Date: 30 October 2020

Abstract

Fungal diseases are estimated to cause over 1.6 million deaths annually and over one billion people suffer from fungal diseases. Currently, there are limited antifungal drugs approved for use in humans and the emergence of antifungal resistance is endangering the already limited treatments options. The development of novel antifungal compounds may help overcome the problem of growing antifungal resistance. Recently, there has been increased interest in antifungal peptides (AFPs) as a promising approach for discovery and development of novel antifungal agents. However, prior to clinical use there needs to be an understanding of how resistance may develop towards these antifungal peptides. This thesis describes the development and mechanisms of fungal resistance towards a selection of AFPs and compares them to resistance to clinical antifungal drugs. The prototypic plant defensin NaD1, aprotinin, also known as BPTI, and the clinical antifungal caspofungin were serial passaged with the model fungus *Saccharomyces cerevisiae* at sub-lethal concentrations of each of the molecules. The yeast strain did develop tolerance to NaD1, but it occurred more slowly than resistance to BPTI and caspofungin. Resistance to NaD1 developed via an accumulation of single nucleotide mutations and had a fitness penalty associated with it. Full genome sequencing of resistant lines revealed that resistance to BPTI resulted from deleterious mutations in either the *ptk2* or *sky1* genes. Resistance to AFPs was investigated further using the human pathogen *Candida albicans. C. albicans* was serially passaged in the presence of NaD1 and the human cathelicidin LL-37, as well as the clinical small molecule antifungal, itraconazole. Resistance to NaD1 and LL-37 developed more slowly and to a lower fold-MIC compared to itraconazole. Unlike resistance to itraconazole, no “hot-spot” mutations were identified in any of the NaD1 or LL-37-resistant strains. Overall, these data indicate that it is more difficult to generate resistance to antimicrobial peptides compared to small molecule antifungals and therefore their clinical application should be investigated further.

Chapter One

1. Review of literature

The findings of this chapter have been drafted as a manuscript for publication. The manuscript can be found in the Appendix section of this thesis.

McColl AI, Bleackley MR, Parisi K, Anderson MA, (2020) The Mechanisms of Resistance to Antifungal Peptides.

Kathy Parisi conceptualised and drafted part of section 1.4.3. Screening of deletion libraries for resistance.

1.1 The development of resistance to clinical antifungals

Fungal diseases are estimated to cause over 1.6 million deaths annually and over one billion people globally suffer from fungal diseases (Fisher *et al.*, 2020). However, most estimates of the incidence of fungal disease are conservative because public health surveillance of these diseases is generally not compulsory (Casadevall., 2017). Fungal diseases are also a major threat to food security because damage to crop plants causes major losses in yield and food quality (Avery *et al.*, 2019; Fisher *et al.*, 2012; Fones *et al.*, 2020). Today, crop-destroying fungi account for perennial yield losses of ~20% worldwide, with a further 10% loss postharvest (Fisher *et al.*, 2020).

Currently, only a limited number of antifungal drugs have been approved for use in humans (Pianalto & Alspaugh, 2016). These antifungals are generally grouped into five classes based

on their site of action (Fuentefria *et al.*, 2018). They are the polyenes, which interact with fungal membrane sterols; echinochandins, which affect the cell wall by inhibiting (1,3)-β-D-glucan synthases (Chen & Sorrell, 2007); pyrimidine analogues such as flucytosine; and the azoles and allylamines which inhibit ergosterol synthesis (the main fungal sterol). Unfortunately, new species of multidrug-resistant pathogenic fungi are emerging and are of great concern in the clinical setting (Fisher *et al.*, 2018). Resistance evolves via natural selection through random mutation, or by application of an evolutionary stress on a population (Revie *et al.*, 2018). Pathogens that acquire a beneficial mutation that allows them to survive the stress will live on to reproduce, whereas the bulk of the population will have their growth retarded or will be killed. The organisms with the beneficial mutation then pass this trait to their offspring generating a fully resistant population. A pathogen with a range of resistance genes conferring resistance to more than one drug, is called multi-drug resistant or, informally, a superbug. Examples of this are clinical isolates of *Candida auris* which have acquired resistance to all the major classes of antifungals and isolates of *Aspergillus fumigatus* which are resistant to all primary azole treatments (Perlin *et al.*, 2017; Pinto *et al.*, 2018). Factors contributing towards the development of resistance include incorrect diagnosis, unnecessary prescriptions, and improper use by patients (e.g., when dosages are too low and do not lead to rapid killing, or when treatment courses are not long enough or used for long periods prophylactically) (Pea & Lewis., 2018). The use of fungicides in agriculture for prevention and treatment of fungal diseases in crops can also contribute to resistance in people exposed to those fungicides (Berger *et al.*, 2017). Most human pathogens also have environmental niches, implying that the agricultural use of fungicides with similarities to medically approved drugs impose the concrete risk of fostering drug resistance via environmental exposure imparting selective pressures that benefit resistant strains (Berger *et al.*, 2017; Wiederhold, 2017). Emergence of antifungal resistance can endanger the already limited treatments options, with

calamitous effects for treatment outcomes (Perlin *et al.*, 2017). New antifungal compounds with different mechanisms of action are needed to overcome the problem of growing antifungal resistance. Novel antifungal agents should have broad-spectrum activity, target specificity, low toxicity, a diverse mode of action and no antagonistic effects with other medications (Aoki & Ueda, 2013; Ciociola *et al.*, 2016). Although new drugs may not fulfil all these criteria, these properties should be used as guidelines in drug discovery (Wang, 2014). Recently, there has been an increased interest in antimicrobial peptides as a promising approach for discovery and development of novel antifungal agents (Mookherjee *et al.*, 2020). In this context, peptides have favourable properties, such as moderate immunogenicity as described below, strong antimicrobial activity, high specificity and affinity for targets, distinct mechanisms of action, good organ and tissue penetration and broad-spectrum activity (Aoki & Ueda., 2013; Ciociola *et al.*, 2016). However, before a new antifungal agent is adopted, we need to understand how resistance may develop prior to clinical use. This will help in determining which peptides should be used in combination therapy with existing antifungal drugs and in prioritising development of antifungal peptides with a lower propensity for resistance.

1.2 Mechanisms of Resistance to clinical fungicides

Resistance to current antifungals can develop in multiple ways and is broadly characterised by the mechanism by which resistance occurs. These mechanisms include drug target alteration or overexpression, upregulation of multidrug transporters, and activation of stress responses (Table 1) (Cowen *et al.*, 2014; Ghannoum & Rice, 1999; Mookherjee *et al.*, 2020).

Mode of resistance	Antifungal class	Detailed mechanism	Isolates	Reference
Drug target alteration /overexpression	Azoles (e.g. Itraconazole)	Mutation in *ERG11* Gain of function mutation in *UPC2* Mutations in *CYP51A*	*Candida parapsilosis* and *C. tropicalis C. albicans, C. glabrata* and more recently *C. auris Cryptococcus species*, and *Aspergillus species*	(Arendrup & Patterson, 2017; Castanheira *et al.*, 2020; Halliday *et al.*, 2017; Heilmann *et al.*, 2010; Marichal *et al.*, 1999; Revie *et al.*, 2018; Zoran *et al.*, 2018)
	Echinochandins (eg. Caspofungin)	Mutation in *FKS1*	*C. albicans, C. auris*, and *C. glabrata*	(Balashov *et al.*, 2006; Chaabane *et al.*, 2019)
	Polyenes	Loss of function mutation in *ERG* genes	*Candida species*, *Fusarium species*, *Scedosporium apiospermum*, and *Sporothrix schenckii*	(Ellis, 2002; Hull *et al.*, 2012; O'Shaughnessy *et al.*, 2009)
Efflux pump overexpression	Azoles	Overexpression of *CDR1*, *CDR2*, Mdr-like pumps, *MDR1–4*, and ABC transporters *AFR1* and *ATRF*	*C. albicans, C. neoformans*, and *A. fumigatus*	(Chen *et al.*, 2010; Posteraro *et al.*, 2003; Rosana *et al.*, 2015; Slaven *et al.*, 2002; Tsao *et al.*, 2009)
Cellular stress response pathways	Azoles Echinocandins Polyenes	Inhibition of Hsp90 and downstream signalling cascades	*Candida* and *Aspergillus species*	(Cowen *et al.*, 2014; Cowen *et al.*, 2009; Lamoth *et al.*, 2015; Lamoth *et al.*, 2013; Singh *et al.*, 2009; Vincent *et al.*, 2013)

Table 1.1. Summary of mechanisms of resistance to small molecule drugs and species in which resistance has been identified.

1.2.1 Drug target alteration/overexpression

The most common way for resistance to occur is through the alteration of a protein target site that the antifungals bind to (Hokken *et al.*, 2019). When fungi grow, mutations in their genome can occur due to replication error. These mutations can alter the amino acid sequence of the target site, therefore changing its structure and reducing the likelihood of binding by a fungicide (Sierotzki *et al.*, 2000). A common mechanism of azole resistance in *C. albicans* involves amino acid substitutions in the drug target, Erg11p, which leads to lower drug-binding affinity (Revie *et al.*, 2018). Over 140 amino acid substitutions in Erg11 have been associated with azole resistance, with the majority of these substitutions clustered into hot-spot regions (Arendrup & Patterson, 2017; Castanheira *et al.*, 2020; Halliday *et al.*, 2017; Marichal *et al.*, 1999). Azole resistance has also been identified in *Cryptococcus* and *Aspergillus* species with mutations identified in the Lanosterol 14-α Sterol Demethylase Gene *CYP51A* (Zoran *et al.*, 2018).

Overexpression of *ERG11* is also common in azole-resistant clinical isolates of *C. albicans* and *A. fumigatus* and contributes directly to increased target abundance, ultimately lowering drug susceptibility (Pinto *et al.*, 2018; Robbins *et al.*, 2017b). In *C. albicans*, the transcriptional activator, Upc2p, is a crucial regulator of many ergosterol biosynthesis genes, including *ERG11*. Gain-of-function mutations in *UPC2* cause the constitutive overexpression of ergosterol biosynthesis genes, a higher ergosterol content and a reduction in fluconazole susceptibility (Heilmann *et al.*, 2010).

Resistance to polyenes such as amphotericin B is uncommon. However, in the rare incidence that it does occur, it is mediated by alterations in enzymes that reduce drug-binding affinity or deplete ergosterol from the membrane (Ellis, 2002; O'Shaughnessy *et al.*, 2009). In *C. albicans*, reduced amphotericin B susceptibility can occur through mutations in several ergosterol biosynthesis enzymes, including *ERG2*, *ERG3*, *ERG5*, and *ERG11* (Ellis, 2002; O'Shaughnessy *et al.*, 2009). Likewise, for *C. glabrata*,

mutations in *ERG2*, *ERG6*, and *ERG11* have been documented in polyene-resistant clinical isolates (Hull *et al.*, 2012).

Another less commonly used class of antifungal is flucytosine, which is a nucleoside analogue that inhibits nucleic acid synthesis. In all *Candida* species, flucytosine resistance has been linked to mutations in *FUR1*, a gene involved in 5-FC metabolism (Chaabane *et al.*, 2019).

Echinocandin resistance is primarily mediated by mutations in the *FKS* genes which encode the catalytic subunit of 1,3-β-glucan synthase, which is necessary for production of 1,3-β-D-glucan, an essential component of all fungal cell walls. In *C. albicans*, mutations that confer echinocandin resistance occur at hot-spot regions in the essential gene, *FKS1* (Balashov *et al.*, 2006). More recently, sequencing of 38 *C. auris* strains led to the discovery of an additional hot-spot mutation in *FKS1* that leads to a S639F amino acid substitution that is correlated with pan-echinocandin resistance (Chaabane *et al.*, 2019).

1.2.2 Upregulation of multidrug transporters

Another prominent mechanism of resistance is drug efflux. The main class of efflux pumps implicated in azole drug resistance is the ATP-binding cassette (ABC) superfamily. ABC transporters possess two transmembrane-spanning domains and two cytoplasmic nucleotide-binding domains (NBDs) (Coleman & Mylonakis, 2009). The NBD drives the movement of substrates across the fungal membrane via ATP hydrolysis (Coleman & Mylonakis, 2009). In *C. albicans*, overexpression of two homologous ABC transporters, Cdr1p and Cdr2p, has been frequently implicated in azole resistance (Tsao *et al.*, 2009). The second class of efflux pumps implicated in azole resistance is the major facilitator (MF) superfamily. Like the ABC superfamily, MF transporters also possess transmembrane-spanning helices but use the proton gradient generated across the plasma membrane to drive

MF-mediated translocation (Coleman & Mylonakis, 2009). Fluconazole resistance has been linked to Mdr1 (multidrug resistance 1) overexpression (Rosana *et al.*, 2015). In *C. neoformans* and *A. fumigatus*, the ABC transporters responsible for azole efflux are Afr1 and AtrF, respectively (Posteraro *et al.*, 2003; Slaven *et al.*, 2002)

1.2.3 Activation of stress responses

The diverse and dynamic niches that fungal pathogens inhabit are subject to a variety of environmental fluctuations, including temperature, pH, and nutrient levels which are capable of perturbing cellular homeostasis and imposing significant stress on the fungal cell (Brown *et al.*, 2017). Antifungal agents represent a chemical stressor these pathogens must recognize, respond to, and adapt to in order to survive (Cowen & Steinbach, 2008; Robbins *et al.*, 2017a). Consequently, fungal pathogens have evolved broad stress-response circuitry that enables them to thrive in the presence of diverse cellular insults (Hayes *et al.*, 2014). A global cellular regulator that governs stress responses in diverse fungal pathogens is the essential molecular chaperone, heat shock protein 90 (Hsp90) (O'Meara *et al.*, 2017). Hsp90 is highly abundant, and its function is tightly coupled to environmental perturbations. It interacts with over 20 co-chaperones that facilitate the recognition of specific client proteins, which are enriched in kinases, signal transducers, and transcription factors, many of which serve as hubs in regulatory networks (Taipale *et al.*, 2010). Thus, Hsp90 regulates basal tolerance and resistance to azole, echinocandin and polyene antifungals through multiple signalling cascades (Cowen *et al.*, 2014; Cowen *et al.*, 2009; Singh *et al.*, 2009; Vincent *et al.*, 2013).

In depth reviews have been completed on the disadvantages of clinical drug resistance (Cowen *et al.*, 2014; Lee *et al.*, 2020; Robbins *et al.*, 2017a). The rapidly increasing resistance toward conventional antifungals that is presented in these reviews has led to the conclusion that without urgent action previously effective therapeutic strategies for fungal diseases are not sustainable. Due to the limited number of available antifungals, and the similarities in their activity spectrum as well as mode of action, intensive nonclinical and clinical research needs to be conducted to identify new antifungal therapies. Interestingly, the antimicrobial peptides (AMPs) are gaining more attention as novel drug candidates (Kosikowska & Lesner, 2016; Mookherjee *et al.*, 2020). AMPs have been found in virtually all organisms and they display remarkable structural and functional diversity, which make them especially interesting compounds for the development of novel therapeutics.

1.3 Introduction to Antimicrobial Peptides

Antimicrobial peptides (AMPs) are naturally occurring molecules that provide a first line of defence against pathogenic microorganisms. AMPs exhibit a broad range of inhibitory activity, protecting their host by either directly killing or retarding the growth of pathogens, or by activating the immune system, wound healing or the inflammatory response in higher eukaryotes (Mookherjee *et al.*, 2020). Plants, mammals and insects all release AMPs in response to infections from bacteria or fungi. Microorganisms also release AMPs to kill competing microorganisms (Magana *et al.*, 2020; Tobias *et al.*, 2017). AMPs can be divided into several different classes with different mechanisms of action and different microbial targets including yeast, fungi, bacteria, and parasites (Zhang & Gallo, 2016). Peptides have promising properties for clinical development, such as moderate immunogenicity, strong antimicrobial activity, high specificity and affinity for targets, distinct mechanisms of action, good organ and tissue penetration and broad-spectrum activity (Aoki & Ueda, 2013;

Ciociola *et al.*, 2016). Against a background of rapidly increasing resistance development to conventional antimicrobials all over the world, efforts to bring AMPs into clinical use are accelerating. There are encouraging examples of AMPs already introduced into the market, and many AMPs have been or are currently being tested in clinical trials (Fox, 2013; Mahlapuu *et al.*, 2016; Mookherjee *et al.*, 2020), which provides a reason for investigation into the mechanisms of action and resistance of the most promising candidates.

1.3.1 Structure and diversity of AMPs

AMPs are typically 10- 50 amino acids in length, are generally positively charged and often contain hydrophobic residues, forming unique amphipathic structures (Pasupuleti *et al.*, 2012). The amphipathicity of AMPs facilitates their interactions with microbial membranes while their cationic nature makes them ideal for targeting the negatively charged surface of bacteria and fungi (Hancock & Sahl, 2006). AMPs can be classified based on their secondary structure, these include α-helical and β-sheet peptides as well as peptides with an extended/random-coil structure (Nguyen *et al.*, 2011; Takahashi *et al.*, 2010; van der Weerden *et al.*, 2013) (Figure 1.1).

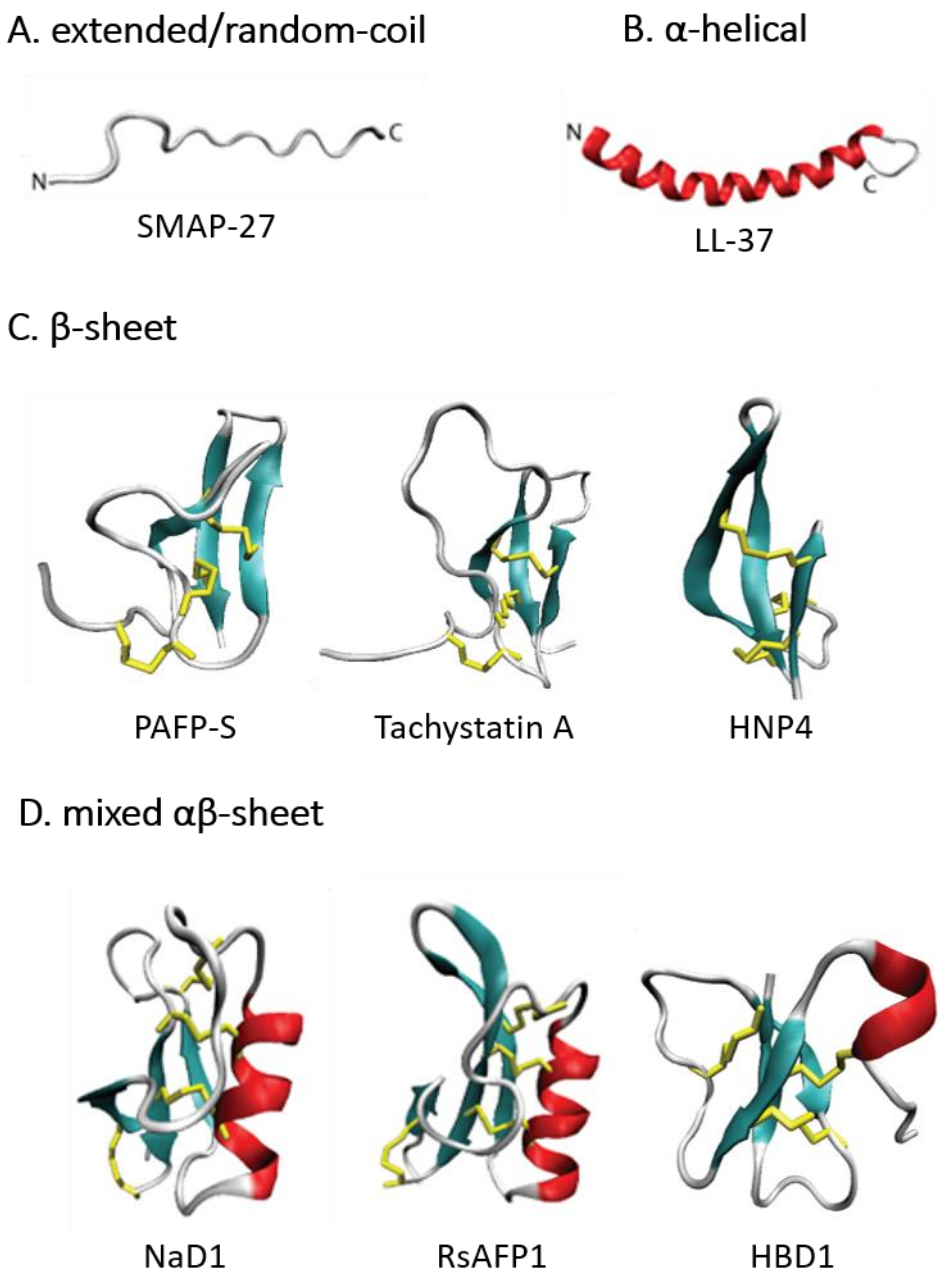


Figure 1.1. Four structural classes of AMPs. (A) extended coil structure of SMAP-27 (PDB code 1FRY); **(B)** α -helical structure of human cathelicidin LL-37 (PDB code 2K6O); **(C)** β – sheeted PAFP-S (PDB code 1DKC), tachystatin A (PDB code 1CIX) and HNP4 (PDB code 1ZMM); **(D)** mixed αβ structures of the plant defensins NaD1 (PDB code 1MR4) and RsAFP1 (PDB code 2N2R), and the human beta defensin HBD1 (PDB code 1IJU).

α-helical peptides are unstructured in aqueous solutions, but when they encounter a biological membrane, they form an amphipathic helical structure (Pasupuleti *et al.*, 2012; Yeaman & Yount, 2003). The most studied examples of α-helical peptides are the human cathelicidin LL-37, and the histidine-rich peptides from human saliva, histatin 3 and 5 (Dong *et al.*, 2003; Fitzgerald *et al.*, 2003; Vandamme *et al.*, 2012). β-sheet peptides usually have two or more β-strands that are stabilized with inter-strand disulfide bonds. Due to their rigid structure, these peptides do not undergo drastic conformational changes in aqueous solution or when interacting with biological membranes (Nguyen *et al.*, 2011). The knottin-type peptides such as PAFP-S, from *Phytolacca americana*, are an example of β-sheet peptides, which as the name suggests, have a cystine-stabilized, "knotted" topology, defined by two parallel disulfide bonds threaded by a third disulfide bond (Gao *et al.*, 2001; Gracy & Chiche, 2011). α-defensins such as human HNP4 (Ashrafi *et al.*, 2017) have 6 conserved cysteine residues which form three disulfide bonds in the three stranded β-sheet fold. There are also examples of more complex peptides, that contain a combination of α-helical and β-sheet structures (Kovaleva *et al.*, 2020; Rees & Lipscomb, 1982). They include vertebrate β-defensins and defensins from insects, invertebrates, and molluscs. This group of defensins includes human HBD2 (Krishnakumari *et al.*, 2009). β-defensins have six conserved cysteines forming three disulfide bonds and differ from α-defensins in the connectivity of disulfide bonds and the addition of a short α helix. Despite their extensive structural and functional similarities, the vertebrate β-defensins and defensins from insects, invertebrates and molluscs actually consist of two evolutionarily independent superfamilies (trans and cis-defensins) having arisen by convergent evolution (Shafee & Anderson, 2019; Shafee *et al.*, 2016; Shafee *et al.*, 2017). Most *trans*-defensins occur in vertebrates (fish, reptiles, birds and mammals) (Shafee *et al.*, 2016). The larger superfamily is named the *cis*-defensins and is dominated by plant defensins (Shafee & Anderson, 2019). Plant defensins have very different sequences and mechanisms of action but have a common fold consisting

of a triple-stranded, anti-parallel β-sheet connected to an α-helix by three disulfide bonds forming a cystine-stabilized αβ motif (Parisi *et a.l*, 2018; van der Weerden *et al.*, 2013). A fourth disulfide bond joins the N- and C-termini creating an extremely stable protein (van der Weerden *et al.*, 2013).

Lastly, there are a small number of AMPs that lack a secondary structure and often contain a high content of arginine, proline, tryptophan, and/or histidine residues (Nguyen *et al.*, 2011; Takahashi *et al.*, 2010), that have an extended/random coil structure. A well characterised peptide from this class is indolicidin a 13 amino acid cationic peptide from bovine neutrophils whereby it is globular and amphipathic in solution and changes to a wedge like shape on interaction with a membrane (Hsu *et al.*, 2005).

AMPs are universal; they are produced by organisms in all kingdoms of life. According to the DRAMP (Data repository of antimicrobial peptides) (Last updated on 2020-9-14), there are currently 20592 entries, 5877 of which are general AMPs (containing natural and synthetic AMPs), 14739 patent AMPs and 76 AMPs in drug development (preclinical or clinical stage) (Kang *et al.*, 2019). AMPs can be constitutively expressed or induced after exposure to stress or invading pathogens. In mammals, AMPs have been isolated from exposed tissue areas such as the skin, eyes, ears, mouth and urinary and intestinal tracts. An example is the α-helical peptide, human cathelicidin LL-37 and lactoferrin from human tears (Tsai *et al.*, 2014b; Wang, 2014) and the histidine-rich peptides from human saliva, histatin 3 and 5 (Dong *et al.*, 2003; Fitzgerald *et al.*, 2003; Vandamme *et al.*, 2012). In humans, AMPs have other roles which is why they are also called Host Defence Peptides. These functions are immune modulation, apoptosis, and wound healing (Mookherjee *et al.*, 2020). Microbes also produce AMPs, usually to limit the growth of competing microorganisms such as other bacteria or fungi. An example of a microbial AMP is polymyxin B from *Bacillus polymyxa*, which kills Gram-negative bacteria, and vancomycin which is a non-cationic peptide that kills Gram-positive bacteria. Both of these AMPs have

been developed into FDA approved antibiotics (Mahlapuu *et al.*, 2016). AMPs are also produced by plants and insects as a first line of defence against potentially damaging pathogens, for example, the plant defensin NaD1, from *Nicotiana alata,* is active against both filamentous fungi and yeast (Hayes *et al.*, 2013; van der Weerden *et al.*, 2008). In this thesis I will be focusing on peptides that have antifungal properties known as Antifungal peptides (AFPs).

1.3.2 Mechanism of action of Antimicrobial peptides

The modes of action of AMPs are diverse and can be difficult to categorise (Amerikova *et al.*, 2019; Raheem & Straus, 2019; van der Weerden *et al.*, 2013). Membrane disruptive mechanisms involve cell membrane penetration or the combination of AMPs with the membrane component of the microorganism. The characteristics of their amino acid composition, amphipathicity, and electric charge allow these peptides to induce membrane permeabilsation or disruption, leading to membrane depolarisation, loss of vital organelles and other cellular components, and cell lysis and death (Barbosa Pelegrini *et al.*, 2011; Bondaryk *et al.*, 2017; Rautenbach *et al.*, 2016a). However, these peptides can also act via membrane non-disruptive mechanisms, by interacting with intracellular targets after crossing the plasma membrane or disrupting key cellular processes (Rautenbach *et al.*, 2016a).

1.3.2.1 Membrane-disruptive mechanisms

AMPs are able to inhibit fungal growth by the disruption of cellular membrane integrity resulting in the leakage of ions and other molecules (Yeaman & Yount, 2003), together with a general loss of membrane functionality. Various models and modes of action have

been proposed for the disruption of membrane integrity via pore formation, particularly in bacteria (Seyfi *et al.*, 2020). These models are discussed in a number of reviews on the mode of action of AMPs (Bechinger & Lohner, 2006; Hollmann *et al.*, 2018; Nguyen *et al.*, 2011; Sengupta *et al.*, 2008; Seyfi *et al.*, 2020) and therefore will not be discussed in detail in this review. Most of these models of membrane permeabilisation and pore formation are based on studies with bacterial membranes (Mattila *et al.*, 2008). However, some of these modes of action may not be valid for fungi as the structure of fungal membranes differs significantly from bacterial membranes and fungal membranes have unique components such as the sterol ergosterol and fungal specific sphingolipids (Nguyen *et al.*, 2011; Sevcsik *et al.*, 2007; Singh & Prasad, 2011). Other modes of membrane permeabilisation include binding with membrane lipids and fungal specific membrane components (Cools *et al.*, 2017), changes in membrane potential due to peptide accumulation in outer leaflet, or association with small anions leading to electroporation (Chan *et al.*, 2006) and dissipated membrane potential (Gifford *et al.*, 2005).

Examples of AMPs that act by membrane permeabilization are the cathelicidins from sheep (SMAP-29) and cow (BMAP-27, BMAP-28) which form amphipathic α-helices in a hydrophobic environment. They have C-terminal hydrophobic domains, with strong membrane permeabilization activities (Benincasa *et al.*, 2006; Skerlavaj *et al.*, 1999). SMAP-29 concentrates on the plasma membrane of treated cells and causes propidium iodide uptake provided the cells are metabolically active (Lee *et al.*, 2002). In a hydrophobic environment, PMAP-23 (from pigs) forms two short α-helices joined by a flexible region (Park *et al.*, 2002). This peptide binds to the plasma membrane of treated cells and is active against *C. albicans* protoplasts indicating interaction with the cell wall is not required for inhibitory activity (Lee *et al.*, 2001). The β-hairpin peptide protegrin and the extended, tryptophan-rich peptide indolicidin (both from pigs), also exhibit candidacidal activity through membrane permeabilization (Benincasa *et al.*, 2006).

LL-37

So far, only one cathelicidin, LL-37, has been identified in humans and will be one of the peptides investigated further in this thesis (Kai-Larsen & Agerberth, 2008). It forms an amphipathic α-helix and binds to the cell wall and plasma membrane of treated cells (den Hertog *et al.*, 2005). It disrupts the *C. albicans* cell membrane completely and allows leakage of small nucleotides such as ATP and proteins of up to 40 kDa into the medium (den Hertog *et al.*, 2005). Insertion of LL-37 into membranes is equally dependent on hydrophobic interactions between the peptide and acyl chains of the membrane lipids as it is on electrostatic interactions with lipid head groups (Henzler-Wildman *et al.*, 2004). LL-37 is known to associate with cell wall components of *C. albicans*, (Burton and Steel, 2009) such as Xog1p and inhibit cell adhesion (Tsai *et al.*, 2014a; Tsai *et al.*, 2011). Analysis by live-cell imaging showed that LL-37 peptides kill *C. albicans* rapidly. (Ordonez *et al.*, 2014). The strong membrane binding of LL-37 during the killing process led to the suggestion that direct membrane permeabilization is the main mechanism of action. However, small amounts of LL-37 peptides were detected intracellularly, even before PI influx and vacuolar expansion took place. Therefore, the authors hypothesised that a relatively high concentration of peptides is required at the membrane for the peptides to traverse the cell boundaries (Ordonez *et al.*, 2014).

Extensive research on plant defensin–target interactions revealed two groups of fungal lipid targets, namely sphingolipids and phospholipids (PLs) (Cools *et al.*, 2017). Lipid rafts containing glycosphingolipids (GSLs) and ergosterol are present at the growth tip of budding yeast and hyphae (including filamentous fungal hyphae), making this a vulnerable target (Martin & Konopka, 2004; Takeshita *et al.*, 2008). The binding of a peptide to GSLs could have a major influence on cell function as these lipids participate in signal

transduction, and delivery of glycerophosphatidylinositol (GPI)-linked cell wall proteins in the lipid rafts (Aerts *et al.*, 2007; Bagnat *et al.*, 2000; Thevissen *et al.*, 2004). The GPI anchoring on the outer membrane leaflet results in presentation of these GPI-linked cell wall proteins on the surface of the cell. The antifungal plant defensin from *Dahlia merckii*, *Dm*AMP1, interacts with the fungal specific sphingolipid $M(IP)_2C$ from *S. cerevisiae*, leading to a biphasic permeabilisation of the fungal membrane (Thevissen *et al.*, 2003). Furthermore, the presence of another fungal specific lipid, ergosterol, together with $M(IP)_2C$, enhanced *Dm*AMP1 activity (Thevissen *et al.*, 2003). The plant defensins NaD1 and TTP3, as well as human β-defensin 2 binds to the membrane phospholipid phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) directly causing membrane permeabilisation in tumour cells and potentially in fungal hyphae (Baxter *et al.*, 2015; Järvå *et al.*, 2018b; Payne *et al.*, 2016; Poon *et al.*, 2014). Furthermore, these researchers have high-resolution structural evidence for the formation of a carpet-like configuration by NaD1 during the initial stages of membrane encounter with target phospholipids (Järvå *et al.*, 2018a).

While most AMPs interact with and influence the integrity of microbial membranes, it is not fully understood whether membrane permeabilization is always a lethal event or whether the membrane is the only site of action (Cools *et al.*, 2017) Indeed in some cases, loss of membrane integrity may occur after cell death has been induced by another mechanism (Figure 1.2).

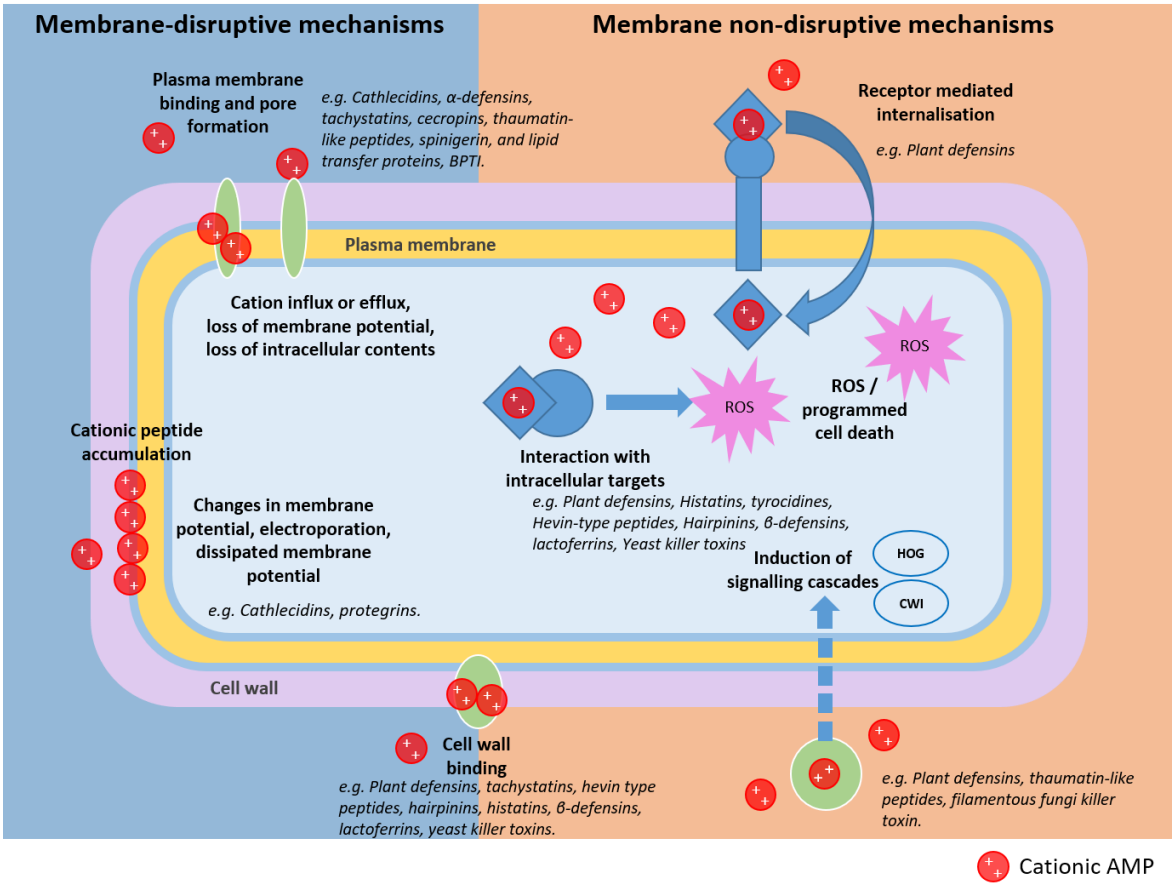


Figure 1.2. Mechanism of action of AMPs on Fungi. The mechanism of action of AMPs can be categorised into membrane disruptive and membrane non-disruptive mechanisms. Membrane disruptive mechanisms include plasma membrane binding and cell wall binding leading to membrane permeabilization and loss of intracellular contents, or cationic peptide accumulation on the plasma membrane leading to change in membrane potential. Membrane non-disruptive mechanisms include entering the cell via binding to cell wall polysaccharides or receptor mediated internalisation, once inside the cytoplasm they bind to intracellular targets which can cause ROS production or programmed cell death. A result of cationic peptide interaction with fungal cells is the induction of signalling cascades such as the HOG and CWI pathway. This an updated version of van der Weerden's 2013 figure (van der Weerden *et al.*, 2013).

1.3.2.2 Membrane-nondisruptive mechanisms

As stated earlier, the most studied mechanism of action of cationic AMPs has been the interaction with, and disruption of, the cytoplasmic membrane. More recently a number of cationic AMPs have been reported to act on internal targets, either as their major mechanism of action following their translocation across the membrane or as an additive effect, combined with (often incomplete) membrane disruption (Figure 1.2).

BPTI

Another peptide that will be investigated in this thesis is bovine pancreatic trypsin inhibitor BPTI also known as aprotinin which inhibits growth of *S. cerevisiae* and the human pathogen *C. albicans*. Bleackley and co-workers (2014) revealed by screening of the yeast heterozygous essential deletion collection that the magnesium transporter Alr1p was the likely BPTI target. This was confirmed when BPTI treatment of wild type cells resulted in a lowering of cellular Mg^{2+} levels and the observation that populations treated with BPTI had fewer cells in the S -phase of the cell cycle and a corresponding increase of cells in the G_0/G_1 and G_2 phases. Furthermore, the same patterns of cell cycle arrest obtained with BPTI were also obtained with the magnesium channel inhibitor hexamine (III) cobalt chloride (Bleackley, 2014). Analysis of the growth inhibition of *C. albicans* revealed that BPTI acts via the same mechanism in the two yeast species (Bleackley, 2014).

It has been proposed more recently that cationic AMPs have a 'multitarget'-mechanism of action (Hale & Hancock, 2008; Kumar *et al.*, 2018), whereby they are able to interact with multiple anionic targets. Certainly, there are many examples of cationic AMPs with more than one non-membranous target site within a fungal cell (Lee & Lee, 2018; Puri & Edgerton, 2014; van der Weerden *et al.*, 2013; Yeaman *et al.*, 2018).

NaD1

NaD1 is one of the best characterised AMPs with a multi-site mechanism of action, which makes it an advantageous peptide to study in this thesis. NaD1 a plant defensin from the ornamental tobacco *Nicotiana alata* is active against several pathogenic fungi (Dracatos *et al.*, 2014; Lay *et al.*, 2003; van der Weerden *et al.*, 2008) and functions to protect the floral tissues against damage from potential fungal pathogens (Lay *et al.*, 2012). Treatment of fungal cells with NaD1 led to damage of the inner leaflet of the cell membrane and cell death within 10 min of exposure to NaD1 (Hayes *et al.*, 2014; Payne *et al.*, 2016; van der Weerden *et al.*, 2010). The mechanism of cell death is complicated and involves interactions with multiple fungal components (Parisi *et al.*, 2018). Firstly, NaD1 requires the presence of the cell wall polysaccharide 1,3-β-glucan to initiate its specific and lethal effect on fungal cells (Bleackley *et al.*, 2019; van der Weerden *et al.*, 2008). As mentioned earlier, NaDI forms dimers that bind tightly to phosphatidylinositol 4, 5-bisphosphate ($PI(4,5)P_2$) which is located on the inner leaflet of the plasma membrane (Poon *et al.*, 2014). However, interaction with $PI(4,5)P_2$ may not be essential for the antifungal mechanism because NaD1 variants that do not bind $PI(4,5)P_2$ still kill fungal cells (Bleackley *et al.*, 2016). In *C. albicans*, NaD1 accumulates on the surface of the plasma membrane and traverses via endocytosis (Hayes *et al.*, 2018). Once NaD1 enters the cytoplasm it interacts with intracellular targets leading to the production of ROS, permeabilisation of the plasma membrane, granulation of the cytoplasm and cell death (Hayes *et al.*, 2018; Hayes *et al.*, 2013; van der Weerden *et al.*, 2010; van der Weerden *et al.*, 2008). Activation of the HOG1 pathway in response to NaD1 mediated osmotic and oxidative stress along with other stressors has also been reported to be protective at sub-lethal concentrations of NaD1 (Hayes *et al.*, 2014).

The histatins are a well characterised group of histidine-rich peptides from human saliva that are active against several *Candida* species (den Hertog *et al.*, 2005; Pathirana *et al.*,

2018). Histatin 5, a cleavage product of histatin 3, is the most potent of these molecules and as such, the most well studied. The heat shock protein Ssa2p, a 70 kDa cell wall protein in *C. albicans*, is the binding site for histatin 5 (Li *et al.*, 2006; Sun *et al.*, 2008). The presence of the Ssa2p is required for susceptibility of *C. albicans* to histatin 5 and internalization of histatin 5 into cells (Li *et al.*, 2006; Sun *et al.*, 2008). Uptake of histatin 5 into *C. albicans* cells is dependent on the presence of two polyamine transporters, Dur3 and Dur31, which usually function in spermidine uptake (Kumar *et al.*, 2011). Internalization must occur by translocation, not endocytosis, for histatin to act as an antifungal molecule against *C. albicans* (Jang *et al.*, 2010). Upon internalization, histatin 5 travels to the mitochondria providing respiration is underway and causes a loss of mitochondrial membrane integrity (Komatsu *et al.*, 2011; Puri & Edgerton., 2014)

MtDef4 a 47 amino acid protein from the model legume *Medicago truncatula* is active against the filamentous fungal pathogen, *F. graminearum* and *N. crassa* (Ramamoorthy et al., 2007; Sagaram et al., 2011). Sagaram and coworkers (2013) discovered that the loop 5 sequence (RGFRRR) binds to phosphatidic acid (PA) and is essential for MtDef4 entry into fungal cells. MtDef4 also disrupts Ca^{2+} signalling and/or homeostasis leading to inhibition of hyphal growth and fusion (Muñoz *et al.*, 2014; Spelbrink *et al.*, 2004). Once in the cytoplasm MtDef4 interferes with internal cellular targets of *F. graminearum* that are still unknown (Sagaram *et al.*, 2013; Sagaram *et al.*, 2011). In a later study, it was discovered that membrane permeabilization is required for the antifungal activity of MtDef4 against *F. graminearum* but not against *N. crassa*. MtDef4 appears to translocate into *F. graminearum* autonomously using a partially energy-dependent pathway (El-Mounadi *et al.*, 2016). By contrast, internalization of MtDef4 in *N. crassa* is energy-dependent and involves endocytosis (El-Mounadi *et al.*, 2016). This led to the suggestion that the plasma membrane localized phospholipase D, involved in the biosynthesis of PA, is needed for entry of this defensin into *N. crassa*, but not into *F. graminearum* (El-Mounadi *et al.*, 2016).

ROS production is a component of the antifungal activity of a number of peptides (Basso *et al.*, 2020), including the tyrocidines which induced the generation of ROS in *C. albicans* (Troskie *et a.l*, 2014). However, ROS formation was not essential for the activity of the tyrocidines, as their antifungal activity was surprisingly significantly higher in the presence of the antioxidant, ascorbic acid (Troskie *et al.*, 2014). As the tyrocidines induce rapid permeabilisation of fungal membranes, ROS are probably a consequence of osmotic stress and/or binding to GlcCer-ergosterol rich lipid rafts (Rautenbach *et al.*, 2016b). GlcCer-dependent activity of the plant defensin *Pv*D1 directly induces the formation of ROS in *C. albicans* and *F. oxysporum*, as well as nitric oxide in *C. albicans* (de Medeiros *et al.*, 2014; Mattila *et al.*, 2008). Furthermore, *Pv*D1 activity on *C. albicans* leads to disorganisation in the cytoplasm and plasma membrane. The ROS formation by *Pv*D1 action likely enhances the delayed *Pv*D1 membrane permeabilisation, as well as induction of programmed cell death in *C. albicans* and *F. oxysporum* (Mello *et al.*, 2011). *Rs*AFP2 a plant defensin from radish inhibits *C. albicans* by binding to GlcCer, inducing endogenous ROS, which explains the *Rs*AFP2-induced biphasic membrane leakage (Aerts *et al.*, 2007; Thevissen *et al.*, 2012). Following initial interaction with GlcCer, downstream signalling pathways lead to the induction of ROS, membrane permeabilisation and programmed cell death (Rego *et al.*, 2014). Therefore, suppression of ROS production would increase fungal resistance to these antifungal peptides. This was indeed the case with *Rs*AFP2 as the presence of ascorbic acid led to a significant loss in fungicidal activity, linking the involvement of ROS induction to *Rs*AFP2's activity against *C. albicans* (Aerts *et al.*, 2007).

Because of their broad spectrum of activity, AMPs are considered promising alternatives to conventional antimicrobial agents. Therefore, understanding the mechanism of action of AMPs is a crucial step in understanding the mechanisms of resistance, and in turn assessing their potential for clinical use.

1.4 Mechanism of resistance to antimicrobial peptides

Some researchers have reported that development of microbial resistance to AMPs is unlikely to occur (Mahlapuu *et al.,* 2016). This has led to the hypothesis that AMPs have larger interactive surfaces with the targets in the pathogen than can be achieved with small molecule drugs (Tsomaia., 2015) and hence single amino acid substitutions are unlikely to lead to rapid evolution of resistance. Furthermore, AMPs have complicated mechanisms of action and often interact with more than a single target in the fungus so that multiple mutations are needed for full resistance. However, some bacteria and fungi have developed mechanisms to resist the toxic effects of AMPs or developed ways of evading host immune response and detection, explaining why commensal organisms and pathogens survive the arsenal of AMPs presented by epithelial and mucosal surfaces and neutrophils.

1.4.1 Mechanisms of resistance to antibacterial peptides

In order to understand the potential for resistance to develop to AMPs in fungi, it is important to understand the mechanisms that have already been identified in bacteria (Abdi *et al.,* 2019; Band & Weiss, 2015; Joo *et al.,* 2016). Common AMP resistance mechanisms in bacteria are repulsion, sequestration, removal, and degradation (Figure 1.3).

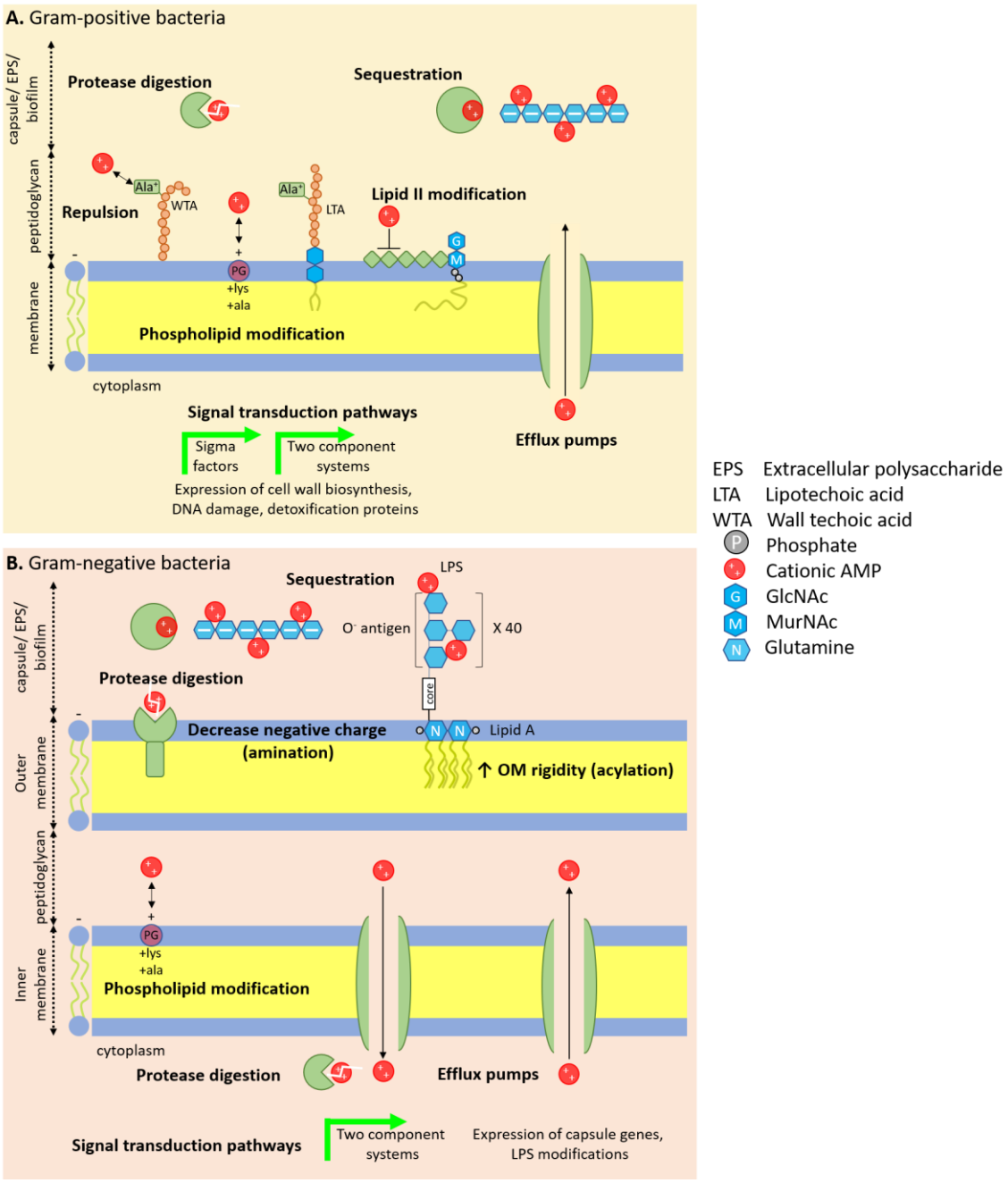


Figure 1.3. Mechanisms of resistance to Antibacterial peptides. (A) Gram-positive bacteria. **(B)** Gram-negative bacteria. The mechanisms of resistance are highlighted in bold in the figure and include: **Protease digestion**: degradation by secreted proteases, outer membrane (OM) proteases or cytosolic proteases. **Sequestration** by secreted proteins, anionic polysaccharides or O antigen. **Electrostatic Repulsion,** by alanylated lipoteichoic acid (LTA), wall teichoic acid (WTA) or aminoacylated phospatidylglycerol (PG). **Lipid II modification**, blocking of AMPs binding by altering the pentapeptide on lipid II. **Efflux pumps**, export of AMPs by efflux pumps. **Signal transduction pathway** activation that induces expression of genes that reinforce the wall or

detoxify products of AMP activity. **Lipid A modification** by amine compounds, enhanced membrane rigidity by lipid A acylation. This figure is an adaptation from (Mookherjee *et al.*, 2020).

Repulsion. Gram-positive and Gram-negative bacteria incorporate positive charges into their normally negatively charged cell surfaces (either the membrane or cell wall) to decrease interaction with the cationic AMPs. The modifications vary between bacterial species, but common targets are the most abundant negatively charged molecules on the outer surface such as teichoic acids on Gram-positive bacteria and lipopolysaccharide (LPS) on Gram-negative bacteria. AMP resistance is enhanced by the formation of lysyl-phosphatidyl glycerol (L-PG) by the enzyme multi peptide resistance factor, Mprf, which is present in many Gram-positive and Gram-negative bacteria (Roy, 2009). Bacteria have specific sensors that activate AMP resistance mechanisms when AMPs are present. The regulatory systems have been most extensively studied in the Gram-negative bacterium *Salmonella typhimurium* where the PmrAB, Pho PQ and Rcs regulatory systems mediate many of the LPS modifications that increase surface charge (Andersson *et al.*, 2016). The best studied regulatory system in Gram-positive bacteria is the antimicrobial peptide sensing system of *Staphylococcus epidermidis* which induces D-alanylation of teichoic acids, incorporation of lysyl-phospatidylglycerol into the bacterial membrane coupled with an increase in lysine biosynthesis and activation of the VraFG AMP efflux pump (Li *et al.*, 2007).

Sequestration. This involves secretion of proteins or anionic extracellular polysaccharides into the cell walls, capsules or biofilms that bind the AMPs and restrict access to the cell membrane. Examples are the anionic capsular polysaccharides of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*, the O-antigen of lipopolysaccharide on Gram-negative bacteria and polysaccharide intercellular adhesin (PIA also known as poly-N-acetyl glucosamine) which is produced by a variety of bacteria including Staphylococcal species and *E.coli* and is crucial for biofilm formation. Alginate,

a negatively charged polymer secreted by *Pseudomonas aeruginosa* during biofilm formation, also sequesters AMPs. The intrinsic resistance of biofilms to AMPs is likely to limit the therapeutic potential of AMPs for treatment of biofilm infections.

Removal. AMPs that have inserted into the membrane or entered the cytoplasm can be removed by efflux pumps. The resistance/nodulation/cell division (RND) efflux pumps are present in many Gram-negative bacteria. For example, the MtrCDE pump of *Neisseria gonorrhoeae* enhances tolerance to LL-37 and protegrin (Tzeng & Stephens, 2015).

Degradation. Proteolytic degradation by extracellular proteases, outer membrane proteases or by cytosolic proteases after uptake by transporters. Examples are PgtE in *S. typhimurium, S.aureus* aureolysin and OmT in *E.coli*. The linear AMPs such as LL-37 are more susceptible to degradation than AMPs such as the defensins that have compact scaffolds held together by several disulphide bonds.

Additional resistance mechanisms are modification of the pentapeptide on Lipid II, a prominent AMP target and alteration of the rigidity of the membrane by acylation of Lipid A.

1.4.2 Mechanisms of resistance to Antifungal peptides

Studies on the mechanisms that fungi employ to enhance tolerance to AFPs are limited and have mainly focused on Candida species which also employ repulsion, sequestration, removal by efflux pumps and proteolytic degradation for protection against the human AFPs LL-37, histatin 5, hNP-1, hBD1-3 lactoferrin and hGAPDH (Swidergall & Ernst, 2014) (Figure 1.4).

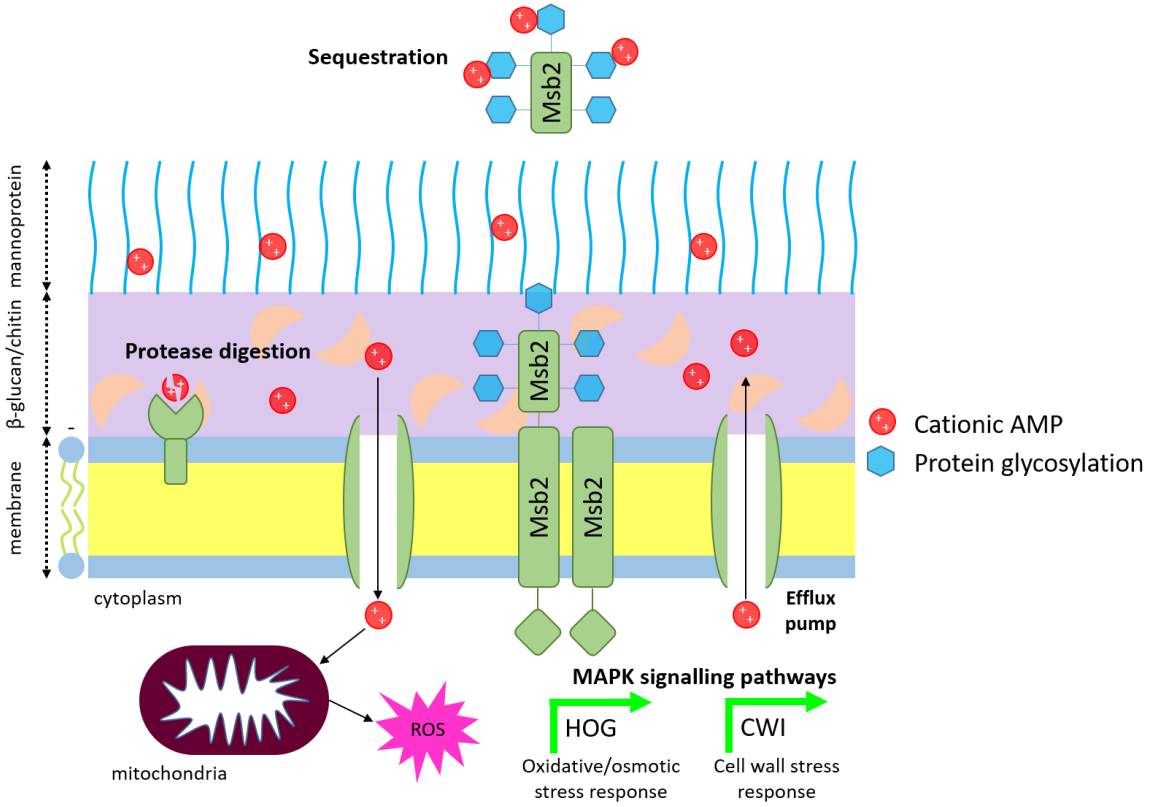


Figure 1.4. **Mechanism of resistance to Antifungal peptides.** The mechanisms of resistance are highlighted in bold in the figure and include: **Sequestration** by secreted proteins, anionic polysaccharides or mannosylphosphate side chains on glycoproteins. **Efflux pumps**, export of AMPs. **Signal transduction pathway** activation that induces expression of genes that reinforce the wall or detoxify products of AMP activity. **Mitogen-activated protein kinase (MAPK) signalling pathways** in fungi for protection against oxidative, osmotic or cell wall stress. This figure is an adaptation from (Mookherjee *et al.*, 2020).

Histatin 5 for example is degraded by the extracellular GPI anchored proteases Sap9/10. Extracellular concentrations of histatin 5 are also limited by sequestration to the outer layer of mannosylated glycoproteins (Harris *et al.*, 2009) and by the soluble domain of the MbS2 membrane sensor which is a broad range protectant against AFPs including histatin 5, LL-37, hNP-1 and hBD1 (Szafranski-Schneider *et al.*, 2012). After traversing the fungal cell wall Histatin 5 gains access to the plasma membrane and enters cells via a polyamine transporter. After reaching threshold levels Histatin 5 stimulates production of reactive

oxygen species and ATP efflux. The Flu1 efflux pump reduces the cytoplasmic concentration of Histatin 5 but it is not upregulated in response to AFP exposure and is not sufficient for complete resistance (Li *et al.*, 2013). The cell responds instead by activation of the High Osmolarity Glycerol (Hog1) MAP kinase pathway which induces transcription of genes that protect against oxidative and osmotic stress. The Hog1 and Mkc1 cell wall integrity (CWI) pathways protect Candida against a variety of AFPs including histatin 5 and hβD2. These pathways are also active in filamentous fungi for example the plant pathogen *Fusarium graminearum* which can survive exposure to plant defensins by inducing transcription of genes that protect against oxidative stress and reinforce the fungal cell (Hayes *et al.*, 2014).

Most AMP resistance studies have been conducted in bacteria and in an *in vitro* setting where there is a high selection for resistance development. These studies are essential to gain more information regarding the ability of microbial pathogens to develop AFP resistance in advance of their practical application. Moreover, development and characterization of resistant microorganisms in the laboratory can provide a way to advance our knowledge regarding the mechanisms of action of AMPs. Thus far, there are few reports describing successful attempts at obtaining such mutants in fungi.

1.4.3 Determining AFP resistance mechanisms via Yeast deletion screens

The development of chemical genomic assays with *S. cerevisiae* mutant libraries has facilitated identification of the mechanism of action of many drugs and novel small molecules. The complete genome sequence of the yeast *S. cerevisiae* was published over 20 years ago and has become an essential tool for small molecule discovery and characterisation (Ho *et al.*, 2011). The genome sequence enabled construction of an almost

complete set of haploid non-essential gene deletion mutants as well as a set of heterozygous deletion mutants in the essential genes (Pierce *et al.*, 2007). These libraries have been used to identify genes that affect the relative fitness of *S. cerevisiae* upon exposure to chemical drugs or selective growth conditions. Initially these collections were screened in 96 well microtiter plates but the development of high density colony arrays and next generation barcode sequencing have substantially improved the throughput and decreased the amount of drug needed to perform these screens (Ho *et al.*, 2011; Shoemaker *et al.*, 1996).

Library screens are scoured for strains with enhanced resistance to assist in determining the mechanism of action of an AMP but also to explore how strains may develop AMP resistance. A screen of the *S. cerevisiae* deletion library determined that cells without the *agp2* gene had enhanced resistance to NaD1 (Bleackley *et al.*, 2014). Cells with a deletion of *agp2* accumulate positively charged molecules at the surface repelling cationic antifungal peptides (Schuber, 1989). In another screen of the *S. cerevisiae* non-essential deletion collection, mutants with increased tolerance to PAF26, a synthetic cationic antifungal hexapeptide were identified with deletions in protein glycosylation genes, indicating the importance of the glycosylation pathway in the mechanism of antifungal activity (Harries *et al.*, 2013). This complemented previous studies demonstrating PAF26 interaction with the outer layers of the fungal cell before translocation into the cell where PAF26 exerts its killing (Munoz *et al.*, 2006).

Barseq screening allowed for yeast deletion screens to be performed on a pooled collection of yeast strains with different deletions. This decreased the length of time and resources that were required to perform these screens and made it much easier to perform multiple screens in parallel. Yeast deletion screens with AFPs have been performed with human salivary peptides. Strains with deletions in genes associated with the RIM101 pathway were more sensitive to the MUC7 human salivary AMP, a 12 mer, leading to the discovery that RIM101 functions in protecting fungi against human salivary AMPs (Lis *et al.*, 2013; Lis

et al., 2009). In addition, in a direct selection technique with the AMP MUC7, the pool of yeast deletion strains was repeatedly treated with MUC7 leading to identification of a clone with elevated survival rates. This clone had a deletion in the *MIS1* gene encoding mitochondrial C_1-tetrahydrofolate synthase (Lis *et al.*, 2009). Another mitochondrial mutant displayed enhanced resistance to MUC7 in a separate screen (Lis *et al.*, 2013). This mutation decreases energy production, a condition known to protect against AMPs (Helmerhorst *et al.*, 1999). Similarly, in a separate screen yeast strains with deletions that decreased ATP transport and export were more resistant to the plant defensins, NbD6, SBI6, DmAMP1 and NaD1, suggesting that a reduction in energy levels protects the pathogen from several plant defensins with different mechanism of action (Parisi *et al.*, 2019). The importance of mitochondrial function has also been identified in a yeast screen aimed at elucidating the mechanism of action of the plant defensin HsAFP1. This screen, together with previous data, revealed that HsAFP1 binds to the cell wall and plasma membrane, penetrates the cytoplasm to target the mitochondria and activates ROS production leading to cell death (Aerts *et al.*, 2011; Parisi *et al.*, 2019; Thevissen *et al.*, 1997).

Screens of yeast deletion libraries have largely focused on determining the mechanisms of action for selected AMPs, but these screens also have the potential to identify likely routes of resistance. However, full gene deletions, in the strains in the non-essential gene collection, are often associated with fitness defects. Resistance caused by gene deletions is likely to be different to the genetic mechanisms for resistance that arise in the clinic or field. Nevertheless, laboratory screens will give insight into the potential biological mechanisms of resistance.

1.4.4 Laboratory based evolution of resistance to antifungal peptides

The yeast deletion screens discussed above focused on loss of gene function. Although this method has been very productive for describing the mode of action of AFPs, it does not provide enough insight into whether mutations of these targets would provide robust resistance in the clinic. Clinical antifungal resistance is often not associated with a total loss of function, but with changes to enzyme active sites or upregulation of drug efflux pumps and/or other genes that counter act the effect of the antifungal (Cowen et al., 2014). Therefore, in order to understand the potential for clinical resistance risk in advance, there needs to be a more comprehensive approach to studying the development of resistance. One tool for the prediction of resistance risk is laboratory selection for resistance.

1.4.4.1 Laboratory selection for AFP resistance via chemical mutagenesis

Chemical mutagenesis involves producing drug resistant strains by randomly introducing DNA mutations by the introduction of a mutagen (usually UV or chemicals such as ethylmethanesulphonate (EMS) (Hawkins & Fraaije, 2016). Mutagenesis enables resistance to emerge more rapidly in the laboratory than in the field but may produce mutations that would not emerge under field conditions (Hawkins & Fraaije, 2016). An example of this is the laboratory selection of resistance to the antifungal peptide MUC7. MUC7 is a 12 amino acid cationic peptide from the N-terminal portion of human mucin MUC7, that has potent antifungal properties (Situ *et al.*, 2003). MUC7-resistant mutants of *C. albicans* were developed by mutagenesis with EMS (Lis & Bobek, 2008). One colony, exhibiting the highest level of MUC7 resistance in a killing assay, was characterised further. This colony, designated mutant #37 had a 6-fold increase in MIC compared to the parental strain. The mutant exhibited lowered ATP content along with a relatively lower rate of oxygen consumption, as well as an inability to grow on nonfermentable carbon

sources. The authors suggested that the resistance is likely to be associated with changes in metabolic regulation, rather than the lack of functional mitochondria, as determined by rhodamine 123 staining (Lis & Bobek, 2008). Therefore, it is possible that the lowered metabolism of the mutant #37 led to its resistance to the MUC7 12-mer. (Situ *et al.*, 2003). However, the lowered level of metabolism and inability to grow on fermentable carbon sources is also likely to decrease the survival of mutant #37 within a viable host. Another example of a mutation leading to resistance that is associated with decreased fitness is the mutants of *Neurospora crassa* that were generated by chemical mutagenesis using EMS and selected for resistance towards the radish defensin RsAFP2 (Ferket *et al.*, 2003). These mutants grew about 10-fold slower than the *N. crassa* wildtype and had lost the ability to form ascospores (Ferket *et al.*, 2003). Characterisation of the lipids in the membranes of the two *N. crassa* mutants revealed structurally different glucosylceramides, novel glycosphingolipids, and an altered level of steryl glucosides (Ferket *et al.*, 2003). Subsequent work revealed that the sphingolipid GlcCer in the fungal plasma membrane and cell wall, is essential for the activity of RsAFP2 (Aerts *et al.*, 2007; Thevissen *et al.*, 2012; Thevissen *et al.*, 2003; Thevissen *et al.*, 2004)

1.4.4.2 Serial passaging to generate AFP resistance

As stated previously, mutagenesis is a way to speed up the development of resistant mutants, although it also produces mutants that are less viable and thus unlikely to survive in a clinical setting. Another tool for analysis of resistance in fungi is to use selection for resistance in the laboratory without the introduction of a mutagen. This method involves sub-culturing, or passaging, the parental fungal strain at sub-inhibitory and/or increasing concentrations of AMP until resistance reaches a certain threshold, usually at least 4-fold more resistant than wildtype. Although there will be a reduced number of mutations, it is

considered more likely that these mutations could occur in a clinical setting (Beardsley *et al.*, 2018).

One of the first attempts at laboratory selection for AFP resistance was used by Thevissen et al. (2000), to determine the mechanism of action of the dahlia defensin DmAMP1. They used selection in liquid medium containing 5 μM DmAMP1 to isolate two independent DmAMP1-resistant *S. cerevisiae* strains (DM1 and DM2) (Thevissen *et al.*, 2000b). Wild-type yeast was sensitive to DmAMP1 at 2 μM, whereas the DmAMP1-resistant yeast mutants were resistant up to 40 μM DmAMP1. A complementation approach followed that consisted of the introduction of clones from a genomic library from the DmAMP1-sensitive wild-type yeast into the DmAMP1-resistant yeast mutant and screening for restored sensitivity to DmAMP1(Thevissen *et al.*, 2000a). The gene that restored DmAMP1 sensitivity to the resistant mutant was *IPT1*, a gene encoding an enzyme involved in the last step of the synthesis of the sphingolipid mannose-(inositol-phosphate)$_2$-ceramide. This revealed DmAMP1's mechanism of action, which is specific interaction with the sphingolipid M(IP)$_2$C from *S. cerevisiae*, leading to a biphasic permeabilisation of the fungal membrane (Thevissen *et al.*, 2003).

The human protein histatin has a complex mechanism of action that involves binding, internalisation, intracellular targeting, and release of ATP (Pathirana *et al.*, 2018; Puri & Edgerton, 2014). Resistant *C. albicans* mutants have been developed by sequential exposure to increasing concentrations of histatin 3 (Fitzgerald *et al.*, 2003). Proteomic analysis was used to identify alterations between the histatin-resistant strains and the histatin-susceptible parent to identify other potential targets (Fitzgerald-Hughes *et al.*, 2007). This proteomic analysis revealed that 59 proteins were differentially expressed

compared to the parental strain. Proteins that were absent from the wildtype but present in the histatin-resistant strains, included isocitrate lyase (Icl1p), fructose biphosphate aldolase (Fba1p), pyruvate decarboxylase (Pdc2p), and ketol-acid reductoisomerase (Ilv5p) which impact metabolic pathways. The resistant strains also decreased rates of oxygen consumption and histatin 3-mediated ATP release compared with wildtype. The authors concluded that these data support an important role for metabolic pathways in the histatin resistance mechanism and suggest that there may be several intracellular targets for histatin 3 in *C. albicans* (Fitzgerald-Hughes *et al.*, 2007).

Only a handful of studies have been completed thus far on laboratory selection for resistance to AFPs. These studies have also focused on determining the mechanism of action of AFPs instead of predicting the mechanisms of resistance for future clinical use. As well as this, some of these studies are up to 20 years old and have relied mostly on phenotype characterisation instead of whole genome sequencing. Due to the prevalence of resistance to all the major classes of clinical antifungals, and the understanding that fungal pathogens also have the potential to develop resistance to AFPs, it is important to further investigate the mechanisms of fungal resistance to a range of AFPs.

1.5 Thesis Aims

This thesis describes the development and mechanisms of resistance in *S. cerevisiae and C. albicans* towards a selection of AFPs and compares them to the development of resistance to current clinical drugs. The studies in this thesis will rely on the use of laboratory selection for resistance development rather than chemical mutagenesis or deletion screens, because they are more likely to replicate the development of resistance in the clinic. Furthermore, it is likely to provide insight into the mechanisms of action and resistance to AFPs that may not be elucidated otherwise. Having a better understanding of the targets for resistance development will help in development of strategies for introduction and sustainable use of AMPs in the clinic and will guide further investigation into the mechanisms of resistance to these peptides. In this thesis the prototypic plant defensin NaD1 and aprotinin, also known as Bovine Pancreatic Trypsin inhibitor (BPTI) were serially passaged with the model fungus *S. cerevisiae* and compared to the clinically used echinocandin caspofungin (Figure 1.5).

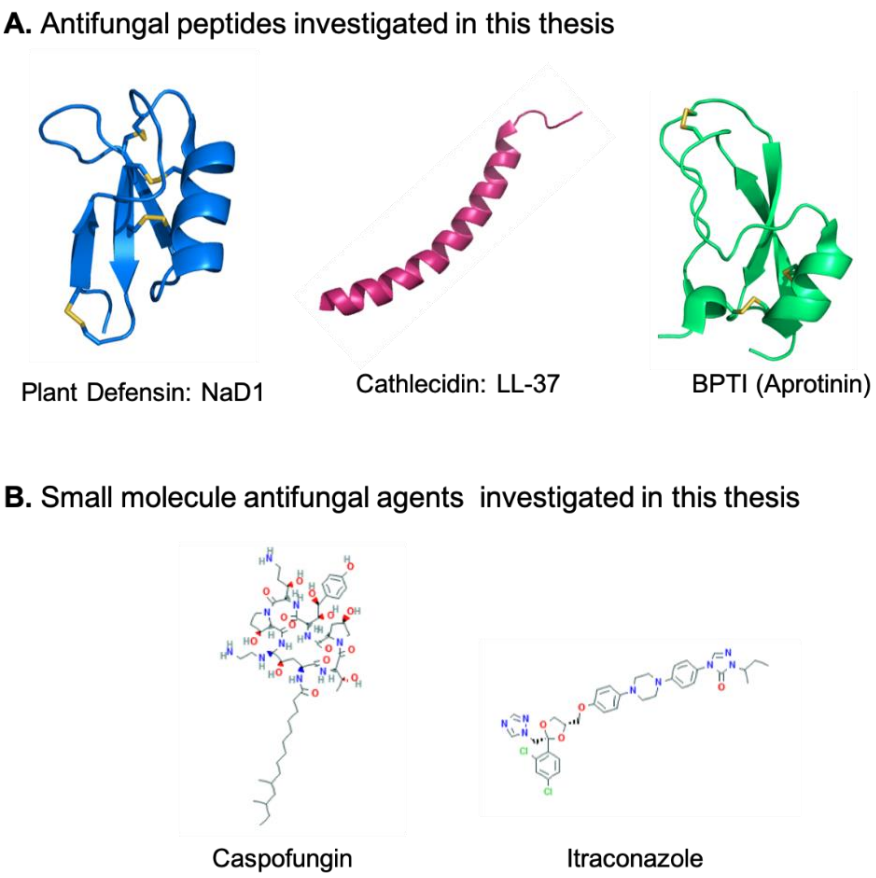


Figure 1.5. Structures of antifungal agents studied in this thesis. (A) 3D structure of AMPs used in this thesis; the plant defensin NaD1 (PDB code 4AAZ), human cathelicdin LL-37 (PDB code 2K60) and bovine pancreatic trypsin inhibitor BPTI (commercial name: Aprotinin) (PDB code 4TPI). **(B)** 2D structure of the small molecule antifungal agents used in this thesis; the echinocandin antifungal caspofungin (PubChem code 2826718) and the azole antifungal itraconazole (PubChem code 55283).

Resistance to AFPs was investigated further in the opportunistic fungal pathogen *C. albicans*. *C. albicans* was serial passaged in the presence of NaD1, the human cathelicidin LL-37 and the clinical azole antifungal itraconazole and the genomes of the resistant strains were compared to identify changes associated with resistance development. It was hypothesised that resistance to AFPs would develop more slowly in these yeast species and that the mechanism of resistance would be more complicated than resistance to small molecule antifungals. Despite the clinical interest in AFP's, laboratory selection for resistance to NaD1, aprotinin (BPTI) and LL-37 has not been conducted before. Therefore,

understanding the mechanisms of resistance of these peptides will further enhance their clinical potential.

Aim of study 1 (Chapter 2) – Mechanisms of resistance to NaD1 in *S. cerevisiae.*

The aim of this study was to determine whether resistance to NaD1 could be developed *in vitro* and to elucidate the mechanisms by which resistance occurs. The aim was to generate three strains of *S. cerevisiae* with at least a 4-fold increase in resistance to NaD1. Initially the NaD1 resistant strains were compared to wildtype *S. cerevisiae* by examining their growth rate in the presence and absence of NaD1 and the size and shape of the resistant strains. Whole genome sequencing of the NaD1-resistant strains was conducted using the Illumina MiSeq method and the changes in the genome were identified by comparison to the wild-type. The NaD1 resistant strains and the parent wt strain were then examined for differences in their response to general stresses such as UV radiation, oxidative stress, and cell permeabilisation. The rate of resistance development to NaD1 was compared to the clinically relevant echinochandin caspofungin to understand the clinical potential of NaD1. It was hypothesised that resistance to NaD1 would occur more slowly compared to caspofungin and due to the complex nature of the mechanism of action of NaD1, the mechanism of resistance would involve more than one target site.

Aim of study 2 (Chapter 3) - Mechanisms of resistance to Aprotinin (BPTI) in *S. cerevisiae*

Using the method described above three *S. cerevisiae* strains with increased tolerance to Aprotinin (BPTI) were generated and the genomes of the resistant strains were sequenced and compared to the NaD1 and caspofungin resistant strains. The aim of this study was to determine whether resistance development to another AFP was also slow and multifactorial

as it was with NaD1, and whether the same mutations were involved in broad spectrum resistance to cationic peptides or whether the mutations were AFP specific reflecting a different mechanism of action. Furthermore, the BPTI and NaD1-resistant strains were both assessed using synergy assays to determine whether fungal inhibition could be rescued with the addition of either NaD1 or BPTI in low doses.

Aim of study 3 (Chapter 4) – Sequence wide characterisation of NaD1 and LL-37-resistance in *C. albicans*

Studies 1 and 2 were conducted with the non-pathogenic organism *S. cerevisiae*. A clinically relevant human pathogen *C. albicans* was chosen for the third study. The aim of this study was to examine development of resistance to the plant defensin NaD1 and the human cathelicidin LL-37 and to compare this with resistance development to the antifungal itraconazole which is used in the clinic. It is important to note that in order to avoid developing a virulent strain of *C. albicans* that was highly resistant to antifungal molecules, we intentionally used a disabled strain (auxotrophic for uridine, histidine and arginine) named BWP17 that would not be viable in a host, and this strain was maintained in isolated PC2 conditions. These precautions were made to ensure the experiments were as safe as possible. *C. albicans* was serially passaged in the presence of each of NaD1, LL-37, itraconazole and a no drug control. To obtain more genetic data, eleven *C. albicans* cultures were set up and passaged for each treatment group, making a total of 44 independent isolates (11 NaD1-resistant, 11 itraconazole-resistant, 11 LL-37 resistant and 11 no treatment control). Whole genome sequencing was completed using the Illumina NextSeq method, and the changes to the genome were compared to the parent wild-type strain. The rate of development and genetic determinants of resistance to NaD1, LL-37 and itraconazole were compared to one another.

Chapter Two

2. STUDY 1 – Mechanism of resistance to NaD1 in *S. cerevisiae*

The findings of this chapter have been published in a peer-reviewed journal, Frontiers in Microbiology, November 2018.

McColl AI, Bleackley MR, Anderson MA, Lowe RGT (2018) Resistance to the Plant Defensin NaD1 Features Modifications to the Cell Wall and Osmo-Regulation Pathways of Yeast. Frontiers in Microbiology 9: 1648

https://www.frontiersin.org/articles/10.3389/fmicb.2018.01648/full

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Statement of Contribution

Amanda McColl has made the following contributions:

- Design and experimental completion of data collection,
- Analysis and interpretation of all data,
- Preparation of methods and figures for the manuscript.
- Drafted and provided all revisions of manuscript prior to submission.

Other authors have made the following contributions:

- Bleackley MR, Anderson MA, and Lowe RGT contributed to the conception and design of the experiments, data analysis and manuscript revisions.

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Resistance to the Plant Defensin NaD1 Features Modifications to the Cell Wall and Osmo-Regulation Pathways of Yeast

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Over the last few decades, the emergence of resistance to commonly used antifungal molecules has become a major barrier to effective treatment of recurrent life-threatening fungal diseases. Resistance combined with the increased incidence of fungal diseases has created the need for new antifungals, such as the plant defensin NaD1, with different mechanisms of action to broaden treatment options. Antimicrobial peptides produced in plants and animals are promising new molecules in the arsenal of antifungal agents because they have different mechanisms of action to current antifungals and are often targeted specifically to fungal pathogens (van der Weerden et al., 2013). A key step in the development of novel antifungals is an understanding of the potential for the fungus to develop resistance. Here, we have used the prototypic plant defensin NaD1 in serial passages with the model fungus *Saccharomyces cerevisiae* to examine the evolution of resistance to plant antifungal peptides. The yeast strains did develop tolerance to NaD1, but it occurred more slowly than to the clinically used antifungal caspofungin. Sequencing the genomes of the strains with increased tolerance failed to identify any 'hotspot' mutations associated with increased tolerance to NaD1 and led to the identification of 12 genes that are involved in resistance. Characterization of the strains with increased tolerance to NaD1 also revealed changes in tolerance to abiotic stressors. Resistance developed slowly via an accumulation of single nucleotide mutations and had a fitness penalty associated with it. One of the genes identified *FPS1*, revealed that there is a common mechanism of resistance to NaD1 that involves the osmotic stress response pathway. These data indicate that it is more difficult to generate resistance to antimicrobial peptides such as NaD1 compared to small molecule antifungals.

Keywords: antifungal, defensin, genome, yeast, resistance, NaD1, cell wall, stress

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INTRODUCTION

Pathogenic fungi have become a serious threat to both agriculture and human health (Murray and Brennan, 2009). In human health, fungal pathogens are detrimental to immunocompromised individuals, such as individuals with HIV, transplant recipients and cancer patients receiving chemotherapy (Ortega et al., 2010). Indeed, some invasive fungal diseases can become

life-threatening in the immunocompromised and mortality can reach up to 80% (Lass-Flörl, 2009). There are very few therapeutic options for systemic fungal infections, and some fungicides are known to be dangerous to human health due to severe side effects such as toxicity (Ortega et al., 2010). Fungicide resistance occurs when a fungal pathogen becomes less susceptible to an antifungal agent. Resistance is broadly characterized by the mechanism by which it occurs. These mechanisms include; alteration of the target site in a protein, detoxification of the fungicide, overexpression of the target site, and the use of efflux pumps to expel the fungicide (Leroux et al., 2002). The increased use of the small molecule antifungal drugs that are currently in the clinic as well as related molecules used in agriculture has led to reports of fungal pathogens resistant to almost all common antifungals (Verweij et al., 2009). There is a need for new antifungal agents to battle the phenomenon of fungal resistance; antifungal proteins are one attractive option for development (Sanglard et al., 1996; van der Weerden et al., 2013).

A wide variety of organisms produce antifungal peptides as part of their innate immunity arsenal (van der Weerden et al., 2013). They are highly represented in plants where defensins are the largest family. Plant defensins are small proteins of 45 to 54 amino acids that are ubiquitous in the seeds, leaves and flowers of all plants examined (Berkut et al., 2014). They are usually produced constitutively as a defense against pathogens, particularly in reproductive tissues and seeds (Lay et al., 2003). They are also expressed in response to infections and environmental stress (Lay et al., 2003; Sagaram et al., 2011). There are thousands of plant defensins in public sequence databases. They share a common structure, but are highly variable in sequence and, not surprisingly, they often have different mechanisms of action (Parisi et al., 2018). The mechanism of action of only a handful of defensins has been elucidated. They often have multistep mechanisms that affect more than one target in the fungus (Parisi et al., 2018). Hence, it is expected that resistance to defensins is likely to develop more slowly than resistance to smaller antifungal molecules that interact with a single site, composed of a few amino acids, on a single protein target. NaD1 is a potent antifungal defensin that accumulates in the flowers of the ornamental tobacco plant *Nicotiana alata*, where it functions to protect the reproductive organs from damage by fungal pathogens (Lay et al., 2003). NaD1 has a well-characterized structure, and several features of its mechanism of action have been well described but not completely elucidated (Lay et al., 2012). NaD1 has at least a three-step mechanism of action that involves: interaction with the fungal cell wall (van der Weerden et al., 2008), movement across the plasma membrane, induction of oxidative stress, and interaction with phosphatidylinositol 4,5 bisphosphate. These processes lead to damage of the inner leaflet of the cell membrane and cell death within 10 min of exposure to NaD1 (van der Weerden et al., 2010; Hayes et al., 2014; Poon et al., 2014; Payne et al., 2016).

In this study, yeast strains were generated that have increased tolerance to NaD1, and genetic mutations linked to the decreased response to NaD1 were identified. Phenotypic characterization of resistant lines revealed slower growth rates, as well as cell wall changes reflected as sensitivity to the anionic detergent SDS and the chitin binding molecule calcofluor white (CFW). That is, there was a fitness trade-off associated with NaD1-resistance. Mutations across twelve genes correlated with NaD1 resistance. These genes were associated with diverse aspects of cellular processes suggesting that NaD1 acts upon multiple cellular targets. Affected locations or processes included the cell wall, transporters and signaling pathways. Mutations in the gene *FPS1* indicate glycerol accumulation may modulate NaD1 antifungal activity. Resistance to NaD1 occurred more slowly than resistance to caspofungin in similar experiments.

MATERIALS AND METHODS

Fungal Strains

The *S. cerevisiae* strain BY4741 (*MATαhis3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) was purchased from Thermo Scientific. Single deletion strains were retrieved from the haploid non-essential deletion collection (Thermo Scientific) (Winzeler et al., 1999). *S. cerevisiae* was routinely cultured on YPD-Agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) medium at 30°C.

Antifungal Molecules

NaD1 and NaD2 were purified from *Nicotiana alata* flowers as described in Lay et al. (2003) and Dracatos et al. (2014). HXP4 and DmAMP1 were expressed in *Pichia pastoris* and purified as described previously (Hayes et al., 2013; Bleackley et al., 2016). CP29 was purchased from GL Biochem (China), BPTI (synonym Aprotinin) was purchased from Astral Scientific (Australia), caspofungin was purchased from Sigma (Australia).

Culturing in the Presence of Antifungal Molecules to Develop Resistance

S. cerevisiae BY4741 was grown overnight at 30°C with agitation in 5 mL of YPD. The overnight culture was then diluted to an OD 600 nm of 0.01 in 50% strength PDB medium (½ PDB) before addition of antifungal molecules. Cultures were initially grown with the antifungal molecules at 0.5x the minimum inhibitory concentration (MIC) or 1x MIC alongside a negative control lacking antifungals. Three independent lines for the test and controls were grown at the same time. The cultures were incubated overnight at 30°C with agitation. The cultures that exhibited growth at the highest concentration of the antifungal molecules were sub-cultured with medium containing a higher concentration of the antifungal molecule. Sub-culturing was stopped once growth occurred at 32 times the original MIC.

Single-Colony Isolation of Resistant Strains

Cultures that were more tolerant to the antifungal molecule were streaked out for single colonies on non-selective YPD agar. Three colonies were picked from each line, and their resistance was re-tested. The colony with the highest resistance to the antifungal was retained for further experimentation. The MIC of pure strains isolated from each culture was broadly equivalent (Supplementary Figure 1).

Antifungal Assay

Antifungal assays were performed as described in Hayes et al. (2013). Briefly, cultures were grown overnight (30°C, 250 rpm) in 5 mL YPD and diluted to an OD600 of 0.01 in ½ PDB. Antifungal molecules were prepared at 10x the assay concentration, and 10 μL was mixed with 90 μL of diluted yeast culture before incubation for 24 h at 30°C. The final OD600 was measured using a SpectraMAX M5e plate reader (Molecular Devices).

Cell Growth Assay

S. cerevisiae BY4741 cultures were grown overnight (30°C, 250 rpm) in 5 mL of YPD and diluted to an OD_{600} of 0.5 in 1 mL YPD and ½ PDB. Each culture (100 μL) was incubated in a SpectraMAX M5e plate reader (Molecular Devices) at 30°C in a 96-well microtiter plate format. Optical density at 600 nm was recorded every 30 min over the 48 h culture period.

Cell Size and Area Measurement

S. cerevisiae BY4741 cultures were grown overnight (30°C, 250 rpm) in 5 mL of YPD and were imaged using an Olympus IX81 brightfield microscope (LIMS Bioimaging Facility). Cell dimensions were measured from images using FIJI software (Schindelin et al., 2012). A minimum of 30 cells was measured for each sample.

Stress Assay With Hydrogen Peroxide, Calcofluor White, NaCl, and SDS

YPD agar medium (25 mL) was amended to a final concentration of hydrogen peroxide (0.625 mM, 1.25 mM, 2.5 mM, 5 mM), CFW (1 μg/mL, 2.5 μg/mL, 5 μg/mL, 10 μg/mL), NaCl (100 mM, 200 mM, 300 mM), or SDS (12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL) just before each plate was poured. Yeast cultures were grown overnight in 5 mL of YPD before dilution to an OD 600 nm of 0.1. A fivefold dilution series of each culture was spotted onto the plate (4 μL per spot) and incubated overnight at 30°C before being photographed.

Stress Assay With Ultraviolet Light

S. cerevisiae cultures were grown overnight in 5 mL YPD and diluted to an OD 600 nm of 0.1 in 1 mL MilliQ-purified water. A fivefold dilution series of each strain (4 μL per spot) was added to the YPD agar plate and allowed to dry, before exposure to UV light (Phillips, 30 W bulb at 50 cm) for 1.2, 2.4, 5.2, or 10.4 min.

Stress Assay With Heat

S. cerevisiae cells were grown overnight in 5 mL YPD and diluted to an OD 600 nm of 0.1 in 1 mL MilliQ-purified water. Diluted cultures (100 μL) were heated (30°C, 37°C, 41°C, or 46°C) for 30 min. Survival was assessed after heat treatment using a spot assay on YPD agar.

DNA Extraction From Wild-Type and Resistant Strains of *S. cerevisiae*

Genomic DNA was extracted using the Qiagen DNeasy® plant miniprep kit. Three individual lines of NaD1-resistant strains and three lines of the no-treatment controls were sequenced. Sequencing was completed at the La Trobe Genomics Platform, using Illumina MiSeq V3 chemistry. One run was performed for all six genomes, generating 25 million 300 bp paired-end reads. The pre-processing and variant discovery steps were performed as described by the GATK best practices and are summarized in McKenna et al. (2010).

Genomic Analysis of Resistant Strains of *S. cerevisiae* Sequence Pre-processing

Picard tools (v.2.4.1) fastqtosam was used to convert raw sequence files into Sam format and to add read group information. Any Illumina adapters were identified and marked using Picard (v.2.4.1) markilluminaadapters. BWA-mem (v.0.7.12) was used to align reads to the reference *S. cerevisiae* (R64-1-1.23) genome (Engel et al., 2014). Alignment files were merged, and duplicate reads were marked using Picard (v.2.4.1) mergebamalignment and markduplicates. Local alignments were optimized, and sequence quality scores were recalibrated using GATK (v.3.6) realignertargetcreater and baserecalibrator.

Variant Discovery

GATK (v.3.6) Haplotypecaller was used to find genome variations that were either SNVs (single-nucleotide variants) or INDELs (insertion/deletion) simultaneously, also using known variants from dbSNP (Sherry et al., 2001). The samples were merged using GATK (v.3.6) combinegvcf, and then GenotypeGVCFs was used to rescore and genotype the combined gVCFs. GATK (v.3.6) VariantFiltration and VariantRecalibrator were used to extract SNVs and indels from the combined call set based on the default quality parameters, the SNVs and indels were then labeled as passed or filtered.

Variant Refinement

The high-quality variants identified during the variant discovery process were annotated using SnpEff (v.2.4) (Cingolani et al., 2012). SnpEff was used to determine whether each mutation was predicted to alter an encoded protein sequence (**Table 3**). Variant effect predictor (VEP) marked any codon changes as either tolerant or deleterious (McLaren et al., 2016). SnpSift (v.2.4) was used to was used to identify SNVs or indels that were present in NaD1 resistant replicates and not in the Control strains (**Table 3**). The variants selected during refinement were inspected manually using IGV (v.2.3.77) to rule out unexpected processing artifacts (Robinson et al., 2011).

Sanger Sequencing of the FKS1 Gene of Caspofungin-Resistant Mutants

The *FKS1* gene from three individual lines of caspofungin-resistant strains and a no treatment control was amplified by PCR using primers TCAAGGAAGGCAAGAAAAGCTA and GAGGCCGATACTGGTGAAAA and NEB Q5 proofreading polymerase according to the manufacturer's directions. Initial denaturation was at 95°C for 2 min, followed by 30 cycles of: 95°C 30 s, 55°C 30 s, 72°C 2 min, and a final extension at 72°C 2 min. Sanger sequencing of the FKS1 amplicon using primers "TCAAGGAAGGCAAGAAAAGCTA"

and "CTGCATTTGCCCCTCTACAT" was completed by the Australian Genome Research Facility (AGRF). Sequence data were analyzed using Geneious software.

RESULTS

Evolution of Resistance to NaD1

Yeast strains with increased tolerance to NaD1 or caspofungin were developed by continuous culture of *S. cerevisiae* in sub-lethal concentrations of each antifungal molecule. Each time the MIC increased, the dose of antifungal was doubled. The starting concentration of NaD1 was 1 μM; it took 20 rounds of sub-culturing for NaD1-R A, 21 rounds for NaD1-R C and 22 rounds for NaD1-R B to achieve growth in 32 μM NaD1 (**Figure 1A**). In contrast, it took only 15 rounds of sub-culture to achieve growth in caspofungin at concentrations 32-fold higher than the initial MIC 10 nM (**Figure 1A**).

Three genetically pure strains of each of the NaD1-resistant and caspofungin-resistant lines were isolated, and their resistance phenotype was confirmed using a standard antifungal growth assay. The colony with the most resistance for each line was used for all further experimentation. The NaD1-resistant isolates were 10-fold more resistant to NaD1 than the no-treatment control lines that had been passaged at the same time, with an MIC of 40 μM compared to an MIC of 4 μM (**Figure 1B** and **Table 1**). The caspofungin-resistant isolates were 25-fold more resistant to caspofungin with an MIC of 500 nM compared to the no treatment control which had an MIC of 20 nM (**Figure 1C** and **Table 1**). In most fungal species, resistance to caspofungin occurs via mutations to the FKS1 gene within a "hot spot" zone affecting residues Phe639 to Pro647 (Katiyar and Edlind, 2009). Sequencing of the entire FKS1 gene of our caspofungin-resistant strains revealed that all three strains contained a single point mutation (F639V) confirming resistance was derived by the most commonly observed mechanism (Supplementary Figure 2).

Resistance to NaD1 Confers Resistance to Some but Not All Antifungal Peptides

The NaD1-resistant lines were tested against a range of antimicrobial molecules to determine if the observed resistance was broad-spectrum or specific to NaD1. The caspofungin-resistant strains were as sensitive to NaD1 as the wild type (**Figure 2A**), and similarly, the NaD1-resistant strains were as sensitive to caspofungin as the wild-type strain (**Figure 2B**).

NaD1-resistant strains were tested against some other plant defensins; NaD2 from *Nicotiana alata,* DmAmp1 from *Dahlia mercki* and the chimeric defensin HXP4. The NaD1-resistant strains were not resistant to NaD2 with an MIC of 20 μM, which was the same as the wild-type (**Figure 3A**). However, they were more resistant to DmAMP1 with an MIC of 20 μM

TABLE 1 | The MIC of NaD1- and caspofungin-resistant lines of *S. cerevisiae*.

Strain	NaD1 MIC (μM)	Caspofungin MIC (nM)
Wild-type	4	20
NaD1-R A	40	25
NaD1-R B	40	25
NaD1-R C	40	25
Caspofungin-R A	4	500
Caspofungin-R B	4	500
Caspofungin-R C	4	500

The minimum inhibitory concentration (MIC) of NaD1 and caspofungin are summarized for NaD1 resistant isolates, caspofungin resistant isolates and the parental wild type line of S. cerevisiae.

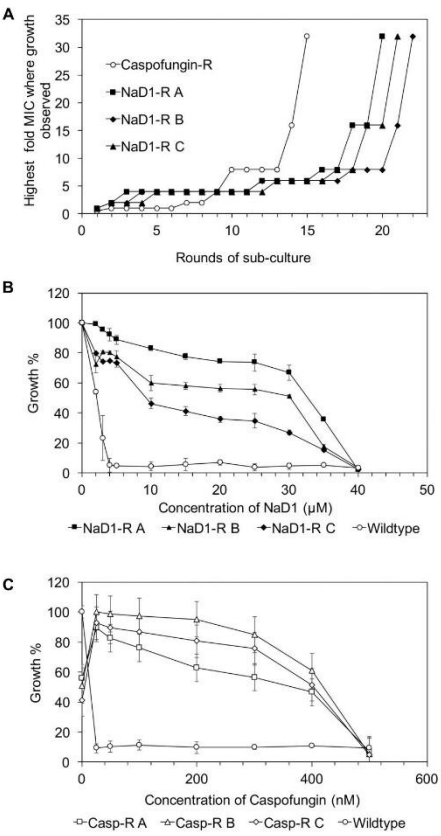

FIGURE 1 | Mild resistance to NaD1 or caspofungin can be evolved *in vitro*. Summary of the development of resistance during sub-culturing in the presence of Caspofungin or NaD1 **(A)**. Three independent strains of NaD1-resistant yeast are shown, along with a representative example of the caspofungin-resistant lines. The antifungal activity of NaD1 **(B)** and caspofungin **(C)** against NaD1-resistant and caspofungin-resistant lines, respectively, is graphed relative to the highest measured OD for each strain. Error bars represent ±standard error of the mean ($n = 3$).

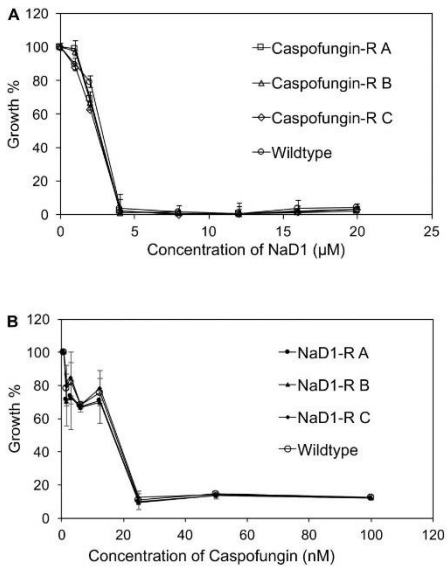

FIGURE 2 | There is no cross activity between caspofungin-resistance and NaD1-resistance. Cross-resistance of caspofungin-resistant **(A)** and NaD1-resistant lines **(B)**, on NaD1 and caspofungin was determined, respectively. **(A)** Average growth percentage is shown, relative to the highest measured absorbance for each strain. Error bars represent ±standard error of the mean (n = 3).

compared to 1.25 μM for the wild type (**Figure 3B**) and to HXP4 with an MIC of 20 μM compared to 10 μM for the wild type (**Figure 3C**). Similarly, NaD1-resistant strains were not resistant to two unrelated cationic antifungal proteins, bovine pancreatic trypsin inhibitor (BPTI) and the insect cecropin CP29. The MIC for BPTI against both the wild type and the NaD1-resistant cultures was 10 μM (**Figure 3D**). When incubated with CP29 the NaD1-resistant strains grew slightly better than wild-type at concentrations below the MIC, but the MIC was the same for all strains tested (**Figure 3E**).

There Is a Fitness Penalty Associated With NaD1 Resistance

The relative fitness of the NaD1-resistant strains was assessed by comparing growth rate over 48 h in two different growth media. NaD1-resistant strains B and C grew slower in YPD for the first 18 h but reached the same culture density as wild-type after 23 h. NaD1-resistant strain A grew marginally slower than the wild type (**Figure 4A**). The growth in ½ PDB was less varied, with only NaD1-resistant strain C growing significantly more slowly than wild-type (**Figure 4B**). The cellular dimensions of NaD1-resistant strains were smaller than the wild type in both length and area (**Figure 5**).

NaD1-Resistant Strains Are Sensitive to Cell Wall Stressors and Are Resistant to Osmotic Stress

Potential alterations to the cell wall and membrane were examined by exposing the NaD1 resistant strains to SDS and CFW. SDS is an anionic detergent that causes cell wall stress, and membrane permeabilization and CFW is a cell wall stressor that binds to chitin. This revealed a significant growth defect of the NaD1-resistant strains in the presence of SDS or CFW (**Figures 6B,C**). Sensitivity of the NaD1-resistant strains was observed at 12.5 μg/mL SDS (Supplementary Figure 3) and at 1 μg/mL CFW (Supplementary Figure 4).

In *Candida albicans,* the HOG1 osmotic stress response pathway is involved in tolerance to NaD1 (Hayes et al., 2013). It was, therefore, important to assess whether the *S. cerevisiae* NaD1-resistant strains had an altered osmotic stress response. NaD1-resistant strains grew better than wild-type at 200 mM NaCl (**Figure 6D** and Supplementary Figure 5). This supported the hypothesis that NaD1-resistance correlates with increased osmotic stress tolerance.

NaD1-Resistant Strains Are Not Resistant to Hydrogen Peroxide, UV Light, or Heat

NaD1 induces ROS production in *Candida albicans,* which is a contributing factor to cell death. However, at low NaD1 levels, *C. albicans* cells cope by activation of the HOG1 pathway and enhancing transcription of genes that protect against oxidative stress (Hayes et al., 2013). Thus, the NaD1-resistant strains were tested for sensitivity to hydrogen peroxide generated oxidative stress. The NaD1-resistant strains grew the same as the wild-type strain in the presence of a range of hydrogen peroxide concentrations (**Figure 7** and Supplementary Figure 6).

NaD1-resistant strains were also tested for resilience to ultraviolet light (UV) that causes DNA damage, as well as their resilience to heat shock.

There was no observable difference in the growth of the NaD1-resistant strains and the wild-type cells after UV light or heat treatment (Supplementary Figure 7).

Genetic Characterization of NaD1 Resistance

The genomes of each of the NaD1-resistant and non-selected control lines were sequenced to identify mutations exclusively found in NaD1 resistant lines. Mutated genes identified in the resistant isolates were compared to the genes in the non-selected wild type (**Table 2**), along with the predicted amino acid changes. There were eight mutated genes found in NaD1-resistant strain A, five mutated genes in strain B, and seven genes mutated in strain C. There were three genes mutated in all three strains (FPS1, TOM1, and RSP5) and two genes were mutated in both NaD1-resistant B and C strains (PHO84 and CWP2) (**Table 2**). The results obtained from the VEP (McKenna et al., 2010), which determines the consequence of DNA variants on

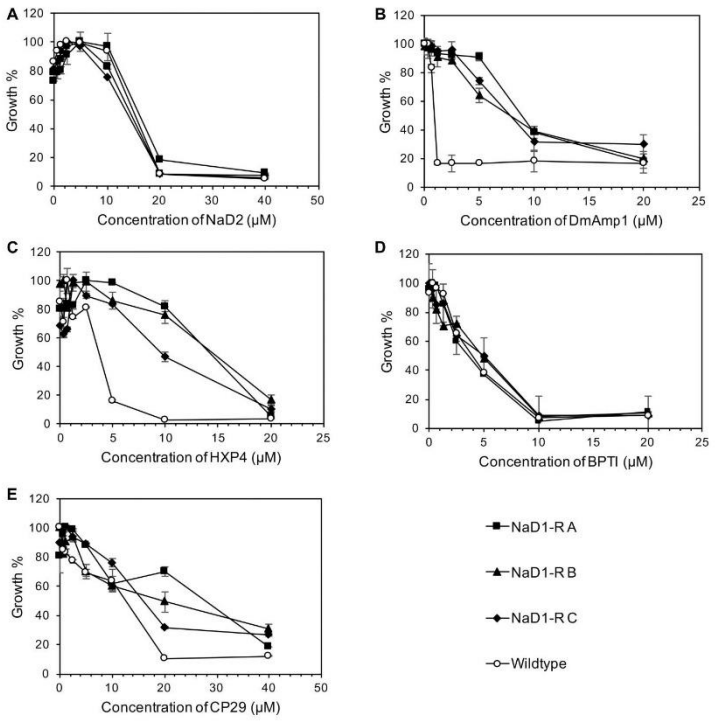

FIGURE 3 | Resistance to NaD1 is not broad spectrum. Growth inhibition of NaD1-resistant strains by a selection of antimicrobial peptides of different origin and mechanisms of action. The peptides examined were: the plant defensins NaD2 **(A)**, DmAMP1 **(B)**, and HXP4 **(C)**, BPTI, a trypsin inhibitor from *Bos Taurus* **(D)**, and the insect-derived cercropin variant CP29 **(E)**. NaD1-resistant strains were also resistant to the plant defensins HXP4 and DmAMP1 but not to NaD2 or the antifungal BPTI. The three NaD1-resistant strains were slightly more resistant to CP29. Average growth percentage is shown, relative to the highest measured absorbance for each strain. Error bars represent ±standard error of the mean (n = 3).

TABLE 2 | Summary of variants that disrupted protein coding regions in NaD1-R strains.

Gene Name	Amino acid change	NaD1-R strains containing variant	Type	Inference
BUD4	p.Asn415Asp	A	SNV	Tolerated missense variant
CWP2	p.Leu92del	B, C	INDEL	Disruptive in-frame deletion
FPS1	p.Phe555fs	A, B, C	INDEL	Disruptive frame shift
MRPS16	p.Pro45Gln	C	SNV	Deleterious missense variant
PHO84	p.Ser183Phep.Val202Ile	BC	SNV	Deleterious missense variant
PMR1	p.Val170Ile	A	SNV	Deleterious missense variant
RAS2	p.Asp112Gly	C	SNV	Deleterious missense variant
RET2	p.Gln12His	A	SNV	Missense variant
RSP5	p.Gly689Cys	A, B, C	SNV	Missense variant
SIR3	p.Glu451*STOP	A	SNV	Disruptive premature stop
SKY1	p.Trp173Leu	A	SNV	Deleterious missense variant
TOM1	p.Ala2381Gly	A, B, C	SNV	Deleterious missense variant

The observed changes to protein coding regions of NaD1-R strains are shown along with the inferred impact on the encoded protein. SNV, single nucleotide variant; INDEL, insertion or deletion. These genes may be viewed on the Saccharomyces Genome Database www.yeastgenome.org (Cherry et al., 2012; Engel et al., 2014).

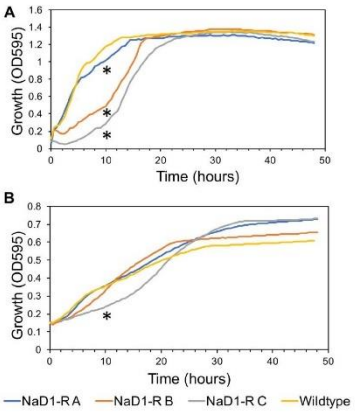

FIGURE 4 | Growth rate of NaD1-resistant strains in YPD and ½ PDB medium compared to wild-type *S. cerevisiae* BY4741. Growth rate in the absence of antifungals was determined for NaD1-resistant strains in YPD medium **(A)** and ½ PDB medium **(B)**. An asterisk (*) denotes a statistically significant difference in growth rate compared to wild-type, by a two-tailed homoscedastic *T*-test ($P < 0.05$, $n = 6$).

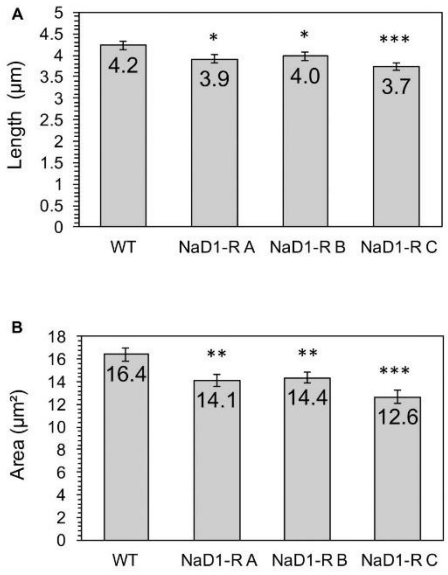

FIGURE 5 | NaD1-resistant strains are smaller in length and area compared to wild type. Cross-sectional length **(A)** and area **(B)** was determined for NaD1-resistant strains and wild-type. Average values are shown ±1 standard error of the mean ($n = 30$). P value determined by two-tailed homoscedastic *T*-test indicated by $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

protein sequence, are listed in **Table 2**. A description of predicted functions for the affected genes is listed in **Table 3**.

Determining the Relative Contributions of Loss of Function Mutations to NaD1 Tolerance

It was considered likely that most of the observed mutations would have resulted in a loss-of-function phenotype for the affected genes. To test this hypothesis, strains with single-gene knockouts for mutated genes were retrieved from the yeast deletion set (Winzeler et al., 1999) and antifungal growth assays were performed to assess whether gene deletion replicated the NaD1-resistant phenotype. The knock-out strains were only selected from non-essential genes. The antifungal assay revealed that none of the single gene knockout mutants (*fps1*Δ, *cwp2*Δ, *mrps16*Δ, *pmr1*Δ, *pho84*Δ, and *sky1*Δ) were as resistant to NaD1 as the three NaD1 resistant strains. Instead, each of the knockout mutations conferred partial resistance to NaD1. The highest level of resistance from a single knock-out occurred with *fps1*Δ, which had an MIC of 9 µM. Compared to the original NaD1-R mutants that had MICs of 40 µM, *cwp2*Δ, *pmr1*Δ, *mrps16*Δ, and *pho84*Δ contributed a smaller amount of resistance with an MIC of 6–7.5 µM while *sky1*Δ had the same MIC as the wild-type and control strains (**Table 4**).

TABLE 3 | Summary of gene functions impacted by NaD1-resistance.

Gene	Functional group	Description
BUD4	Cell wall	Protein involved in bud-site selection.
CWP2	Cell wall	Cell wall mannoprotein.
FPS1	Transport	Aquaglyceroporin, plasma membrane channel.
PHO84	Transport	Inorganic phosphate transporter.
PMR1	Transport	Calcium and manganese transport to the Golgi.
SKY1	Signaling	Regulating cation homeostasis.
RAS2	Signaling	Regulates sporulation and filamentous growth.
TOM1	Ubiquitin ligase	E3 ubiquitin ligase (Hect-domain class)
RSP5	Ubiquitin ligase	E3 ubiquitin ligase (NEDD4 family)
SIR3	Chromatin binding	Chromatin remodeling.
RET2	Unknown	Retrograde transport between Golgi and ER.
MRPS16	Ribosome structure	Mitochondrial ribosomal protein.

The genes that have mutations linked to NaD1 resistance and the description of their role in S. cerevisiae are shown. Gene ontology functional analysis revealed that some of these genes can be grouped by location or function, including: cell wall, transporter, signaling or ubiquitin ligase categories. These genes may be viewed on the Saccharomyces Genome Database www.yeastgenome.org (Cherry et al., 2012; Engel et al., 2014).

DISCUSSION

Resistance to NaD1 Is Slow to Develop

Antimicrobial peptides represent a promising next generation of therapeutics to combat drug-resistant fungi and bacteria (Wang et al., 2016). Peptides provide benefits as pharmaceuticals over small molecule drugs because they bind with high specificity to

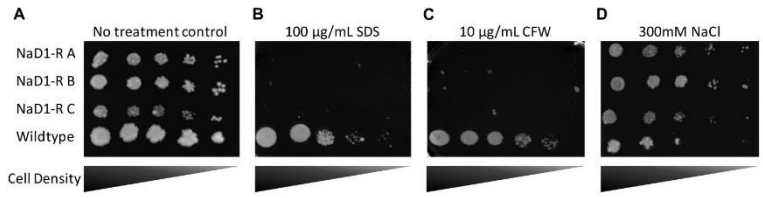

FIGURE 6 | NaD1-resistant strains are sensitive to SDS and calcofluor white (CFW), but are resistant to NaCl. NaD1-resistant and wild-type *S. cerevisiae* BY4741 cells were diluted and spotted onto YPD agar alone **(A)** or YPD supplemented with SDS **(B)**, CFW **(C)**, or NaCl **(D)**. NaD1-resistant strains but not the wild type were inhibited by 100 µg/mL SDS or 10 µg/mL CFW compared to wild type. However, NaD1-resistant strains maintained growth at concentrations of NaCl that the wild type strains could not tolerate. Images are representative of three repeated experiments, all showing similar results.

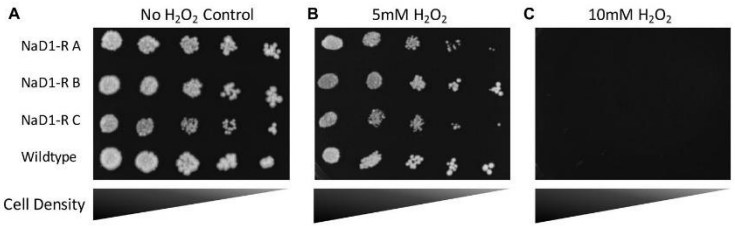

FIGURE 7 | NaD1-resistance does not alter sensitivity to oxidative stress. NaD1-resistant and wild-type *S. cerevisiae* BY4741 cells were diluted and spotted onto YPD agar containing 0 mM **(A)**, 5 mM **(B)**, or 10 mM **(C)** H_2O_2. There was no difference in H_2O_2 sensitivity between the NaD1-resistant strains compared to the wild type. Images are representative of three experiments, all showing similar results.

TABLE 4 | Comparison of NaD1 activity against single-gene deletion strains representing key resistance variants.

Strain of *S. cerevisiae*	NaD1 MIC (µM)	95% CI ±
Wild type	4.5	0.03
NaD1-resistant strain A	40	0.03
NaD1-resistant strain B	40	0.04
NaD1-resistant strain C	40	0.03
Control A	4.5	0.03
Control B	4.5	0.03
Control C	4.5	0.03
FPS1 knockout	9	0.05
CWP2 knockout	6	0.03
MRPS16 knockout	7.5	0.06
PMR1 knockout	6	0.03
PHO84 knockout	6	0.04
SKY1 knockout	4.5	0.02

The minimum inhibitory concentration (MIC) of NaD1 was determined for single gene deletion strains linked to NaD1 resistance is shown, alongside control lines and the NaD1-resistant strains. Average values and a 95% confidence interval (CI) were calculated from three independent experiments.

their targets and require a relatively large interaction interface, which results in fewer off-target side effects (Craik et al., 2013). Plant defensins are known to bind to lipids and polysaccharides (Kvansakul et al., 2016; Payne et al., 2016; Poon et al., 2014). As hypothesized in this report, resistance to the antifungal peptide NaD1 developed more slowly than resistance to the small molecule drug caspofungin (**Figure 1**). The MIC of NaD1-resistant strains was only 10-fold greater than wild type, which was less than the equivalent caspofungin-resistant strains (20-fold greater than wild type) and caspofungin resistance developed more rapidly than NaD1 resistance (**Figure 1**). Our observation is consistent with the reported benefits of peptide drugs, where their larger interaction surface requires more changes to the target before binding is disrupted.

Resistance to NaD1 Did Not Confer Broad-Spectrum Resistance to Other Antifungal Peptides

An example of broad-spectrum resistance to cationic AFPs has been reported for an *agp2*Δ mutant of *S. cerevisiae* whereby resistance was mediated by an accumulation of positive charges at the cell surface that repelled positively charged antifungal peptides (Bleackley et al., 2014b). Therefore, it was important to determine whether evolved NaD1-resistant strains were resistant to other cationic peptides. The NaD1-resistant strains were resistant to the plant defensins HXP4 and DmAMP1 (**Figures 3B,C**). HXP4 is a chimera of NaD1 and NaD2, with a similar mechanism of action of NaD1, and hence was expected to share cross-resistance with NaD1 (Bleackley et al., 2016). DmAmp1 a plant defensin from *Dahlia merckii*, has a different mechanism of action to NaD1 (Parisi et al., 2018) whereby it

binds to sphingolipids in the cell wall and plasma membrane of *S. cerevisiae* to exert antifungal activity (Thevissen et al., 2000). Although DmAMP1 and NaD1 have different mechanisms of action, they each stimulate the high-osmolarity glycerol (HOG) pathway in *C. albicans* (Hayes et al., 2013) and mutants in that pathway (*hog1* or *pbs2*) were more sensitive to NaD1 and DmAmp1. In *S. cerevisiae,* the alteration of the osmotic stress pathway could also affect the sensitivity to DmAmp1. The antifungals BPTI and NaD2 were still effective against the NaD1-resistant strains demonstrating the developed resistance was not broad spectrum (**Figures 3A,D**). BPTI inhibits *S. cerevisiae* growth by targeting a magnesium transporter and blocking the uptake of magnesium. Therefore, it was expected that the NaD1-resistant strains would still be sensitive to BPTI (Bleackley et al., 2014a). The mechanism of action of NaD2 is mostly unknown, but it is known to bind to phosphatidic acid to exert its antifungal activity, unlike NaD1 that binds to both PIP2 and PA (Bleackley et al., 2016; Payne et al., 2016). The cationic peptide CP29 was less effective at sub MIC concentrations, but there was no shift in MIC detected (**Figure 3E**). Taken together this means that the resistance to NaD1 did not occur through a broad-spectrum resistance mechanism against all cationic AFPs. Plant defensins act synergistically with the clinical antifungal caspofungin and boost overall antifungal activity (van der Weerden et al., 2014; Vriens et al., 2015, 2016). We found that NaD1 was still effective against strains resistant to caspofungin (**Figure 2A**). Plant defensins may provide a very robust therapy if delivered in combination with existing clinical antifungals.

Resistance to NaD1 Has a Fitness Penalty

NaD1-resistant strains were tested for physical differences with wild type cells, to establish whether there is a fitness penalty associated with NaD1-resistance. The cell growth assays (**Figure 4**) revealed that NaD1-resistant strains grew more slowly than the wild type strain in the rich medium, YPD. NaD1-resistant strain C grew the slowest in YPD medium, this may be due to the mutation in MRPS16, which is a mitochondrial ribosomal protein and RAS2, which regulates sporulation and filamentous growth. Knockout mutants of MRPS16 have been reported to have decreased vegetative and respiratory growth (Orij et al., 2012; Schlecht et al., 2014). A knockout of RAS2 has also been reported to have decreased fitness in YPD medium (Qian et al., 2012), supporting our observation that the NaD1-resistant strain C had the largest fitness defect. Interestingly, the growth of the NaD1-resistant strains was equivalent to wild type in ½ PDB, supporting the veracity of the antifungal assays that were all performed in this medium. Individual cells of the NaD1-resistant strains were smaller in length and cross-sectional area compared to wild-type cells grown in YPD (**Figure 5**). NaD1-resistant strains were consequently tested against a range of cell wall stressors to investigate whether adaptations to NaD1 resistance had altered the properties of the cell wall and membrane. SDS is a detergent with a negatively charged head group that is commonly used to test the susceptibility of yeast cells to membrane permeabilization and cell wall perturbation (Sirisattha et al., 2004; Gao et al., 2014). NaD1-resistant strains were more sensitive to SDS than the wild type strain (**Figure 6**). This sensitivity suggests that strains with enhanced tolerance to NaD1 have modifications their cell walls or plasma membranes, and may be more susceptible to alternative antifungal drugs. NaD1-resistant strains were also sensitive to CFW, which binds to cell wall chitin and leads to permeabilization (**Figure 6**). NaD1-resistant strains may contain more chitin in their cell wall and therefore increase the binding of CFW (Roncero et al., 1988). Furthermore, CFW relies on a functional Hog1 pathway for its antifungal activity, and thus hyperactive Hog1 signaling to protect against defensin activity may result in heightened sensitivity to CFW (García-Rodriguez et al., 2000). NaD1-resistant strains were also tested against more diverse environmental stresses, but we found no evidence for protection or sensitivity to oxidative stress (**Figure 7**), DNA damage or heat stress. We have established a link between NaD1 resistance and cell wall stress. The observed NaD1 resistance appears limited to the NaD1 mechanism of action, and it does not mitigate oxidative stress, DNA damage or heat shock.

Unlike Resistance to Azoles and Echinocandins, Resistance to NaD1 Occurs via Multiple Quantitative Mutations

Whole genome sequencing revealed multiple genes were linked to NaD1 resistance (**Table 2**). The functional diversity of these genes revealed that the mechanism of NaD1 is likely to involve more than a single protein target. This contrasts with resistance to caspofungin, which can be achieved by a single amino acid alteration in the targeted β-glucan synthase Fks1p (Katiyar and Edlind, 2009), or resistance to fluconazole with mutations to the Erg11p enzyme (Sionov et al., 2012). Our caspofungin-resistant mutants all followed this path to resistance, with each acquiring a single mutation at residue 639 of Fks1p. The NaD1-resistant strains acquired mutations related to the protection from osmotic stress, alteration of the cell wall, solute transport, signaling, and cation homeostasis (**Table 3**). In summary, unlike echinocandin and azole classes of fungicides, resistance to NaD1 did not feature a "hot-spot" for genomic mutations.

The NaD1-resistant strains had accumulated several mutations, and thus no single gene could be identified that was responsible for the resistance phenotype. The relative contribution of each observed mutation was assessed by comparing the level of NaD1 resistance in strains with knockouts of individual genes (**Table 4**). None of the single gene knockouts produced the level of NaD1 resistance obtained in the evolved strains. The *FPS1* knockout had the biggest effect and was mutated in all three of the evolved resistant strains. *PHO84*, *PMR1*, and *CWP2* deletion mutants contributed relatively smaller degrees of NaD1 resistance. In *PHO84* and *PMR1*, mutations in the NaD1-resistant strains were single nucleotide changes with conservative effects; it may be that protein function was only mildly affected. Combinations of mutations were not assessed as we felt that an exhaustive account of these variants was not supported

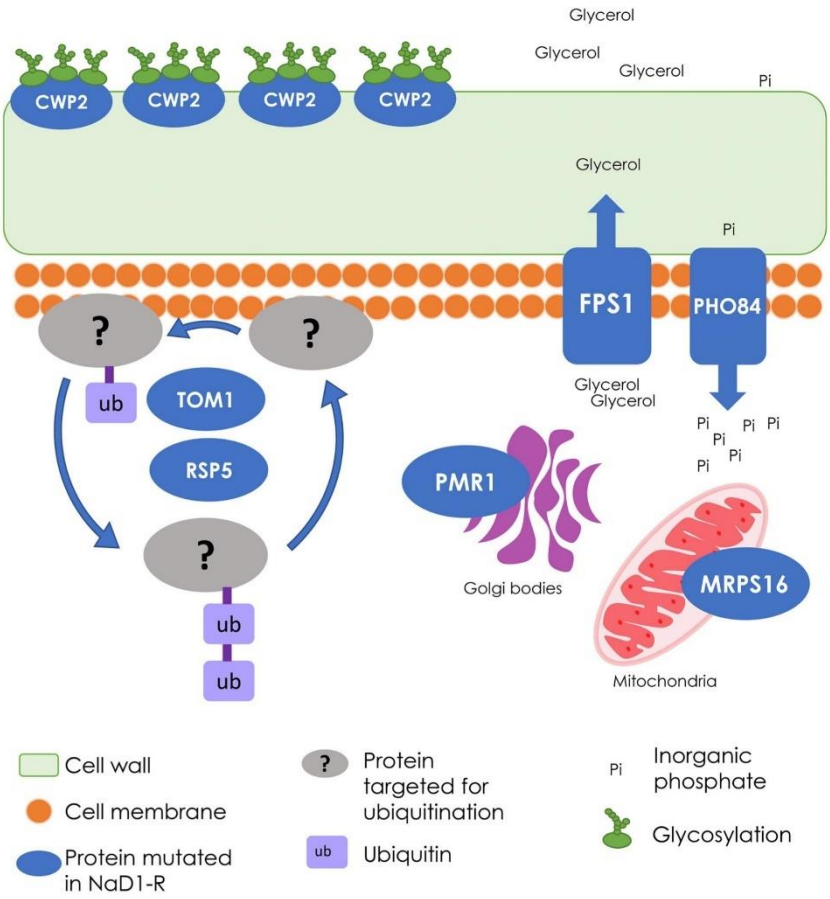

FIGURE 8 | Summary of the wild-type cellular processes that were modified in NaD1-resistant strains. The cellular processes involved in NaD1 resistance included the cell wall, transport, and signaling functions. FPS1 is a glycerol transporter that protects the cell from osmotic shock by exporting or preventing export of glycerol; the FPS1 gene deletion was the most resistant single-gene knockout tested. Other gene deletions that contributed to NaD1 resistance were: CWP2, which is a cell wall mannoprotein, PHO84, which is a phosphate and manganese ion transporter, PMR1, which transports calcium and manganese to the Golgi bodies, and MRPS16, which is a mitochondrial ribosomal protein. TOM1 and RSP5 are essential ubiquitin ligases that were mutated in all NaD1-resistant strains. Ubiquitin ligases target proteins for degradation and regulate a range of processes, including multivesicular body sorting. Overall, these genes regulate a range of cellular processes with a common theme of cell wall and osmo-regulation.

as we did not have enough individual strains to support which combination was evolutionarily more successful. Future work will focus on increasing the number of individual resistant lines studied. This should provide quantitative data on the relative benefit of different combinations of variants.

There were SNV's found in the TOM1 and RSP5 genes of all three NaD1-resistant strains. The SNV's are unlikely to lead to a complete loss of function and instead are likely to represent a partial loss or gain of function. Both TOM1 and RSP5 are E3 ubiquitin ligases, a class of protein that tags protein substrates for destruction. Ubiquitin ligases regulate diverse functions including cell trafficking, DNA repair, and signaling. TOM1 regulates mRNA export from the nucleus and targets excess histones for degradation (Saleh et al., 1998; Singh et al., 2009). RSP5 is an essential gene that regulates a variety of processes including mitochondrion organization and sorting of multivesicular bodies (Katzmann et al., 2004; McNatt

et al., 2007; Kaliszewski and Zoladek, 2008). In *C. albicans*, NaD1 is known to cross the plasma membrane via endocytosis. It is possible that a restriction of multivesicular transport could also restrict NaD1 movement inside the target cell. It has also been reported that decreased function of RSP5 can increase the susceptibility to cell wall stressors such as calcofluor, as was seen for NaD1-resistant lines in our stress assays (**Figure 6**). The identification of genomic variants in essential genes highlights an advantage of natural selection and genome sequencing as a method to identify mechanisms of resistance mechanisms.

Resistance to NaD1 Has a Common Theme of the Osmotic Stress Response

In our study, *FPS1* was mutated in all three of the evolved NaD1-resistant strains. *FPS1* encodes an aquaglyceroporin plasma membrane channel with a role in the efflux of glycerol and xylitol (Luyten et al., 1995). This efflux pump maintains osmotic balance by moderating the passive diffusion of glycerol (Toh et al., 2001). NaD1-resistant strains all contained a frameshift (Phe555fs) that prevents translation of 115 amino acids from the C-terminal regulatory domain of the FPS1 protein (Hedfalk et al., 2004). This could result in substantial modification to its function and cellular osmotic balance because this 115-amino acid region contains seven phosphorylation sites and two ubiquitinylated lysine sites that regulate the function of the channel. It is unclear if loss of this c-terminal region would cause protein instability and a total loss of function, or if it would produce an unregulated glycerol channel. The phenotype of the FPS1 knockout had significant resistance to NaD1, suggesting loss of function is the most likely result of the frameshift mutation. Fps1p is regulated by the HOG pathway in *S. cerevisiae*. In wild-type cells the Fps1p-mediated efflux of glycerol decreases when the cell is under hyper-osmotic (high salt) stress which in turn increases the internal accumulation of glycerol (Hedfalk et al., 2004). In theory, the *FPS1* deletion mutants will be resistant to hyper-osmotic shock as they are always accumulating intracellular glycerol (Toh et al., 2001). This resistance to hyper-osmotic stress was confirmed in the NaCl spot assays where we observed increased growth of NaD1-resistant strains under high salt conditions compared to wild-type cells (**Figure 6D**). We hypothesize that loss of FPS1 activity would prevent the release of excess turgor pressure via glycerol efflux, and result in excess pressure on the cell wall and susceptibility to cell wall stress. This is supported by previous reports that show that a combination of a FPS1 deletion with cell wall weakening mutations in *S. cerevisiae* results in cell lysis and lethality (Tamás et al., 1999). In work by García-Rodriguez et al. (2000), the ability of CFW to inhibit *S. cerevisiae* was dependent on a functional HOG pathway (**Figure 7C**). The work of Hayes et al. (2013) in *Candida albicans* supports this model as NaD1 is known to activate the osmotic stress response, or HOG, pathway in *C. albicans* and permit tolerance of low amounts of NaD1. In addition, *hog1* mutants are more sensitive to NaD1 and DmAmp1 (Hayes et al., 2013). In a similar mechanism, via modification of the osmotic balance of the cell, our yeast mutants gained resistance to plant defensins NaD1 and DmAMP1 and conversely increased their sensitivity to cell wall stressors. The role of *FPS1* in resistance to NaD1 is consistent with NaD1 activation of Hog1p in *C. albicans*, as FPS1p activity is regulated by Hog1p in *S. cerevisiae* (Lee et al., 2013; Muir et al., 2015). One possible mechanism for *FPS1*-mediated NaD1 resistance is that *FPS1* mutants accumulate high intracellular concentrations of glycerol, which stabilizes lipid bilayers and protects the cellular organelles that are targeted by the NaD1 protein.

The NaD1-resistant strains also had mutations in other solute transporters. *PHO84* an inorganic phosphate transporter and low affinity manganese transporter and, *PMR1* a high affinity calcium and manganese transporter (Lapinskas et al., 1995; Jensen et al., 2003). Calcium is known to be involved in the response to osmotic stress, *S. cerevisiae* releases a stretch-activated pulse of calcium ions in response to cellular swelling from hypo-osmotic stress (Batiza et al., 1996; Tong et al., 2004). It is possible the Pmr1p transporter produces this calcium release.

NaD1-resistant strains also had mutations in genes that affect cell wall composition including *CWP2*, *RAS2*, and *BUD4*. *CWP2* encodes a mannoprotein that has a major role in stabilizing the cell wall (Frieman and Cormack, 2003). Mutants with a *cwp2* deletion are more sensitive to CFW and congo red, which are cell wall stressors, providing another explanation for why the NaD1-resistant strains were more sensitive to CFW than the wild type in **Figure 7C** (van der Vaart et al., 1995). Both *RAS2* and *BUD4* affect the structure of the cell wall and are associated with protein localization to the bud neck (Gimeno et al., 1992; Kang et al., 2013). Hence, changes in cell size and growth in the resistant strains (**Figures 5**, **6**) could be linked to the mutations in these genes. In summary, the NaD1-resistant mutants were characterized by mutations that increased resistance to hyperosmotic stress and conversely increased sensitivity of the resistant strains to cell wall stressors such as CFW and SDS. An overall summary of the key changes observed in NaD1-resistant strains is presented in **Figure 8**.

CONCLUSION

In this paper, we described the development of *S. cerevisiae* tolerance to an antifungal plant protein, the defensin NaD1. The overall aim was to compare the rate and mechanism of resistance development of a small protein to a small molecule antifungal of the echinocandin class. This study identified that resistance to the defensin NaD1 was slow to develop and had limited effectiveness compared to caspofungin resistance. A fitness penalty was associated with NaD1 resistance, thus if the selective pressure of NaD1 was removed it is likely that non-resistant strains would outcompete the NaD1 resistant strains. Increased tolerance to NaD1 developed via the accumulation of multiple mutations over time, and not via a single target site modification as with caspofungin. There was no cross resistance observed between NaD1 or caspofungin resistance,

therefore, this study indicates that NaD1, and by extension other plant defensins, may complement existing clinical antifungals due to their resilience and unique mechanism of action.

AUTHOR CONTRIBUTIONS

AM performed the experiments and wrote the manuscript. MB, MA, and RL edited the manuscript and designed the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01648/full#supplementary-material

REFERENCES

Batiza, A. F., Schulz, T., and Masson, P. H. (1996). Yeast respond to hypotonic shock with a calcium pulse. *J. Biol. Chem.* 271, 23357–23362. doi: 10.1074/jbc.271.38.23357

Berkut, A. A., Usmanova, D. R., Peigneur, S., Oparin, P. B., Mineev, K. S., Odintsova, T. I., et al. (2014). Structural similarity between defense peptide from wheat and scorpion neurotoxin permits rational functional design. *J. Biol. Chem.* 289, 14331–14340. doi: 10.1074/jbc.M113.530477

Bleackley, M. R., Hayes, B. M., Parisi, K., Saiyed, T., Traven, A., Potter, I. D., et al. (2014a). Bovine pancreatic trypsin inhibitor is a new antifungal peptide that inhibits cellular magnesium uptake. *Mol. Microbiol.* 92, 1188–1197. doi: 10.1111/mmi.12621

Bleackley, M. R., Payne, J. A. E., Hayes, B. M. E., Durek, T., Craik, D. J., Shafee, T. M. A., et al. (2016). *Nicotiana alata* defensin chimeras reveal differences in the mechanism of fungal and tumor cell killing and an enhanced antifungal variant. *Antimicrob. Agents Chemother.* 60, 6302–6312. doi: 10.1128/aac.01479-16

Bleackley, M. R., Wiltshire, J. L., Perrine-Walker, F., Vasa, S., Burns, R. L., van der Weerden, N. L., et al. (2014b). Agp2p, the plasma membrane transregulator of polyamine uptake, regulates the antifungal activities of the plant defensin NaD1 and other cationic peptides. *Antimicrob. Agents Chemother.* 58, 2688–2698. doi: 10.1128/aac.02087-13

Cherry, J. M., Hong, E. L., Amundsen, C., Balakrishnan, R., Binkley, G., Chan, E. T., et al. (2012). Saccharomyces genome database: the genomics resource of budding yeast. *Nucleic Acids Res.* 40, D700–D705. doi: 10.1093/nar/gkr1029

Cingolani, P., Platts, A., Wang le, L., Coon, M., Nguyen, T., Wang, L., et al. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. *Fly* 6, 80–92. doi: 10.4161/fly.19695

Craik, D. J., Fairlie, D. P., Liras, S., and Price, D. (2013). The future of peptide-based drugs. *Chem. Biol. Drug Des.* 81, 136–147. doi: 10.1111/cbdd.12055

Dracatos, P. M., van der Weerden, N. L., Carroll, K. T., Johnson, E. D., Plummer, K. M., and Anderson, M. A. (2014). Inhibition of cereal rust fungi by both class I and II defensins derived from the flowers of *Nicotiana alata*. *Mol. Plant Pathol.* 15, 67–79. doi: 10.1111/mpp.12066

Engel, S. R., Dietrich, F. S., Fisk, D. G., Binkley, G., Balakrishnan, R., Costanzo, M. C., et al. (2014). The reference genome sequence of Saccharomyces cerevisiae: then and now. *G3* 4, 389–398. doi: 10.1534/g3.113.008995

Frieman, M. B., and Cormack, B. P. (2003). The omega-site sequence of glycosylphosphatidylinositol-anchored proteins in Saccharomyces cerevisiae can determine distribution between the membrane and the cell wall. *Mol. Microbiol.* 50, 883–896. doi: 10.1046/j.1365-2958.2003.03722.x

Gao, Q., Liou, L. C., Ren, Q., Bao, X., and Zhang, Z. (2014). Salt stress causes cell wall damage in yeast cells lacking mitochondrial DNA. *Microb. Cell* 1, 94–99. doi: 10.15698/mic2014.01.131

García-Rodriguez, L. J., Durán, A., and Roncero, C. (2000). Calcofluor antifungal action depends on chitin and a functional high-osmolarity glycerol response (HOG) pathway: evidence for a physiological role of the Saccharomyces cerevisiae HOG pathway under non-inducing conditions. *J. Bacteriol.* 182, 2428–2437. doi: 10.1128/JB.182.9.2428-2437.2000

Gimeno, C. J., Ljungdahl, P. O., Styles, C. A., and Fink, G. R. (1992). Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell* 68, 1077–1090. doi: 10.1016/0092-8674(92)90079-R

Hayes, B. M., Anderson, M. A., Traven, A., van der Weerden, N. L., and Bleackley, M. R. (2014). Activation of stress signalling pathways enhances tolerance of fungi to chemical fungicides and antifungal proteins. *Cell. Mol. Life Sci.* 71, 2651–2666. doi: 10.1007/s00018-014-1573-8

Hayes, B. M. E., Bleackley, M. R., Wiltshire, J. L., Anderson, M. A., Traven, A., and van der Weerden, N. L. (2013). Identification and mechanism of action of the plant defensin NaD1 as a new member of the antifungal drug arsenal against *Candida albicans*. *Antimicrob. Agents Chemother.* 57, 3667–3675. doi: 10.1128/aac.00365-13

Hedfalk, K., Bill, R. M., Mullins, J. G., Karlgren, S., Filipsson, C., Bergstrom, J., et al. (2004). A regulatory domain in the C-terminal extension of the yeast glycerol channel Fps1p. *J. Biol. Chem.* 279, 14954–14960. doi: 10.1074/jbc.M313126200

Jensen, L. T., Ajua-Alemanji, M., and Culotta, V. C. (2003). The Saccharomyces cerevisiae high affinity phosphate transporter encoded by PHO84 also functions in manganese homeostasis. *J. Biol. Chem.* 278, 42036–42040. doi: 10.1074/jbc.M307413200

Kaliszewski, P., and Zoladek, T. (2008). The role of Rsp5 ubiquitin ligase in regulation of diverse processes in yeast cells. *Acta Biochim. Pol.* 55, 649–662.

Kang, P. J., Hood-DeGrenier, J. K., and Park, H. O. (2013). Coupling of septins to the axial landmark by BUD4 in budding yeast. *J. Cell Sci.* 126, 1218–1226. doi: 10.1242/jcs.118521

Katiyar, S. K., and Edlind, T. D. (2009). Role for Fks1 in the intrinsic echinocandin resistance of Fusarium solani as evidenced by hybrid expression in Saccharomyces cerevisiae. *Antimicrob. Agents Chemother.* 53, 1772–1778. doi: 10.1128/AAC.00020-09

Katzmann, D. J., Sarkar, S., Chu, T., Audhya, A., and Emr, S. D. (2004). Multivesicular body sorting: ubiquitin ligase Rsp5 is required for the modification and sorting of carboxypeptidase S. *Mol. Biol. Cell* 15, 468–480. doi: 10.1091/mbc.E03-07-0473

Kvansakul, M., Lay, F. T., Adda, C. G., Veneer, P. K., Baxter, A. A., Phan, T. K., et al. (2016). Binding of phosphatidic acid by NsD7 mediates the formation of helical defensin-lipid oligomeric assemblies and membrane permeabilization. *Proc. Natl. Acad. Sci. U.S.A.* 113, 11202–11207. doi: 10.1073/pnas.1607855113

Lapinskas, P. J., Cunningham, K. W., Liu, X. F., Fink, G. R., and Culotta, V. C. (1995). Mutations in PMR1 suppress oxidative damage in yeast cells lacking superoxide dismutase. *Mol. Cell. Biol.* 15, 1382–1388. doi: 10.1128/MCB.15.3.1382

Lass-Flörl, C. (2009). The changing face of epidemiology of invasive fungal disease in Europe. *Mycoses* 52, 197–205. doi: 10.1111/j.1439-0507.2009.01691.x

Lay, F. T., Brugliera, F., and Anderson, M. A. (2003). Isolation and properties of floral defensins from ornamental tobacco and petunia. *Plant Phys.* 131, 1283–1293. doi: 10.1104/pp.102.016626

Lay, F. T., Mills, G. D., Hulett, M. D., and Kvansakul, M. (2012). Crystallization and preliminary X-ray crystallographic analysis of the plant defensin NaD1.

Acta Crystallogr. F Struct. Biol. Cryst. Commun. 68, 85–88. doi: 10.1107/S1744309111049530

Lee, J., Reiter, W., Dohnal, I., Gregori, C., Beese-Sims, S., Kuchler, K., et al. (2013). MAPK Hog1 closes the *S. cerevisiae* glycerol channel Fps1 by phosphorylating and displacing its positive regulators. *Gene Dev.* 27, 2590–2601. doi: 10.1101/gad.229310.113

Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., et al. (2002). Mechanisms of resistance to fungicides in field strains of Botrytis cinerea. *Pest Manag. Sci.* 58, 876–888. doi: 10.1002/ps.566

Luyten, K., Albertyn, J., Skibbe, W. F., Prior, B. A., Ramos, J., Thevelein, J. M., et al. (1995). Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. *EMBO J.* 14, 1360–1371.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., et al. (2010). The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* 20, 1297–1303. doi: 10.1101/gr.107524.110

McLaren, W., Gil, L., Hunt, S. E., Riat, H. S., Ritchie, G. R. S., Thormann, A., et al. (2016). The ensembl variant effect predictor. *Genome Biol.* 17:122. doi: 10.1186/s13059-016-0974-4

McNatt, M. W., McKittrick, I., West, M., and Odorizzi, G. (2007).). Direct binding to Rsp5 mediates ubiquitin-independent sorting of Sna3 via the multivesicular body pathway. *Mol. Biol. Cell* 18, 697–706. doi: 10.1091/mbc.e06-08-0663

Muir, A., Roelants, F. M., Timmons, G., Leskoske, K. L., and Thorner, J. (2015). Down-regulation of TORC2-Ypk1 signaling promotes MAPK-independent survival under hyperosmotic stress. *eLife* 4:e09336. doi: 10.7554/eLife.09336

Murray, G. M., and Brennan, J. P. (2009). Estimating disease losses to the Australian wheat industry. *Aust. Plant. Pathol.* 38, 558–570. doi: 10.1071/ap09053

Orij, R., Urbanus, M. L., Vizeacoumar, F. J., Giaever, G., Boone, C., Nislow, C., et al. (2012). Genome-wide analysis of intracellular pH reveals quantitative control of cell division rate by pH in Saccharomyces cerevisiae. *Genome Biol.* 13:R80. doi: 10.1186/gb-2012-13-9-r80

Ortega, M., Marco, F., Soriano, A., Almela, M., Martínez, J. A., Pitart, C., et al. (2010). Candida spp. bloodstream infection: influence of antifungal treatment on outcome. *J. Antimicrob. Chemother.* 65, 562–568. doi: 10.1093/jac/dkp495

Parisi, K., Shafee, T. M. A., Quimbar, P., van der Weerden, N. L., Bleackley, M. R., and Anderson, M. A. (2018). The evolution, function and mechanisms of action for plant defensins. *Semin. Cell Dev. Biol.* doi: 10.1016/j.semcdb.2018.02.004 [Epub ahead of print].

Payne, J. A., Bleackley, M. R., Lee, T. H., Shafee, T. M., Poon, I. K., Hulett, M. D., et al. (2016). The plant defensin NaD1 introduces membrane disorder through a specific interaction with the lipid, phosphatidylinositol 4,5 bisphosphate. *Biochim. Biophys. Acta* 1858, 1099–1109. doi: 10.1016/j.bbamem.2016.02.016

Poon, I. K. H., Baxter, A. A., Lay, F. T., Mills, G. D., Adda, C. G., Payne, J. A. E., et al. (2014). Phosphoinositide-mediated oligomerization of a defensin induces cell lysis. *eLife* 3:e01808. doi: 10.7554/eLife.01808

Qian, W., Ma, D., Xiao, C., Wang, Z., and Zhang, J. (2012). The genomic landscape and evolutionary resolution of antagonistic pleiotropy in yeast. *Cell Rep.* 2, 1399–1410. doi: 10.1016/j.celrep.2012.09.017

Robinson, J. T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., et al. (2011). Integrative genomics viewer. *Nat. Biotechnol.* 29, 24–26. doi: 10.1038/nbt.1754

Roncero, C., Valdivieso, M. H., Ribas, J. C., and Durán, A. (1988). Isolation and characterization of Saccharomyces cerevisiae mutants resistant to Calcofluor white. *J. Bacteriol.* 170, 1950–1954. doi: 10.1128/jb.170.4.1950-1954.1988

Sagaram, U. S., Pandurangi, R., Kaur, J., Smith, T. J., and Shah, D. M. (2011). Structure-activity determinants in antifungal plant defensins MsDEF1 and MtDEF4 with different modes of action against Fusarium graminearum. *PLoS One* 6:e18550. doi: 10.1371/journal.pone.0018550

Sanglard, D., Ischer, F., Monod, M., and Bille, J. (1996). Susceptibilities of Candida albicans multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. *Antimicrob. Agents Chemother.* 40, 2300–2305.

Saleh, A., Collart, M., Martens, J. A., Genereaux, J., Allard, S., Cote', J., et al. (1998). TOM1p, a yeast hect-domain protein which mediates transcriptional regulation through the ADA/SAGA coactivator complexes. *J. Mol. Biol.* 282, 933–946. doi: 10.1006/jmbi.1998.2036

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Schlecht, U., Suresh, S., Xu, W., Aparicio, A. M., Chu, A., Proctor, M. J., et al. (2014). A functional screen for copper homeostasis genes identifies a pharmacologically tractable cellular system. *BMC Genomics* 15:263. doi: 10.1186/1471-2164-15-263

Sherry, S. T., Ward, M.-H., Kholodov, M., Baker, J., Phan, L., Smigielski, E. M., et al. (2001). dbSNP: the NCBI database of genetic variation. *Nucleic Acid Res.* 29, 308–311. doi: 10.1093/nar/29.1.308

Singh, R. K., Kabbaj, M. H., Paik, J., Gunjan, A. (2009). Histone levels are regulated by phosphorylation and ubiquitylation-dependent proteolysis. *Nat. Cell Biol.* 11, 925–933. doi: 10.1038/ncb1903

Sionov, E., Chang, Y. C., Garraffo, H. M., Dolan, M. A., Ghannoum, M. A., and Kwon-Chung, K. J. (2012). Identification of a Cryptococcus neoformans Cytochrome P450 lanosterol 14α-demethylase (ERG11) residue critical for differential susceptibility between fluconazole/voriconazole and itraconazole/posaconazole. *Antimicrob. Agents Chemother.* 56, 1162–1169. doi: 10.1128/aac.05502-11

Sirisattha, S., Momose, Y., Kitagawa, E., and Iwahashi, H. (2004). Toxicity of anionic detergents determined by Saccharomyces cerevisiae microarray analysis. *Water Res.* 38, 61–70. doi: 10.1016/j.watres.2003.08.027

Tamás, M. J., Luyten, K., Sutherland, F. C. W., Hernandez, A., Albertyn, J., Valadi, H., et al. (1999). Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol. Microbiol.* 31, 1087–1104. doi: 10.1046/j.1365-2958.1999.01248.x

Thevissen, K., Osborn, R. W., Acland, D. P., and Broekaert, W. F. (2000). Specific binding sites for an antifungal plant defensin from Dahlia (*Dahlia merckii*) on fungal cells are required for antifungal activity. *Mol. Plant Microbe Interact.* 13, 54–61. doi: 10.1094/MPMI.2000.13.1.54

Toh, T. H., Kayingo, G., van der Merwe, M. J., Kilian, S. G., Hallsworth, J. E., Hohmann, S., et al. (2001). Implications of FPS1 deletion and membrane ergosterol content for glycerol efflux from *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 1, 205–211.

Tong, A. H., Lesage, G., Bader, G. D., Ding, H., Xu, H., Xin, X., et al. (2004). Global mapping of the yeast genetic interaction network. *Science* 303, 808–813. doi: 10.1126/science.1091317

van der Vaart, J. M., Caro, L. H., Chapman, J. W., Klis, F. M., and Verrips, C. T. (1995). Identification of three mannoproteins in the cell wall of Saccharomyces cerevisiae. *J. Bacteriol.* 177, 3104–3110. doi: 10.1128/jb.177.11.3104-3110.1995

van der Weerden, N. L., Anderson, M. A., and Payne, J. A. (2014). Agents and Methods of Treatment. U.S. Patent No 20170042966A1. Washington, DC: U.S. Patent and Trademark Office.

van der Weerden, N. L., Bleackley, M. R., and Anderson, M. A. (2013). Properties and mechanisms of action of naturally occurring antifungal peptides. *Cell. Mol. Life Sci.* 70, 3545–3570. doi: 10.1007/s00018-013-1260-1

van der Weerden, N. L., Hancock, R. E. W., and Anderson, M. A. (2010). Permeabilization of fungal hyphae by the plant defensin NaD1 occurs through a cell wall-dependent process. *J. Biol. Chem.* 285, 37513–37520. doi: 10.1074/jbc.M110.134882

van der Weerden, N. L., Lay, F. T., and Anderson, M. A. (2008). The plant defensin, NaD1, enters the cytoplasm of Fusarium oxysporum hyphae. *J. Biol. Chem.* 283, 14445–14452. doi: 10.1074/jbc.M709867200

Verweij, P. E., Snelders, E., Kema, G. H., Mellado, E., and Melchers, W. J. (2009). Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? *Lancet Infect. Dis.* 9, 789–795. doi: 10.1016/s1473-3099(09)70265-8

Vriens, K., Cools, T. L., Harvey, P. J., Craik, D. J., Braem, A., Vleugels, J., et al. (2016). The radish defensins RsAFP1 and RsAFP2 act synergistically with

caspofungin against *Candida albicans* biofilms. *Peptides* 75, 71–79. doi: 10.1016/j.peptides.2015.11.001

Vriens, K., Cools, T. L., Harvey, P. J., Craik, D. J., Spincemaille, P., Cassiman, D., et al. (2015). Synergistic activity of the plant defensin HsAFP1 and caspofungin against Candida albicans biofilms and planktonic cultures. *PLoS One* 10:e0132701. doi: 10.1371/journal.pone.0132701

Wang, C. K., King, G. J., Conibear, A. C., Ramos, M. C., Chaousis, S., Henriques, S. T., et al. (2016). Mirror images of antimicrobial peptides provide reflections on their functions and amyloidogenic properties. *J. Am. Chem. Soc.* 138, 5706–5713. doi: 10.1021/jacs.6b02575

Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., et al. (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* 285, 901–906. doi: 10.1126/science.285.5429.901

Conflict of Interest Statement: MA is the Executive Director and Chief Scientific Officer of Hexima Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter Three

3. STUDY 2 – Mechanism of resistance to BPTI in *S. cerevisiae*

The findings of this chapter have been submitted for publication in a peer-reviewed journal G3: Genes, Genomes, Genetics and is currently under review, June 2020. This paper is also available for public access at BioRxiv.

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Statement of Contribution

Amanda McColl has made the following contributions:

- Design and experimental completion of data collection,
- Analysis and interpretation of all data,
- Preparation of methods and figures for the manuscript.
- Drafted and provided all revisions of manuscript prior to submission.

Other authors have made the following contributions:

- Bleackley MR, Anderson MA, and Lowe RGT contributed to the conception and design of the experiments, data analysis and manuscript revisions.
- McKenna JA completed the initial antifungal assay examining the efficacy of BPTI against *T. rubrum, A. fumigatus, S. cerevisiae, C. albicans* and *C. neoformans* (Table 1)

Resistance to the antifungal activity of Aprotinin occurs through mutations in genes that function in cation homeostasis

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Resistance to the antifungal activity of Aprotinin occurs through mutations in genes that function in cation homeostasis

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Abstract:

An increase in the prevalence of fungal infections is coinciding with an increase of resistance to current clinical antifungals, placing pressure on the discovery of new antifungal candidates. One option is to investigate drugs that have been approved for use for other medical conditions that have secondary antifungal activity. Aprotinin, also known as Bovine Pancreatic Trypsin inhibitor (BPTI), is an antifibrinolytic that has been approved for systemic use in patients in some countries. Bleackley and coworkers (2014) revealed that BPTI also has antifungal activity against *S. cerevisiae* and *C. albicans* and does this by targeting the magnesium transporter *ALR1*. Here we have further investigated the potential for aprotinin to be used as an antifungal by assessing the development of resistance. We used an *in vitro* model to assess the evolution of BPTI resistance/tolerance whereby BPTI was serial passaged with the model organism *S. cerevisiae*. Resistance to BPTI developed more quickly than resistance to the plant defensin NaD1 and the clinical antifungal, caspofungin. Full genome sequencing of resistant lines revealed that resistance to BPTI developed as the result of a deleterious mutation in either the *ptk2* or *sky1* genes. This revealed that cation homeostasis and transport functions were particularly affected in *S. cerevisiae* after exposure to BPTI. Therefore, the mutations in these genes probably decreases release of magnesium and other cations from the cell, protecting the yeast from the limiting intracellular magnesium levels that arise when BPTI blocks the magnesium transporter Alr1p.

Introduction

Aprotinin, also known as bovine pancreatic trypsin inhibitor (BPTI), has been used as a drug to reduce bleeding during cardiopulmonary bypass (CPB) and was sold by Bayer under the trade name Trasylol (Bidstrup *et al*, 1989). Aprotinin slows down fibrinolysis, the process that leads to the breakdown of blood clots (Royston *et al*, 1987). In 1987, a small study was published that reported a reduction of blood loss and need for transfusions in patients treated with Aprotinin which resulted in wide use of the drug in cardiac, hepatic and orthopedic surgery (Royston *et al.*, 1987). However, subsequent large scale clinical studies revealed potential safety concerns associated with the use of Aprotinin, including an increased risk of renal failure, myocardial infarction, heart failure, stroke, and encephalopathy (Karkouti *et al*, 2006; Mangano *et al*, 2006). As a result, marketing of Aprotinin was suspended in 2007, but the results of these studies have been challenged and in 2012 Aprotinin was reintroduced in Canada and the European Medical Association also recommended a lift of the ban (Furnary *et al*, 2007; McMullan & Alston, 2013). Therefore, it may be time to rethink the potential uses for Aprotinin, including those outside of reducing blood loss during surgery.

In a previous study, Bleackley and co workers, discovered that Aprotinin (BPTI), also inhibits the growth of *Saccharomyces cerevisiae* and the human fungal pathogen *Candida albicans* (Bleackley *et al*, 2014). It does this by binding to the magnesium transporter Alr1p, blocking magnesium uptake into the cell and causing cell cycle arrest (Bleackley *et al.*, 2014). It also works synergistically with the well characterised antifungal plant defensin, NaD1 against *Fusarium graminearum*, *Colletotrichum graminicola* and *Candida albicans*, although the exact mechanism of synergy is unclear (Bleackley *et al*, 2017). Aproptinin can be administered to patients via injection under strict guidelines and benefit- risk assesment, therefore aprotinin is a potential treatment for systemic fungal infections that requires further

investigation. One important consideration in the use of a drug to treat microbial infections is the potential for the pathogenic microbes to develop increased tolerance and eventually, resistance to the drug.

In this study, we generated yeast strains that have increased tolerance to Aprotinin (BPTI), and identified the genetic mutations linked to the decreased sensitivity to BPTI. We found that resistance to BPTI developed more quickly than resistance to the plant defensin NaD1 and to the clinical antifungal caspofungin. Resistance was caused by single point mutations that inactivated either the *sky1* or *ptk2 genes* that function in ion transport. These mutations are likely to increase magnesium accumulation in the cells and prevent cell cycle arrest. We did not find mutations in the gene encoding the magnesium transporter Alr1p that BPTI is known to bind to. Interestingly synergy between NaD1 and BPTI was not affected in yeast strains that were more resistant to either one of the two peptides. That is, sensitivity to each peptide was restored by the addition of subinhibitory levels of the synergy partner.

Material and methods

Fungal Strains

The *S. cerevisiae* strain BY4741 (*MAT*a *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) was purchased from Thermo Scientific. Single deletion strains were retrieved from the haploid non-essential deletion collection (Thermo Scientific)(Winzeler *et al*, 1999). Production of NaD1 and caspofungin-resistant lines of *S. cerevisiae* are described in (McColl *et al*, 2018).

The *C. albicans* strain ATCC 90028 was purchased from In Vitro Technologies Pty. Ltd. *C. neoformans* KN99 was purchased from The Fungal Genetics Stock Centre. The yeasts *S. cerevisiae, C. albicans and C. neoformans* were routinely cultured on YPD medium at 30°C

A. fumigatus ATCC MYA-3626 was purchased from In Vitro Technologies Pty. Ltd.

A patient isolate of *T. rubrum* was acquired from the National Mycology Reference Centre (Adelaide, SA). The filamentous fungi *A. fumigatus* and *T. rubrum* were cultured at 28°C on V8 agar.

Antifungal molecules

Aprotinin (BPTI) was purchased from Astral Scientific (Australia), NaD1 and NaD2 were purified from *Nicotiana alata* flowers as described in ((Dracatos *et al*, 2014; Lay *et al*, 2003). Caspofungin was purchased from Sigma (Australia).

LL-37 was synthesized by GL Biochem (China) and Bac2a was synthesized by GenScript (China).

Culturing in the presence of antifungal molecules to develop resistance.

The culturing method to develop resistance to BPTI was performed as described in (McColl *et al.*, 2018) whereby, *S. cerevisiae* BY4741 was grown overnight at 30°C with agitation in 5 mL of yeast extract-peptone-dextrose (YPD). The overnight culture was then diluted to an OD 600 nm of 0.01 in 50% strength potato dextrose broth (½ PDB) before addition of antifungal molecules. Cultures were initially grown with the BPTI at 0.5x the mimimum inhibitory concentration (MIC) (5 μM) or 1x MIC (10 μM) alongside a negative control lacking antifungals. Three independent lines for the BPTI resistant and untreated controls were grown in parallel. The cultures were incubated overnight at 30°C with agitation. The cultures that exhibited growth at the highest concentration of BPTI were then used to seed cultures at a higher concentration of BPTI. Sub-culturing was stopped once growth occurred at 32 times the original MIC.

Single Colony Isolation of Resistant Strains

Cultures resistant to the antifungal molecule were streaked out to isolate single colonies on non-selective YPD agar. Three colonies were picked from each line and their resistance was re-tested, the colony with the highest resistance to the antifungal was used in subsequent experiments.

Antifungal Assays

Antifungal assays were performed as described in (Hayes *et al*, 2013). Briefly, yeast cultures *S. cerevisiae, C. albicans and C, neoformans* were grown overnight (30°C, 250 rpm) in 5 mL YPD and diluted to an OD600 of 0.01 in ½ PDB. Antifungal molecules were prepared at 10x the assay concentration and 10µL was mixed with 90 µL of diluted yeast culture before incubation for 24h at 30°C.

Filamentous fungi *T. rubrum and A. fumigatus* were cultured for 3 weeks (28°C) on V8 agar. To prepare the inoculum for the filamentous fungi, the spores were obtained by flooding plates with sterile water. Hyphal matter was removed by filtration through sterile facial tissue. Spores were quantified by hematocytometer and diluted to 50,000 spores/mL in 1/2 PDB before use. Antifungal molecules were prepared at 10x the assay concentration and 10µL was mixed with 90 µL of diluted fungal spores before incubation for 48 h at 28°C. The final OD600 was measured using a SpectraMAX M5e plate reader (Molecular Devices).

Synergy Assays

Synergy assays were performed as described in (Bleackley *et al.*, 2017) whereby, *S. cerevisiae* was cultured in liquid YPD overnight at 30°C beforecells were diluted to an OD600 of 0.01 in ½PDB. Antifungals were prepared at 10 times the assay concentration.

Aliquots of the test molecules (10 µl) were placed into the wells of a 96-well microtiter plate in a standard checkerboard array. Cells (80 µl) were then added to all the wells of the plate followed by incubation at 30°C for 24 h. Growth was monitored by measuring the optical density at 595 nm using a SpectraMax M5e plate reader (Molecular Devices). Synergistic interactions were identified using the fractional inhibitory concentration (FIC) calculation with a cutoff of 0.5 indicative of synergy (FIC = MIC_Acombination/MIC_Aalone + MIC_Bcombination/MIC_Balone where MIC_Acombination is the MIC of agent A in combination and MIC_Aalone is the MIC of agent A alone) as described previously by (Bleackley *et al.*, 2017). If a test molecule was not inhibitory when used in isolation, the MIC was set to two times the top concentration tested.

Cell Growth Assay

S. cerevisiae BY4741 cultures were grown overnight (30°C, 250 rpm) in 5 mL of YPD and were diluted to an OD_{600} of 0.5 in 1 mL YPD or ½ PDB before 100 µL was dispensed into each of 8 wells of a 96 well microtiter plate. The plate was then incubated in a SpectraMAX M5e plate reader (Molecular Devices) at 30 °C. Optical density at 600nm was recorded for each well every 30 min over the 48h culture period and averaged across the 8 replicates.

Stress assay with hydrogen peroxide, calcofluor white, NaCl and SDS

Hydrogen peroxide (0.625, 1.25, 2.5 or 5 mM), calcofluor white (1, 2.5, 5 or 10 µg), NaCl (100, 200 or 300 mM), or SDS (12.5, 25, 50 or 100 µg) were diluted in YPD agar media (25 mL) just before the plates were poured. Yeast cultures were grown overnight in 5mL of YPD before they were diluted to an OD600nm of 0.1 in 1 mL of MilliQ-purified water. A five-fold dilution series of each yeast strain was spotted (4 µL per spot) onto the agar plates with the

added stress factors and incubated overnight at 30°C. Plates were imaged using a ChemiDoc (Biorad).

Stress Assay with Ultraviolet Light

S. cerevisiae cultures were grown overnight in 5 mL YPD and diluted to an OD600nm of 0.1 in 1 mL MilliQ-purified water. A five-fold dilution series of each strain (4 µL per spot) was then spotted onto a YPD agar plate and allowed to dry before exposure to UV light (Phillips, 30w bulb at 50 cm) for 1.2, 2.4, 5.2 or 10.4 min.

DNA extraction from wild-type and resistant strains of *S. cerevisiae*

Genomic DNA was extracted using the Qiagen DNeasy® plant mini kit. Three independent lines of BPTI-resistant strains and three lines of the no-treatment controls were sequenced. Sequencing was completed at the La Trobe Genomics Platform, using the Illumina MiSeq V3 chemistry. One run was performed for all 6 strains, generating 25 million 300 bp paired-end reads. The pre-processing and variant discovery steps were performed as described by the GATK best practices as summarized in (McKenna *et al*, 2010).

Genomic analysis of resistant strains of *S. cerevisiae:*

Genomic analysis was completed as described in (McColl *et al.*, 2018) and was as follows:

Sequence pre-processing

The raw sequence reads were converted to Sam format and illumine adapters were identified using Picard tools (v.2.4.1) fastqtosam and markilluminaadapters. The reads were then aligned to the *S. cerevisiae* (R64-1-1.23) reference genome using BWA-mem (v0.7.12) (Engel *et al*, 2014). The aligned read files were merged using Picard (v.2.4.1)

mergebamallignment and subjected to quality control and filtering using Picard (v2.4.1) markduplicates and GATK (v.3.6) realignertargetcreater and baserecalibrator.

Variant Discovery

Variations that were either SNVs (single-nucleotide variants) or INDELs (insertion/deletion) were discovered using GATK (v.3.6) Haplotypecaller (Sherry *et al*, 2001). The samples were merged using GATK (v.3.6) combinegvcf and then joint genotyping was performed using GATK (v.3.6) GenotypeGVCFs. SNV's and indels were extracted based on default quality parameters using GATK (v.3.6) VariantFiltration and VariantRecalibrator.

Variant Refinement

Variants were annotated using SnpEff (v.2.4) (Cingolani *et al*, 2012). VEP (variant effect predictor) marked any codon changes as either tolerant or deleterious (McLaren *et al*, 2016). SnpSift (v.2.4) was used to identify SNVs or indels that were present in the BPTI-resistant replicates and not in the Control strains. The variants were then inspected manually using IGV (v.2.3.77) (Robinson *et al*, 2011).

Data Availability statement

Strains are available upon request. The authors affirm that all data necessary for confirming the conclusions of this article are represented fully within the article and its tables and figures. The publically available *S. cerevisiae* (R64-1-1.23) reference genome was obtained from the NCBI SRA database with accession number PRJNA43747. Raw sequencing data for NaD1-R and Controls from (McColl *et al.*, 2018) are publicly available from the NCBI SRA database with accession number PRJNA434021. BPTI-R sequencing data has been uploaded to the NCBI SRA database with the submission number SUB7621764 and is confidentially available to the editors and reviewers upon request. This submission will be made publically available once paper is accepted.

257

258 # Results

259 **BPTI inhibits growth of *S. cerevisiae*, *C. albicans and C. neoformans*.**

260 The antifungal activity of BPTI was tested in a growth assay with the yeast *S. cerevisiae*, *C.*
261 *albicans* and *C. neoformans* as well as the filamentous fungi *T. rubrum* and *A. fumigatus.*
262 BPTI was effective against *S. cerevisiae* with an MIC of 12.5 µg/mL and *C. neoformans* with
263 an MIC of 9.4 µg/mL (Table 1). It was less effective against *C. albicans* with an MIC of 100
264 µg/mL and was ineffective against both filamentous fungi, *T. rubrum* and *A. fumigatus* at
265 concentrations up to 200 µg/mL (Table 1).

266

267 **Evolution of resistance to BPTI.**

268 Yeast strains with increased tolerance to BPTI were developed by continuous culture of *S.*
269 *cerevisiae* at sub-inhibitory concentrations. Each time the MIC increased; the dose of
270 antifungal was doubled. The starting concentration of BPTI in the continuous cultures was 5
271 µM. After 12 rounds of sub-culturing strains were able to grow in 160 µM BPTI, a 32-fold
272 increase in the MIC (Figure 1A).

273

274 Once selection pressure was removed, the MIC of the resistant strains can decrease.
275 Therefore, the three BPTI-resistant cultures were streaked onto agar and a pure strain for each
276 culture was isolated. Their resistance phenotype was confirmed using a standard antifungal
277 assay (Figure 1B). These strains were used for all further experimentation. The BPTI-
278 resistant strains were 4-fold more tolerant to BPTI than wildtype, with an MIC of 40 µM
279 compared to the MIC of 10 µM for wildtype (Figure 1B).

280

281 **Resistance to BPTI confers resistance to some but not all antifungal peptides.**

We examined whether the enhanced tolerance of the BPTI resistant strains was broad-spectrum or specific to BPTI by comparing the susceptibility of BPTI-resistant lines and wildtype to a set of antimicrobial molecules. The BPTI-resistant strains were more resistant to the human cathelicidin LL-37 with an MIC of 20 μM compared to wildtype inhibition at 5 μM (Figure 2A) and they were also more resistant to NaD2, another plant defensin from *Nicotiana alata*, with an MIC of 20 μM compared to 10 μM for the wild type (Figure 2B). However, this enhanced tolerance did not extend to all AFPs tested. NaD1 (Figure 2C) and caspofungin (Figure 2D) had similar levels of activity against the BPTI resistant strains as the wild-type strain and BPTI-resistant strains were more sensitive than wildtype strains to the peptide Bac2a, which is a loop swap variant of bactenecin, a bovine cathelicidin (Figure 2E).

BPTI-resistance is associated with cation homeostasis and cell wall stress.

As the BPTI-resistant strains displayed differential sensitivity to AMPs, we assessed whether the BPTI-resistant strains differed to wildtype in susceptibility to a selection of abiotic stresses. The BPTI-resistant strains grew better than wildtype at elevated NaCl concentrations (Figure 3A). Similarly, BPTI-resistant strains were more resistant to calcofluor white (CFW) than wildtype yeast. BPTI-resistant strain B was also more tolerant to hydrogen peroxide compared to wildtype cells but not BPTI resistant strains A and C which had the same sensitivity as wildtype cells (Figure 3A). BPTI-resistant strains and wildtype cells were equally sensitive to UV and to SDS (Supplementary figure A).

We reported in an earlier paper that BPTI blocks the magnesium transporter Alr1p and restricts growth of *S. cerevisiae* (Bleackley, 2014). Therefore, we assessed whether the BPTI-resistant strains in this study had defects in magnesium transport by examining whether they were more tolerant to Hexamine (III) cobalt chloride (HCC), a magnesium channel inhibitor.

307 The BPTI-resistant strains were 4-fold more tolerant to HCC with an MIC of 10 µM HCC
308 compared to wildtype which was inhibited at 2.5 µM HCC (Figure 3B)
309
310 The fitness of the BPTI-resistant strains was assessed relative to wildtype using media with
311 no antifungal molecules. The growth rates of the BPTI-resistant strains were similar to the
312 wildtype, with strains A and C growing slighter slower (Figure 3C).
313
314 **Genetic characterisation of BPTI resistance.**
315 Mutations associated with BPTI resistance, were identified by sequencing the genomes of the
316 BPTI-resistant and non-selected control lines. Five genes (Erg3 Ser24Lue, Gda1 Cys462Phe,
317 Nrp1 Asn444Ser, Ptk2 Gly469*stop, Sky1 Gln65*stop) had mutations in their protein coding
318 regions leading to amino acid substitutions or truncated proteins. Four other genes (Gex1,
319 Sok1, tT(XXX)Q2, YBR298C-A) had mutations in the upstream non-coding region (Table
320 2). A description of the predicted functions of the affected genes is also listed in Table 2.
321
322 **Confirmation of mutation resistance**
323 It was considered likely that the predicted missense or disruptive mutations in coding regions
324 would have resulted in a loss of gene function. To test this hypothesis, single-gene knockouts
325 of the mutated genes were retrieved from the yeast deletion set (Winzeler *et al.*, 1999) and
326 antifungal assays were performed to assess whether gene deletion replicated the BPTI-
327 resistant phenotype. Antifungal assays revealed that the single gene knockout mutants of
328 *ptk2*Δ and *sky1Δ* were as resistant to BPTI as the BPTI-resistant isolates with an MIC of 40
329 µM. The knockout strains *gad1*Δ and *erg3*Δ were more sensitive to BPTI. The knockout
330 *nrp1*Δ was inhibited at the same level as wildtype with an MIC of 10 µM (Table 4).
331

BPTI and NaD1 act synergistically to inhibit BPTI and NaD1-resistant strains

BPTI and NaD1 have been reported to act synergistically in the inhibition of a range of pathogens (Bleackley *et al.*, 2017). Therefore, we assessed whether the NaD1-BPTI synergy still occurred if the strains were resistant to one of the peptides. The NaD1 and BPTI-resistant strains were tested against NaD1 and BPTI in a synergy assay and compared to wildtype (Figure 4). Wildtype had an average FIC synergy value of 0.23. The BPTI-resistant strains had an average FIC synergy value of 0.13 (Figure 4C), and the NaD1-resistant strains had an average FIC value of 0.19 (Figure 4F). Interestingly, the resistance of the BPTI resistant strains to BPTI was abolished upon addition of the lowest concentration of NaD1 (0.16 μM) (Figure 4A and C). Similarly, the resistance of the NaD1 strains to NaD1 was abolished in the presence of 0.16 μM BPTI (Figure 4D and F).

Discussion

Aprotinin, also known as BPTI, was used during surgery to prevent blood loss in patients (Bidstrup *et al.*, 1989). Although BPTI is approved in some countries, it is restricted and the technological advances in medicine have produced better antifibrinolytic drugs (Van der Linden *et al*, 2001). We have been investigating another potential medical application for BPTI. BPTI has antifungal activity against the yeast S. *cerevisiae* with an MIC of 12.5 μg/mL, *C. neoformans* with an MIC of 9.4 μg/mL, and *C. albicans* with an MIC of 100 μg/mL (Table 1). BPTI did not have activity against the filamentous fungi *T. rubrum* and *A. fumigatus* at concentrations up to 200 μg/mL (Table 1). These results prompted the question of whether BPTI has the potential to be used as a treatment for systemic yeast infections. Such an application would depend on whether the yeast is able to develop resistance to BPTI quickly and how this would be managed. Here we showed that yeast do become resistant to BPTI after serial passaging in the presence of increasing amounts of peptide. Indeed

357 resistance to BPTI developed more quickly than resistance to caspofungin and the plant
358 defensin NaD1 that we described in an earlier publication (McColl *et al.*, 2018). Some BPTI
359 resistant strains were cross resistant to other antifungal peptides, specifically LL37 and
360 NaD2. However, the BPTI-resistant strains were still as susceptible to both NaD1, and
361 caspofungin and they were more sensitive to Bac2a. That is, the resistance to BPTI was not
362 due to a general improvement in fitness. The three strains that were resistant to BPTI were
363 also more resistant to osmotic stress from elevated NaCl concentrations, and cell wall stress
364 induced by calcofluor white. One strain was more resistant to hydrogen peroxide. Sequencing
365 the genomes of the BPTI resistant strains and follow-up experiments using whole gene
366 deletion strains revealed that mutations in *ptk2* and *sky1* are likely to be the primary source of
367 resistance to BPTI.
368
369 BPTI exerts its antifungal activity by blocking Mg^{2+} uptake in to the cell and inhibiting
370 growth (Bleackley *et al.*, 2014). Hexamine (III) cobalt chloride (HCC), a well characterized
371 CorA Mg^{2+} transport inhibitor, inhibits cells at the same stages of the cell cycle as BPTI
372 (Bleackley *et al.*, 2014) and results in a similar drop in cellular Mg^{2+} levels. We thus
373 assessed whether BPTI-resistant strains generated in this study were more tolerant to
374 inhibition of magnesium uptake by assessing the effect HCC on their growth. The BPTI-
375 resistant strains were 4-fold more tolerant to HCC with an MIC of 10 μM HCC compared to
376 the wildtype strain which was fully inhibited at 2.5 μM. This 4-fold increase in resistance to
377 HCC parallels the increase in resistance to BPTI observed in these strains. The similarities in
378 the resistance to HCC indicate that resistance to BPTI is related to magnesium transport
379 inhibition.
380
381

Yeast develop resistance to BPTI more quickly than other antifungals.

Resistance to BPTI developed quickly with 12 rounds of sub-culturing compared to 15 rounds for caspofungin and 21 rounds for resistance in NaD1 (Figure 1A). We attributed this relatively rapid development of resistance to two factors. The first is that BPTI is fungistatic, that is, it inhibits fungal growth but does not actively kill the fungus (Bleackley *et al.*, 2014) leaving a larger pool of living cells to develop mutations that confer resistance. Both NaD1 and caspofungin are fungicidal molecules. Fungicidal drugs are usually the preferred choice of treatment in the clinic because they act quickly and kill almost all cells (Kumar *et al*, 2018).The second reason is that BPTI inhibits fungal growth by blocking magnesium uptake by the membrane transporter Alr1p (Bleackley *et al.*, 2014). In contrast, NaD1 has a complex mechanism of action that involves: interaction with the fungal cell wall (van der Weerden *et al*, 2008), movement across the plasma membrane, induction of oxidative stress, and interaction with phosphatidylinositol 4,5 bisphosphate (Parisi *et al*, 2019). These processes lead to damage of the inner leaflet of the cell membrane and cell death within 10 min of exposure to NaD1 (Hayes *et al*, 2014; Payne *et al*, 2016; van der Weerden *et al*, 2010). Resistance to NaD1 develops more slowly compared to caspofungin because resistance to caspofungin can be achieved through point mutations in specific "hot spot" regions in the *fks1* gene, whereas resistance to NaD1 occurs through an accumulation of mutations in different genes (McColl *et al.*, 2018). It is likely that resistance to BPTI developed more quickly because there is only one component of the inhibitory mechanism, that is magnesium transport.

BPTI resistant strains are also resistant to LL37 and NaD2 but not other antifungals

To assess how specific the resistance to BPTI was, the BPTI-resistant strains were tested against a range of other antifungals including; the antimicrobial plant defensin NaD1, another

plant defensin from *Nicotiana alata* NaD2 (Dracatos *et al.*, 2014), the echinocandin caspofungin which is used in the clinic (McCormack & Perry, 2005), the human cathelicidin LL-37(Ordonez *et al*, 2014), and a linear variant of the bovine antimicrobial peptide bactenecin, Bac-2a (Hilpert *et al*, 2005) (Figure 2). There was no cross resistance associated with caspofungin, NaD1 or Bac2a. Therefore, resistance to BPTI would not provide cross protection against NaD1 or caspofungin as BPTI acts predominantly by blocking magnesium transport, which is a very different mechanism of action to NaD1 and caspofungin. However, the BPTI-resistant strains were more resistant to LL-37 as well as NaD2, but to a lesser extent. LL-37 associates with cell wall components of *C. albicans*, leading to cell membrane disruption (Burton & Steel, 2009; Tsai *et al*, 2014). We found that BPTI-resistant strains are also resistant to osmotic stress and calcofluor white, a cell wall stressor that binds to chitin (Figure 3A) which could explain the cross resistance to LL37. Similarly, identification of mutations in genes *sky1* and *ptk2,* which are responsible for the regulation of cation ion transport and homeostasis, would impact the ion gradients across the plasma membrane and therefore place stress on the membrane which in turn could influence susceptibility to LL-37. This pattern of cross resistance was not observed with the NaD1-resistant strains which were also resistant to other plant defensins, DmAMP1 and HXP4 (McColl *et al.*, 2018).

Resistance to BPTI developed as the result of a deleterious mutation in one of two genes

Whole genome sequencing and SNV calling revealed a set of mutations that were present in the resistant strains, these were in the genes *sky1, ptk2, gad1, erg3,* and *nrp1*. It is likely that some of the mutations would result in a loss of gene function, particularly the mutations that were identified as deleterious. To test this hypothesis, single-gene knockouts corresponding to the mutated genes were retrieved from the yeast deletion set (Winzeler *et al.*, 1999) and antifungal assays were performed to assess whether gene deletion replicated the BPTI-

resistant phenotype. Indeed, the single gene knockout mutants of of *ptk2*Δ and *sky1Δ* were as resistant to BPTI as the BPTI-resistant isolates at 40 μM. The BPTI-resistant strains A and C have a deleterious *ptk2* mutation (Gly469 to STOP) and BPTI-resistant strain B has a deleterious *sky1* mutation (Gln65 to STOP) (Table 1). The single gene knockout mutants of *gad1*Δ and *erg3Δ* were more sensitive to BPTI, this is likely because the mutations were either tolerated missense mutations or the mutations had an up-regulatory effect. The knockout *nrp1*Δ was inhibited the same as wildtype at 10 μM as expected because the mutations in nrp1p were unlikely to affect gene function. It was thus considered likely that all the resistance to BPTI was contributed by the mutations in *ptk2* or *sky1* because the *gad1*Δ, *erg3Δ* and *nrp1*Δ knockouts were not more resistant to BPTI. The mutations in *ptk2* and *sky1* both introduced an early stop codon, and thus would have the same effect as a gene knockout. Interestingly the BPTI-resistant strains also exhibited phenotypes that have been reported previously for *ptk2*Δ and *sky1Δ* strains. For example knockouts of *sky1* have been reported to be more resistant to hydrogen peroxide and calcofluor white (Brown *et al*, 2006), and we found that the BPTI-resistant strain B, with the *sky1* mutation, was more resistant to hydrogen peroxide and that this strain together with the other two BPTI-resistant strains were resistant to CFW (Figure 3A). Another screen of a yeast deletion library revealed that *sky1* and *ptk2* deletion mutants are resistant to sodium chloride, as we observed with the BPTI-resistant strains (Yoshikawa *et al*, 2009). Apart from the observed resistance to osmotic and cell wall stress, the BPTI-resistant strains had no other apparent fitness defects even in the presence of several abiotic stressors. This contrasts with the NaD1-resistant strains which had a decreased growth rate and were more sensitive to Calcofluor white and SDS (McColl *et al.*, 2018).

Bleackley and coworkers reported that BPTI interacts with the transporter Alr1p, blocking magnesium uptake into *S. cerevisiae* (Bleackley *et al.*, 2014). Surprisingly, we did not find

any mutations in the *alr1* gene in the BPTI-resistant strains. *ALR1* is an essential gene and it is possible that mutations that would block BPTI binding are also deleterious to the essential function of the gene product in cellular Mg^{2+} uptake and therefore are not viable or have a serious fitness penalty. However, Ptk2p and Sky1p both function in cation homeostasis and transport, the mutations in these genes likely prevent the release of magnesium and other cations out of the cell, protecting the yeast from limiting intracellular magnesium levels that would be caused by BPTI blocking of Alr1p. Mutations in *sky1* and *ptk2* may be easier routes to increase magnesium levels, without a serious impact on cell vitality.

BPTI and NaD1 retain synergy on resistant strains

Combination therapy with more than one antimicrobial is a potential mechanism to prevent the development of resistance in pathogenic microorganisms. In some cases, there is the added advantage that the two molecules work in synergy, reducing the inhibitory concentrations of the antimicrobial molecules below the levels predicted from their additive effect. We have reported previously that BPTI and NaD1 act synergistically to kill fungi (Bleackley *et al.*, 2017). Further investigation into the mechanism of synergy between NaD1 and BPTI revealed that the protease inhibitory activity of BPTI was not required for synergy and that BPTI also acted synergistically with other non-defensin antifungals (Bleackley *et al.*, 2017). It was hypothesized that protease inhibitors such as BPTI, influence stress response pathways that alter the ability of the fungus to respond to NaD1, and synergy could result from overloading of these stress response pathways when exposed to both NaD1 and BPTI. We therefore assessed whether the BPTI and NaD1-resistant strains were susceptible to the synergistic activity of NaD1 and BPTI. The strains that were resistant to NaD1 or BPTI had their sensitivity to the respective antifungal restored once a small amount of the opposing molecule was added (Figure 4). This is likely because the mutations associated with

resistance are associated with mitigating the stress responses that occur after exposure to NaD1 or BPTI (Hayes *et al.*, 2014; McKenna, 2012). However, once the other peptide is added, these stress response pathways that normally enhance resistance are no longer effective. NaD1 resistant strains were sensitive to the cell wall stressor CFW (McColl *et al.*, 2018), whereas BPTI-resistant strains were resistant to CFW, this opposition in stress response may explain the rescue of antifungal synergy between BPTI and NaD1.

Conclusion

Antimicrobial peptides are often touted as an attractive alternative to small molecule antimicrobials because they are more robust in terms of the potential for resistance to emerge (Mookherjee *et al*, 2020). Rapid development of resistance to the antifungal activity of BPTI would seem to contradict this idea but is likely to be a reflection of the superiority of fungicidal molecules as antifungals compared to fungistatic molecules. The fact that the resistance that was developed to either NaD1 or BPTI could be reverted by the addition of very low concentrations of the partner peptide in synergy assays indicates that if AMPs were developed for clinical use any increase in pathogen tolerance may be easy to combat through the use of combinatorial therapies that inactivate the stress response related resistance mechanisms.

References

504 Bidstrup BP, Royston D, Sapsford RN, Taylor KM (1989) Reduction in blood loss and blood
505 use after cardiopulmonary bypass with high dose aprotinin (Trasylol). *The Journal of*
506 *thoracic and cardiovascular surgery* 97: 364-372

507 Bleackley MR, Dawson CS, McKenna JA, Quimbar P, Hayes BME, van der Weerden NL,
508 Anderson MA (2017) Synergistic Activity between Two Antifungal Proteins, the Plant
509 Defensin NaD1 and the Bovine Pancreatic Trypsin Inhibitor. *mSphere* 2: e00390-00317

510 Bleackley MR, Hayes BM, Parisi K, Saiyed T, Traven A, Potter ID, van der Weerden NL,
511 Anderson MA (2014) Bovine pancreatic trypsin inhibitor is a new antifungal peptide that
512 inhibits cellular magnesium uptake. *Mol Microbiol* 92: 1188-1197

513 Bleackley MR, Hayes, B. M., Parisi, K., Saiyed, T., Traven, A., Potter, I. D., van der
514 Weerden, N. L. and Anderson, M. A. (2014) Bovine pancreatic trypsin inhibitor is a new
515 antifungal peptide that inhibits cellular magnesium uptake. *Molecular Microbiology* 92:
516 1188–1197

517 Brown JA, Sherlock G, Myers CL, Burrows NM, Deng C, Wu HI, McCann KE, Troyanskaya
518 OG, Brown JM (2006) Global analysis of gene function in yeast by quantitative phenotypic
519 profiling. *Molecular Systems Biology* 2: 2006.0001

520 Burton MF, Steel PG (2009) The chemistry and biology of LL-37. *Natural Product Reports*
521 26: 1572-1584

522 Cherry JM, Hong EL, Amundsen C, Balakrishnan R, Binkley G, Chan ET, Christie KR,
523 Costanzo MC, Dwight SS, Engel SR *et al* (2012) Saccharomyces Genome Database: the
524 genomics resource of budding yeast. *Nucleic Acids Res* 40: D700-705

525 Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM
526 (2012) A program for annotating and predicting the effects of single nucleotide
527 polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-
528 2; iso-3. *Fly* 6: 80-92

529 Dracatos PM, van der Weerden NL, Carroll KT, Johnson ED, Plummer KM, Anderson MA
530 (2014) Inhibition of cereal rust fungi by both class I and II defensins derived from the flowers
531 of *Nicotiana alata*. *Molecular Plant Pathology* 15: 67-79

532 Engel SR, Dietrich FS, Fisk DG, Binkley G, Balakrishnan R, Costanzo MC, Dwight SS, Hitz
533 BC, Karra K, Nash RS *et al* (2014) The reference genome sequence of Saccharomyces
534 cerevisiae: then and now. *G3 (Bethesda, Md)* 4: 389-398

535 Furnary AP, Wu Y, Hiratzka LF, Grunkemeier GL, Page US, 3rd (2007) Aprotinin does not
536 increase the risk of renal failure in cardiac surgery patients. *Circulation* 116: I127-133

537 Hayes BM, Anderson MA, Traven A, van der Weerden NL, Bleackley MR (2014) Activation
538 of stress signalling pathways enhances tolerance of fungi to chemical fungicides and
539 antifungal proteins. *Cell Mol Life Sci* 71: 2651-2666

540 Hayes BME, Bleackley MR, Wiltshire JL, Anderson MA, Traven A, van der Weerden NL
541 (2013) Identification and Mechanism of Action of the Plant Defensin NaD1 as a New
542 Member of the Antifungal Drug Arsenal against Candida albicans. *Antimicrob Agents*
543 *Chemother* 57: 3667-3675

544 Hilpert K, Volkmer-Engert R, Walter T, Hancock RE (2005) High-throughput generation of
545 small antibacterial peptides with improved activity. *Nature biotechnology* 23: 1008-1012

546 Karkouti K, Beattie WS, Dattilo KM, McCluskey SA, Ghannam M, Hamdy A, Wijeysundera
547 DN, Fedorko L, Yau TM (2006) A propensity score case-control comparison of aprotinin and
548 tranexamic acid in high-transfusion-risk cardiac surgery. *Transfusion* 46: 327-338

549 Kumar A, Zarychanski R, Pisipati A, Kumar A, Kethireddy S, Bow EJ (2018) Fungicidal
550 versus fungistatic therapy of invasive Candida infection in non-neutropenic adults: a meta-
551 analysis. *Mycology* 9: 116-128

552 Lay FT, Brugliera F, Anderson MA (2003) Isolation and Properties of Floral Defensins from
553 Ornamental Tobacco and Petunia. *Plant Physiol* 131: 1283-1293

554 Mangano DT, Tudor IC, Dietzel C (2006) The risk associated with aprotinin in cardiac
555 surgery. *The New England journal of medicine* 354: 353-365

556 McColl AI, Bleackley MR, Anderson MA, Lowe RGT (2018) Resistance to the Plant
557 Defensin NaD1 Features Modifications to the Cell Wall and Osmo-Regulation Pathways of
558 Yeast. *Frontiers in Microbiology* 9

559 McCormack PL, Perry CM (2005) Caspofungin: a review of its use in the treatment of fungal
560 infections. *Drugs* 65: 2049-2068

561 McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K,
562 Altshuler D, Gabriel S, Daly M *et al* (2010) The Genome Analysis Toolkit: A MapReduce
563 framework for analyzing next-generation DNA sequencing data. *Genome Research* 20: 1297-
564 1303

565 McKenna JA, 2012. In planta activity of the antifungal defensin NaD1 and molecules that act
566 in synergy with NaD1, School of Botany. The University of Melbourne, Melbourne.

567 McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GRS, Thormann A, Flicek P, Cunningham F
568 (2016) The Ensembl Variant Effect Predictor. *Genome Biology* 17: 122

569 McMullan V, Alston RP (2013) III. Aprotinin and cardiac surgery: a sorry tale of evidence
570 misused. *British journal of anaesthesia* 110: 675-678

571 Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ (2020) Antimicrobial host
572 defence peptides: functions and clinical potential. *Nature Reviews Drug Discovery* 19: 311-
573 332

574 Ordonez SR, Amarullah IH, Wubbolts RW, Veldhuizen EJA, Haagsman HP (2014)
575 Fungicidal Mechanisms of Cathelicidins LL-37 and CATH-2 Revealed by Live-Cell
576 Imaging. *Antimicrobial Agents and Chemotherapy* 58: 2240

577 Parisi K, Shafee TMA, Quimbar P, van der Weerden NL, Bleackley MR, Anderson MA
578 (2019) The evolution, function and mechanisms of action for plant defensins. *Seminars in*
579 *Cell & Developmental Biology* 88: 107-118

580 Payne JA, Bleackley MR, Lee TH, Shafee TM, Poon IK, Hulett MD, Aguilar MI, van der
581 Weerden NL, Anderson MA (2016) The plant defensin NaD1 introduces membrane disorder
582 through a specific interaction with the lipid, phosphatidylinositol 4,5 bisphosphate.
583 *Biochimica et biophysica acta* 1858: 1099-1109

584 Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP
585 (2011) Integrative Genomics Viewer. *Nature biotechnology* 29: 24-26

586 Royston D, Bidstrup BP, Taylor KM, Sapsford RN (1987) Effect of aprotinin on need for
587 blood transfusion after repeat open-heart surgery. *Lancet (London, England)* 2: 1289-1291

588 Sherry ST, Ward M-H, Kholodov M, Baker J, Phan L, Smigielski EM, Sirotkin K (2001)
589 dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* 29: 308-311

590 Tsai P-W, Cheng Y-L, Hsieh W-P, Lan C-Y (2014) Responses of Candida albicans to the
591 human antimicrobial peptide LL-37. *Journal of Microbiology* 52: 581-589

592 Van der Linden P, De Hert S, Daper A, Trenchant A, Jacobs D, De Boelpaepe C, Kimbimbi
593 P, Defrance P, Simoens G (2001) A standardized multidisciplinary approach reduces the use
594 of allogeneic blood products in patients undergoing cardiac surgery. *Canadian journal of*
595 *anaesthesia = Journal canadien d'anesthesie* 48: 894-901

596 van der Weerden NL, Hancock REW, Anderson MA (2010) Permeabilization of Fungal
597 Hyphae by the Plant Defensin NaD1 Occurs through a Cell Wall-dependent Process. *J Biol*
598 *Chem* 285: 37513-37520

599 van der Weerden NL, Lay FT, Anderson MA (2008) The Plant Defensin, NaD1, Enters the
600 Cytoplasm of Fusarium Oxysporum Hyphae. *J Biol Chem* 283: 14445-14452

601 Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, Bangham R,
602 Benito R, Boeke JD, Bussey H *et al* (1999) Functional characterization of the *S. cerevisiae*
603 genome by gene deletion and parallel analysis. *Science* 285: 901-906

604 Yoshikawa K, Tanaka T, Furusawa C, Nagahisa K, Hirasawa T, Shimizu H (2009)
605 Comprehensive phenotypic analysis for identification of genes affecting growth under
606 ethanol stress in Saccharomyces cerevisiae. *FEMS yeast research* 9: 32-44
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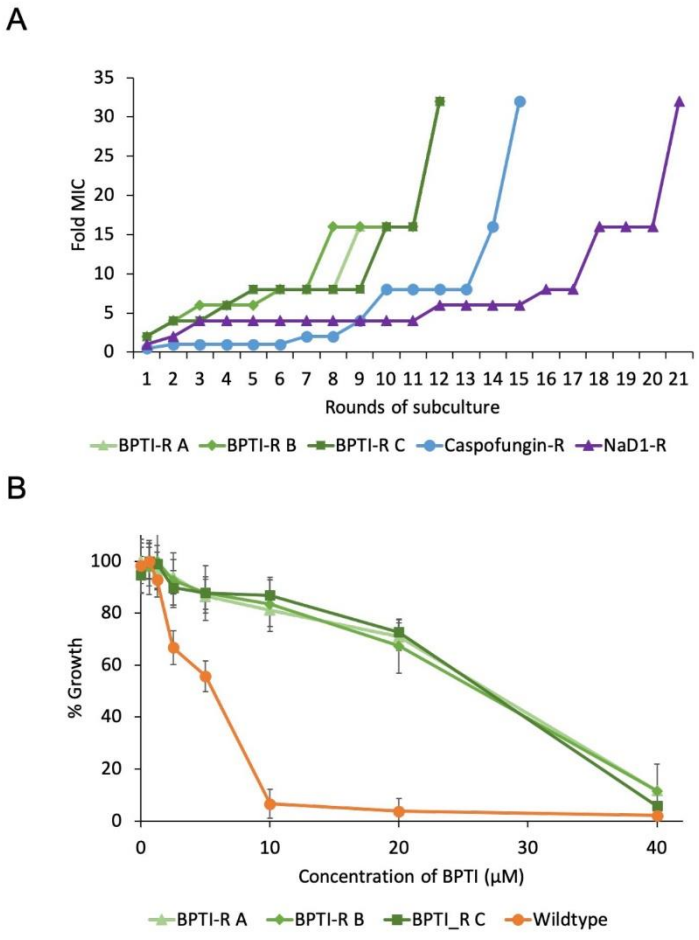
608 ## Figures and legends



609

610 **Figure 1. (A) Resistance to BPTI develops more quickly than resistance to caspofungin or NaD1.** Three
611 independent cultures of BPTI-resistant yeast are shown, along with a representative example of the caspofungin
612 and NaD1-resistant lines described in our previous paper (McColl *et al.*, 2018). Resistance to BPTI developed

steadily with the MIC increasing 32-fold after 12 rounds of growth. It took 15 rounds of subculturing to produce an equivalent level of resistance to caspofungin and 21 rounds for NaD1.

(B) Confirmation of BPTI resistance. Growth of BPTI-resistant strains of *S. cerevisiae* once selection pressure was removed and independent isolates were selected, at various concentrations of BPTI compared to the BPTI inhibition of the wildtype *S. cerevisiae* control. BPTI-resistant strains were 4-fold more resistant than wildtype. Growth % is relative to the untreated control for each strain. Error bars represent +/- standard error of the mean ($n = 3$).

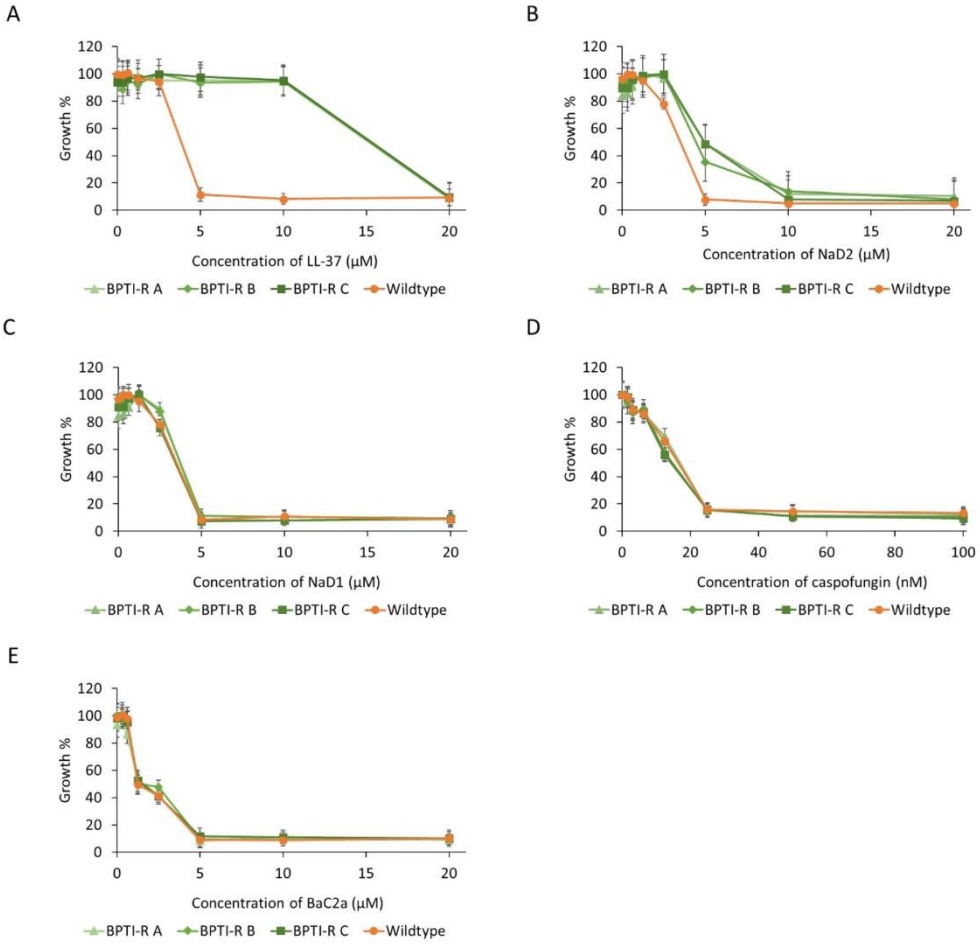


Figure 2. **Resistance to BPTI is not broad spectrum.** Growth inhibition of BPTI-resistant strains by a selection of antimicrobial peptides of different origin and mechanisms of action. The peptides examined were **(A) LL-37, (B) NaD2, (C) NaD1, (D) caspofungin** and **(E) Bac2a**. BPTI-resistant strains were more tolerant than the wildtype cells to the antifungal peptides LL-37 (A) and NaD2 (B), but not to the antifungals NaD1 (C), caspofungin (D) and Bac2a (E). Growth % is relative to the untreated control for each strain. All experiments were performed in triplicate. Error bars represent +/- one standard error of the mean (n= 3).

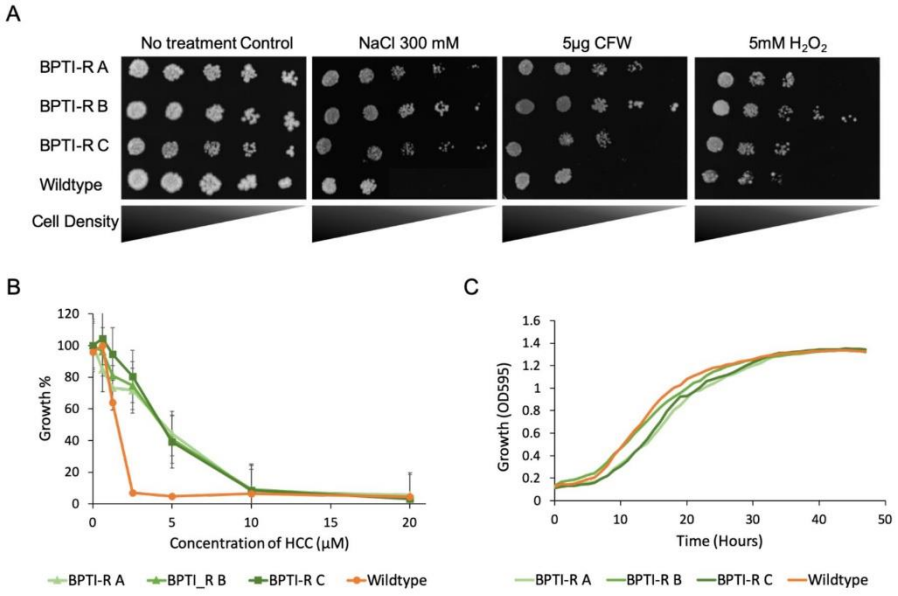


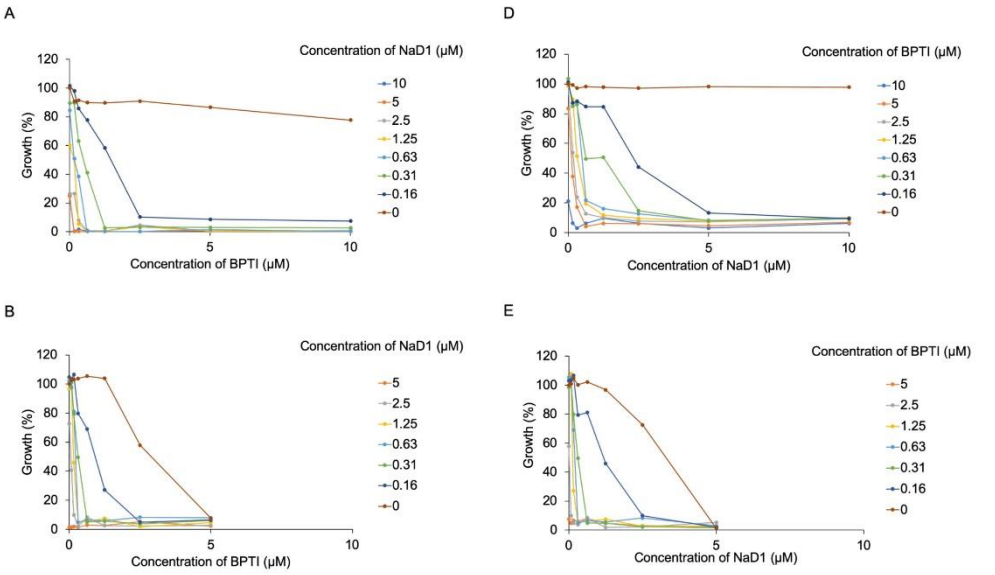
Figure 3. **Fitness of BPTI resistant strains in the presence of various abiotic stressors.**

(A) BPTI-resistant strains were more tolerant to sodium chloride, calcofluor white and hydrogen peroxide than wildtype cells. BPTI-resistant and wild-type *S. cerevisiae* BY4741 cells were diluted and spotted out on to YPD Agar containing different concentrations of abiotic stressors. A no treatment control was prepared using the same cell preparations and at the same time as the treatment plates. The three BPTI-resistant strains grew better than wildtype in the presence of 300mM of NaCl BPTI-resistant strains were more resistant to 5µg/mL CFW compared to wild type. BPTI-resistant strain B grew better than wildtype in the presence of

5mM hydrogen peroxide whereas BPTI-resistant strains A and C had similar growth to wildtype. Images are representative of three replicate experiments.

(B) BPTI-resistant strains were more tolerant to Hexamine (III) cobalt chloride than wildtype. Growth inhibition of BPTI-resistant strains by Hexamine (III) cobalt chloride (HCC) relative to wildtype control. BPTI-resistant strains were more tolerant to the magnesium channel inhibitor HCC with an MIC of 10 µM compared to wild type at 2.5 µM. Growth % is relative to the highest measured absorbance for each strain and the wild-type *S. cerevisiae* BY4741. Error bars represent +/- standard error of the mean (n= 3).

(C) Cell growth of BPTI-resistant strains in YPD media compared to wild-type *S. cerevisiae* BY4741. There was no difference in growth of the BPTI-resistant strains in YPD compared to wildtype.



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Strain	MIC BPTI (μM)		FIC
	0mM NaD1	0.16mM NaD1	
Wildtype	5	2.5	0.23
BPTI-R	>10	2.5	0.13

F

Strain	MIC NaD1 (μM)		FIC
	0mM BPTI	0.16mM BPTI	
Wildtype	5	2.5	0.23
NaD1-R	>10	5	0.19

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650

651 Figure 4. **Synergy between NaD1 and BPTI on BPTI and NaD1 resistant strains is similar to wildtype**.

652 In the absence of NaD1, growth of BPTI-R was not inhibited by BPTI at concentrations up to 10 uM ((A) brown line) whereas growth of wildtype was fully inhibited at 5 uM
653 BPTI ((B) brown line). However, when NaD1 was added at concentrations as low as 0.16 uM the growth inhibition of BPTI-R ((A) dark blue line) reverted to that observed
654 for wildtype ((B) dark blue line). The MIC and FIC values for wildtype and BPTI-R in the presence and absence of the synergy partner are presented in a table (C). In the
655 absence of BPTI, growth inhibition of NaD1-R was not inhibited by NaD1 at concentrations up to 10 uM ((D) brown line) whereas growth of wildtype was fully inhibited at
656 5 uM NaD1 ((E) brown line). When BPTI was added at concentrations as low as 0.16 uM the growth inhibition of NaD1-R ((D) dark blue line) reverted to that observed for
657 wildtype ((E) dark blue line). The MIC and FIC values for wildtype and NaD1-R in the presence and absence of the synergy partner in are presented in a table (F). A
658 comparison of the FIC, which is indicative of the strength of synergy, between wildytype, NaD1-R and BPTI-R (C and F) revealed that the level of synergy between NaD1
659 and BPTI is not affected by the mutations that lead to increased tolerance to each protein alone.

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Table list

Strain	Rep 1 MIC (μg/mL)	Rep 2 MIC (μg/mL)	Average MIC (μg/mL)
S. cerevisiae	12.5	12.5	12.5
C. neoformans	12.5	6.25	9.4
C. albicans	100	100	100
T. rubrum	>200	N/A	>200
A. fumigatus	>200	N/A	>200

Table 1. **BPTI is effective against yeast but not filamentous fungi.** The activity of BPTI was tested against *S. cerevisiae*, *C. neoformans*, *C. albicans*, *T. rubrum* and *A. fumigatus*. BPTI was more effective against *S. cerevisiae* and *C. neoformans* than *C. albicans* and was not effective against *T. rubrum* or *A. fumigatus*. Standard error is based on 2 biological replicates for each yeast and 1 biological replicate for filamentous fungi.

Gene Name	Amino Acid Change	BPTI-R strain containing variant	Inference	Biological function/pathway
ERG3	Ser24 to Leu	A	Missense variant	Ergosterol biosynthesis
GDA1	Cys462 to Phe	A	Missense variant	Transport of GDP-mannose into Golgi
GEX1	upstream	A and B	Upstream gene variant	Exports glutathione across the vacuole and plasma membrane.
NRP1	Asn444 to Ser	B	Tolerated missense variant	Putative RNA binding protein of unknown function
PTK2	Gly469 to STOP	A and C	Disruptive premature stop	Regulation of ion transport across plasma membrane
SKY1	Gln65 to STOP	B	Disruptive premature stop	Regulate mRNA metabolism and cation homeostasis
SOK1	upstream	C	Upstream	Protein of unknown function
tT(XXX)Q2	upstream	C	Upstream INDEL	Mitochondrial threonine tRNA
YBR298C-A	upstream	A,B,C	Upstream	Unknown

Table 2. **Summary of variants present in BPTI-R strains.** The genes that have mutations linked to BPTI resistance and the description of their role in *S. cerevisiae*. The program Variant effect predictor was used to discover the impact of mutations on gene function. Variants were selected if mutations impacted the protein coding region and were present in the resistant strains and absent from the control strains. Upstream gene variants were not selected for further analysis. These genes may be viewed on the Saccharomyces Genome Database www.yeastgenome.org (Cherry *et al*, 2012; Engel *et al.*, 2014).

683

Strain	MIC (μM)
BPTI-R A	40
BPTI-R B	40
BPTI-R C	40
Wildtype	10
ERG3 KO	2.5
GDA1 KO	5
NRP1 KO	10
PTK2 KO	40
SKY1 KO	40

684

685 Table 3. **Comparison of BPTI activity against single-gene deletion strains representing key resistance**
686 **variants.** The activity of BPTI against BPTI-resistant strains A, B and C, wild-type *S. cerevisiae* BY4741 and
687 the gene knock outs of ERG3, GDA1, NRP1, PTK2 and SKY1 in ½ PDB. This is a representative example of
688 three independent experiments, the MIC was the same across all experiments.

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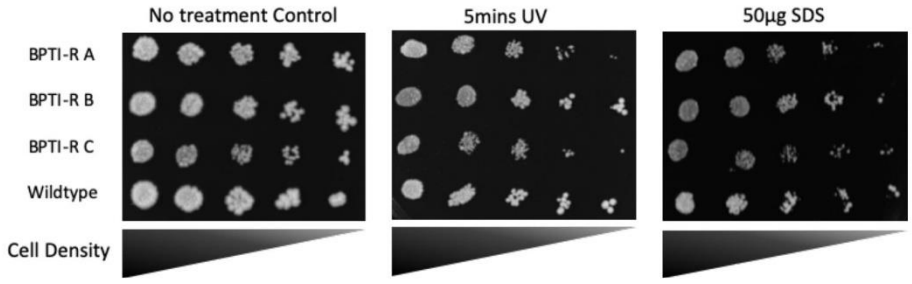
700 # Supplementary figure list



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702 Supplementary figure A. **BPTI-resistant strains showed no difference in sensitivity to UV or SDS to the**
703 **wildtype strain.** (left) No treatment control plate that was incubated at the same time as the treatment plates.
704 (middle) BPTI-resistant and wild-type *S. cerevisiae* BY4741 cells were diluted and spotted out on to YPD agar,
705 before exposure to UV for 5min. There was no difference in sensitivity between the BPTI-resistant strains,
706 control plate, and wild type, when exposed to UV. (right) There is no difference in sensitivity of the BPTI-
707 resistant strains to SDS compared to the controls and wild type.

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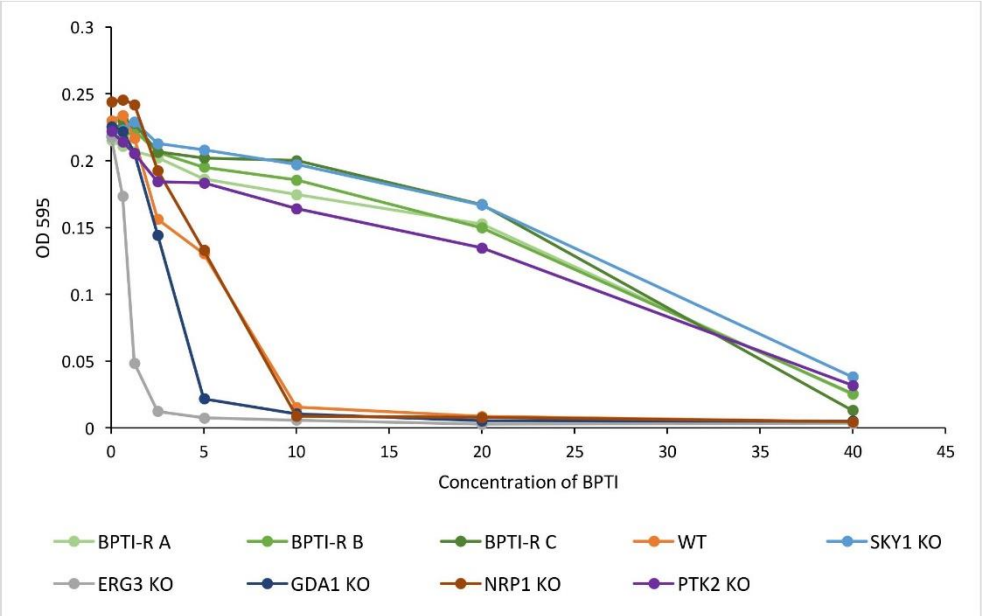


Supplementary figure B. **Comparison of BPTI activity against single-gene deletion strains representing key resistance variants.** The activity of BPTI against BPTI-resistant strains A, B and C, wild-type *S. cerevisiae* BY4741 and the gene knock outs of ERG3, GDA1, NRP1, PTK2 and SKY1, the antifungal assay was performed in ½ PDB with the data being a representative example of three independent experiments, with all experiments having similar MIC.

Chapter Four

4. STUDY 3 – Mechanism of resistance to NaD1 and LL-37 in *C. albicans*

The findings of this chapter have been drafted as a manuscript ready for submission. This manuscript has not yet been submitted to a journal.

McColl AI, Lowe RGT, Anderson AA, Bleackley MR (Unpublished), Sequence wide characterisation of resistance to AMPs in *C. albicans.*

Statement of Contribution

Amanda McColl has made the following contributions:

- Design and experimental completion of data collection
- Analysis and interpretation of all data,
- Preparation of methods and figures for the manuscript.
- Drafting of manuscript prior to submission

Other authors have made the following contributions:

- Bleackley MR, Anderson MA, and Lowe RGT contributed to the conception and design of the experiments, data analysis and manuscript revisions.

Sequence wide characterisation of resistance to antimicrobial peptides in *Candida albicans.*

Authors: **Amanda McColl, Rohan Lowe, Marilyn Anderson, Mark Bleackley.**

Abstract

Treatment of *C. albicans* infections primarily uses antifungal agents from four distinct major classes; azoles, polyenes, pyrimidine analogues, and echinocandins (Revie *et al*, 2018). Resistance to almost all major antifungal agents, has been reported in clinical isolates of *C. albicans* (Robbins *et al*, 2017). Resistance combined with the increased incidence of fungal diseases has created the need for new antifungals with different mechanisms of action to broaden treatment options. Antimicrobial peptides produced by plants and animals are promising new agents because they have different mechanisms of action to current antifungals and are often targeted specifically to fungal pathogens (van der Weerden *et al*, 2013). A key step in the development of novel antifungals is their sustainable use and hence an understanding of the potential for fungi to develop resistance. In this study, *Candida albicans* was serially passaged in the presence of two cationic AMPs, the plant defensin NaD1 and the human cathelicidin LL-37, as well as the azole antifungal itraconazole. Resistance to NaD1 and LL-37 developed more slowly and to a lower magnitude compared to itraconazole. Characterisation of the resistant strains via whole genome sequencing revealed that resistance to itraconazole was attributable to mutations in *ERG11* that were present in all 11 resistant isolates. In contrast, several different genes were mutated in the NaD1 and LL-37-resistant isolates. Resistance to LL-37 was linked to mutations that impacted extracellular cation accumulation, increased efflux of LL-37 via transmembrane transporters and affected beta-glucan masking. There is a potential multi-step mechanism to NaD1 resistance involving beta-glucan masking, metal ion transport and cation homeostasis,

DNA damage mediation and the prevention of binding to potential intracellular targets such as the Golgi or mitochondria. This confirms the hypothesis that resistance to antimicrobial peptides occurs through an accumulation of multiple mutations in different genes whereas resistance to small molecule drugs often arises from mutations in a single target.

Introduction

Infections due to *Candida* species are major causes of morbidity and mortality and are associated with a wide variety of clinical manifestations ranging from superficial and mucosal infections to widely disseminated and bloodstream infections (Pfaller & Diekema, 2010). Invasive candidiasis is estimated to occur in more than three-quarters of a million patients globally every year (Bongomin *et al*, 2017). Antifungal resistance in *C. albicans* has been reported after long-term antifungal use and treatment of recurrent infections, such as treatments of chronic mucocutaneous candidiasis or recurrent oropharyngeal candidiasis in patients with uncontrolled human immunodeficiency virus infection (Fisher et al, 2018; Pfaller et al, 2010). Within the limited antifungal treatment options, the azole antifungals are the most frequent class used to treat *Candida* infections. Azole antifungals such as fluconazole and itraconazole are often the preferred treatment for *Candida* infections because they are inexpensive, easily manufactured, and are available for oral administration (de Oliveira Santos *et al*, 2018) Azoles act by inhibiting the fungal-mediated synthesis of ergosterol, via inhibition of lanosterol 14α-demethylase (Ghannoum & Rice, 1999). Despite their regular administration, there is extensive documentation of intrinsic and developed resistance to azole antifungals among several *Candida* species (Revie *et al.*, 2018). Emergence of antifungal resistance can endanger the already limited treatment options, with serious effects on patient treatment outcomes (Perlin *et al*, 2017). There is an increased

interest in peptides as a promising approach in discovery and development of novel antifungal agents (Mookherjee *et al*, 2020). Peptides have promising properties, such as moderate immunogenicity as described below, strong antimicrobial activity, high specificity and affinity for targets, distinct mechanisms of action, good organ and tissue penetration and broad-spectrum activity (Raheem & Straus, 2019; van der Weerden *et al.*, 2013). The mechanism of action of AMPs is still being elucidated (Mookherjee *et al.*, 2020). They often have multistep mechanisms that affect more than one target in the fungus (Mookherjee *et al.*, 2020; Parisi *et al*, 2018). Hence, it is expected that resistance to AMPs is likely to develop more slowly than resistance to smaller antifungal molecules that interact with a single site, composed of a few amino acids, on a single protein target (Leeuw *et al*, 2010; Magana *et al*, 2020; McColl *et al*, 2018; Schneider *et al*, 2010).

NaD1 is a potent antifungal defensin that accumulates in the flowers of the ornamental tobacco plant *Nicotiana alata*, where it functions to protect the reproductive organs from damage by fungal pathogens (Lay *et al*, 2003). NaD1 has a well-characterized structure, and several features of its mechanism of action have been well described but not completely elucidated (Lay *et al*, 2012; Parisi *et al.*, 2018). NaD1 has at least a three-step mechanism of action that involves: interaction with the fungal cell wall (van der Weerden *et al*, 2008), movement across the plasma membrane, induction of oxidative stress, and interaction with phosphatidylinositol 4,5 bisphosphate. These processes lead to damage of the inner leaflet of the cell membrane and cell death within 10 min of exposure to NaD1 (Hayes *et al*, 2014; Payne *et al*, 2016; Poon *et al*, 2014; van der Weerden *et al*, 2010). Previous studies on the mechanisms of resistance to NaD1 have been conducted with the model organism *S. cerevisiae*. Screening of an *S. cerevisiae* knockout collection identified an *agp2*Δ mutant that conferred resistance to NaD1 and other cation peptides, whereby resistance was mediated by an accumulation of positive charges at the cell surface that repelled positively charged

antifungal peptides (Bleackley, 2014). Resistance to NaD1 has also been assessed by serial passaging *S. cerevisiae* in the presence of sub-lethal amounts of NaD1 (McColl *et al.*, 2018). The yeast strains slowly developed tolerance to NaD1 via an accumulation of single nucleotide mutations, but there was a fitness penalty associated with tolerance. Sequencing the genomes of the NaD1 tolerant strains failed to identify any 'hotspot' mutations associated with the increased tolerance and led to the identification of 12 genes that contributed to the tolerance. One of the genes identified, *FPS1*, revealed that there is a common mechanism of resistance to NaD1 that involves the osmotic stress response pathway. LL-37 is the only human cathelicidin (Gennaro & Zanetti, 2000). It forms an amphipathic α-helix and binds to the cell wall and plasma membrane of *C. albicans* (den Hertog *et al*, 2005). One of the potential targets for LL-37 in *C. albicans* is the β-1,3-exoglucanase Xog1p (Tsai *et al*, 2011; Tsai *et al*, 2014). Binding of LL-37 leads to complete disruption of the *C. albicans* cell membrane leakage of proteins of up to 40 kDa into the medium (den Hertog *et al.*, 2005; Tsai *et al.*, 2014). The kinetics of permeabilization are very rapid with complete lysis occurring within 5 min, supporting the idea that membrane disruption is the main direct mechanism of cathelicidin activity (den Hertog *et al.*, 2005; Shahmiri *et al*, 2016).

In this study, we compare the development of resistance in *C. albicans* to the cationic AMPs NaD1 and LL-37, and to the clinical small molecule itraconazole. Eleven resistant mutants for each antifungal and 11 no treatment control lines were obtained and characterised further. Resistance to NaD1 and LL-37 developed more slowly requiring 20 rounds of selection to reach a 20-fold increase in MIC and 16 rounds to reach 40-fold increase in MIC, compared to itraconazole where a 60-fold increase in MIC was obtained after only 12 rounds of selection. Whole genome sequencing of all resistant isolates revealed that resistance to itraconazole could be attributed to mutations located directly upstream and within protein coding regions of *ERG11* that were present in all 11 resistant isolates. In contrast, all of the NaD1 or LL-37-

resistant isolates did not share the same mutations, supporting the hypothesis that resistance to antimicrobial peptides occurs through an accumulation of mutations in different genes, whereas small molecule resistance is often associated with a single mutation that directly affects the drug target.

Materials and Methods

Fungal Strains

Candida albicans BWP17 was maintained on YPD-Agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) medium at 30°C. Starter cultures were grown in liquid YPD at 30°C.

Antifungal molecules

The NaD1 defensin was extracted and purified from *N. alata* flowers as described by van der Weerden and colleagues (Van Der Weerden et al., 2008). The protein was purified further using reversed phase high-performance liquid chromatography (RP-HPLC) with a C8 Agilent column as described previously (Lay et al., 2003). LL-37 was purchased from Genscript (Hong Kong). Itraconazole was purchased from Sigma (Australia).

Serial passaging of *Candida albicans* in the presence of antifungal molecules

C. albicans BWP17 was grown overnight at 30°C with agitation in 20 mL of YPD. The overnight culture was then diluted to an OD 600 nm of 0.01 in 50% strength PDB (Potato Dextrose Broth) medium (½ PDB) and 80uL were aliquoted into each of the wells in four 96 well plates. Each plate represented one treatment group; NaD1, LL-37, itraconazole or the no

121 treatment control. Each column represented an individual biological (1-11) replicate with a
122 two-fold serial dilution series of the antifungal molecules with a lowest concentration of 0.5x
123 MIC (as determined in preliminary antifungal assays) on the bottom row and the highest at
124 the top (A-H). The plates were incubated for 48h at 30°C with agitation. Cells that grew at
125 the highest concentration of each of the antifungal molecules were diluted to an OD 600 nm
126 of 0.01 in 50% strength PDB medium (½ PDB) and 80 uL were sub-cultured into a fresh
127 column of wells in a 96 well plate with a higher concentration range of the antifungal
128 molecule. Sub-culturing was stopped once growth occurred at a minimum of 40 times the
129 original MIC or passaging had reached 20 rounds.

130

131 **Single Colony Isolation of Resistant Strains**

132 Cultures from each biological replicate (11 for each treatment group) that were more tolerant
133 to the antifungal molecule were streaked out for single colonies on non-selective YPD agar.
134 Three colonies were picked from each replicate, and their resistance was re-tested in a
135 microbroth dilution assay as described below. The colony with the greatest resistance to the
136 respective antifungal was retained for further experimentation. In total, there were 11 NaD1-
137 resistant isolates, 11 LL-37-resistant isolates, 11 Itraconazole-resistant isolates and 11 No-
138 treatment controls.

139

140 **Antifungal Assay**

141 Antifungal assays were performed as described in Hayes et al. (2013). Briefly, cultures were
142 grown overnight (30°C, 250 rpm) in 5 mL YPD and diluted to an OD600 of 0.01 in ½ PDB.
143 Antifungal molecules were prepared at 10x the assay concentration, and 10 µL was mixed
144 with 90 µL of diluted yeast culture before incubation for 30 h at 30°C. The final OD600 was

145 measured using a SpectraMAX M5e plate reader (Molecular Devices). Percentage growth is
146 calculated for each treatment group at each concentration range using the formulae
147 (Maximum growth value - Growth value at *x* concentration)*100.

148

149 **DNA extraction, libarary preparation and whole genome sequencing**

150 Genomic DNA was extracted using the Qiagen DNeasy® plant miniprep kit. Eleven
151 individual lines of NaD1, LL-37, itraconzole-resistant strains and eleven lines of the no-
152 treatment controls were processed. Genomic libraries were prepared using the NebNext
153 Multiplex library kit following standard protocol. Sequencing was completed at the La Trobe
154 Genomics Platform, using Illumina NextSeq V3 chemistry. One run on four lanes was
155 performed for all 44 genomes, generating 20 gigabase's total with 150 bp paired-end reads.

156

157 **Genomic Analysis**

158 The pre-processing and variant discovery steps were performed as described by the GATK
159 best practices and are summarized in (McKenna *et al*, 2010) and are as follows:

160 **Pre-processing**

161 Picard tools (v.2.4.1) fastqtosam was used to convert raw sequence files into Sam format and
162 to add read group information. Any Illumina adapters were identified and marked using
163 Picard (v.2.4.1) markilluminaadapters. BWA-mem (v.0.7.12) was used to align reads to the
164 wildtype parent strain of BWP17, the *C. albicans* SC5134 Assembly 22 (version A22-s07-
165 m01-r125) reference genome (Skrzypek *et al*, 2017). Alignment files were merged, and
166 duplicate reads were marked using Picard (v.2.4.1) mergebamalignment and markduplicates.

Local alignments were optimized, and sequence quality scores were recalibrated using GATK (v.3.6) realignertargetcreater and baserecalibrator.

Variant Discovery

GATK (v.3.6) Haplotypecaller was used to find genome variations within the diploid genomes that were either SNVs (single-nucleotide variants) or INDELs (insertion/deletion) simultaneously, also using known variants from dbSNP (Sherry *et al*, 2001). The samples were merged using GATK (v.3.6) combinegvcf, and then GenotypeGVCFs was used to rescore and genotype the combined gVCFs. GATK (v.3.6) VariantFiltration and VariantRecalibrator were used to extract SNVs and indels from the combined call set based on the default quality parameters, the SNVs and indels were then labelled as passed or filtered.

Variant Refinement

The high-quality variants identified during the variant discovery process were annotated using SnpEff (v.2.4) (Cingolani *et al*, 2012). SnpEff was used to determine whether each mutation was predicted to alter an encoded protein sequence. Variant effect predictor (VEP) marked any codon changes as either tolerant or deleterious (McLaren *et al*, 2016). SnpSift (v.2.4) was used to identify SNVs or indels that were present in antifungal-resistant replicates and not in the control strains. Due to the number of mutations present, homozygous mutations were prioritised and assessed further. The variants selected during refinement were inspected manually using IGV (v.2.3.77) to rule out unexpected processing artifacts (Robinson *et al*, 2011). CGD Batch (Skrzypek *et al.*, 2017) was used to identify the names and functions of the mutated genes. The distribution of genes with similar functions across all resistant strains was determined and graphed using Jvenn (Bardou *et al*, 2014). AmiGo (Carbon *et al*, 2009)

functional characterisation was used to determine groups of genes that impacted similar functions in NaD1 and LL-37-resistant strains.

Results

Resistance to antimicrobial peptides develops slowly

C. albicans strains with increased tolerance to NaD1, LL-37 or itraconazole were developed by continuous culture in sub-lethal concentrations of each antifungal molecule. The rate of resistance development varied with each of the antifungal drugs. A forty-fold increase in MIC was achieved in all 11 isolates for each drug treatment after 8 rounds of sub-culture in itraconazole, 13 rounds for LL-37 and 20 rounds for NaD1. Resistance to itraconazole continued to increase relatively rapidly reaching a 140-fold increase in MIC after 13 rounds of subculture. Resistance to LL-37 developed more slowly reaching a 60-fold increase in MIC after 16 rounds of subculture. Resistance development was slowest with NaD1 which was not taken past 20 rounds of subculture.

Eleven single isolate strains of each of the NaD1, LL-37 and itraconazole-resistant lines were isolated after subculture rounds 20, 16 and 14 respectively (figure 1), and their resistance phenotype was confirmed using a standard antifungal growth assay. Once selection pressure was removed it was expected that the MIC of the resistant isolates would decrease. The colony with the most resistance for each line was used for all further experimentation. The NaD1-resistant isolates were resistant to a 20-fold higher concentration of NaD1 than the no-treatment control lines that had been passaged at the same time, with an MIC of 80 μM compared to the original MIC of 4 μM (Figure 2A). The LL-37-resistant isolates had a 30-

213 fold higher MIC of 200 μM compared to the no treatment control with an MIC of 6 μM
214 (Figure 2B). Finally the itraconazole-resistant isolates were resistant to a 50-fold higher
215 concentration of itraconazole with an MIC of 50 μM compared to the MIC of 1 μM for the no
216 treatment control (Figure 2C). The no-treatment control lines did not obtain any resistance
217 during selection.

218

219 **General genetic characterisation of resistant lines**

220 The genomes of each of the NaD1, LL-37, itraconazole-resistant and non-selected control
221 lines were sequenced to identify mutations that were exclusive to the resistant lines. SNVs or
222 indels were identified by mutations that were present in the antifungal-resistant strains and
223 not in the no-treatment control strains or the starting BWP17 strain that was sequenced at the
224 same time.

225

226 When assessing total number of variants across all 11 strains, itraconazole-resistant strains
227 that had developed resistance the fastest had the least number of mutations 134 after 14
228 rounds, next was LL-37 that had 166 mutations after 16 rounds, and NaD1 which developed
229 resistance the slowest had the highest number of mutations, 213 at 20 rounds (Table 1). In
230 this study, we focused on mutations that were both upstream and within the protein coding
231 regions of genes and excluded mutations downstream and within intergenic regions of genes
232 (Table 1). This was done because the impact of downstream and intergenic regions on protein
233 function is more difficult to predict.

234

235 The mutations in all the resistant strains were compared to identify genes that had been
236 impacted in all strains and thus were likely to be involved in enhancing protective general
237 stress responses rather than response to a specific class of antifungals. The impacted upstream
238 and protein coding regions of genes present across all resistant strains were identified using
239 Jvenn (Figure 3). There were four genes that had mutations in all treatment-resistant strains
240 (*PGA18, OFD1, ADE6* and *CR_04760C*). The NaD1 and LL-37-resistant group were most
241 similar, with 13 genes that were impacted by mutations across both strains (Table 2).

242

243 CGD gene function annotation revealed that more than 60% of the genes that were identified
244 were either unannotated, annotated as encoded proteins of unknown function, or did not have
245 orthologs in other species. These genes were excluded from further genetic characterisation
246 and functional annotation, because their role in enhancing resistance against a broad range of
247 antifungals could not be elucidated without knowledge of their function.

248

249 **Genetic characterisation of the LL-37-resistant strains**

250 LL-37-resistant lines had a total of 37 annotated mutated genes across all strains (Figure 4).
251 The mutations mostly impacted cell wall biosynthesis and structure, transmembrane
252 transporters, and cation transport. Unlike small molecule resistance, there were no "hot-spot"
253 mutations identified in any of the genes in the LL-37-resistant strains. Further functional
254 characterisation using AmiGo revealed common functions in the genes that were mutated in
255 the strains with LL-37 resistance (Figure 5). Mutations were identified upstream of genes
256 *PDR16, C1_13280C, ALR1, C3_04260W, CTP1, C4_02510W, ATM1, CR_04760C,* and
257 *CR_08200C* all of which function in cation transport. Mutations upstream of genes
258 *C1_13280C, ALR1, CTP1, C4_02510W, SGE1, C6_01400W, ATM1, HGT18, CR_04760C,*

and *CR_08200C_A* are likely to have an impact on transmembrane transporter activity. Mutations upstream of genes *CTP1, C4_02510W, CR_04760C*, and *CR_08200C* impact carboxylic acid, organic anion, and organic acid transmembrane transporter activity. Mutations upstream of *HOC1* and *ALG2* impact alpha-1-6-mannosyltransferase activity. The AmiGo annotations and P values are presented in Table 4.

Genetic characterisation of NaD1-resistant strains

There were 40 annotated mutated genes across the NaD1-resistant isolates (Figure 6). These mutations mostly impacted genes with functions in the cell wall, metal ion transport, mitigation of DNA damage, and trafficking to the Golgi or mitochondria. There were no "hot-spot" mutations identified in any of the NaD1 resistant strains. Further functional characterisation using AmiGo revealed that some genes impact similar functions (Figure 7). Mutations upstream of genes *C3_01680C, ALR1, C3_04260W, RBT5* and *ECM7* all impact metal ion transport function. *RHD1, SMP3, HOC1, ALG2, MNT4* impact glycosyl-transferase activity. Mutations in *RHD1, SMP3, HOC1* and *ALG2* impact mannosyltransferase activity. Mutations in genes *PSY2* and *PPH3* impact the regulation of the glucose -mediated signalling pathway, regulation of DNA damage checkpoint and DNA double -stranded break repair. The AmiGo annotations and P values are listed in Table 5.

Genetic characterisation of Itraconazole-resistant strains

SNV and Indel assessment of the Itraconazole resistant lines revealed mutations both upstream and within the protein coding region of *ERG11*. These are presented in Figure 8. The mutations were located at Y132H in strains A, B, and F, *S279F* (C, I, J), *S405F* (E, G),

and *R467K* (D, H, K) (Figure 10). There were also mutations in other ergosterol biosynthesis genes upstream of *ERG251* and *ERG26* that have not been documented previously (Figure 8).

Discussion

A key step in the development of novel antifungals is an understanding of the potential for fungi to develop resistance. We have serially passaged *C. albicans* in the presence of two cationic AMPs, the plant defensin NaD1 and the human cathelicidin LL-37, as well as the azole antifungal itraconazole to compare the rate and nature of resistance development. Resistance to NaD1 and LL-37 developed more slowly and to a lower magnitude compared to itraconazole. Characterisation of the resistant strains via whole genome sequencing revealed that resistance to itraconazole could be attributed mostly to mutations in *ERG11*. In contrast, several different genes were mutated in the NaD1 and LL-37-resistant isolates, with no hot spot regions identified. This confirms the hypothesis that resistance to antimicrobial peptides occurs through an accumulation of multiple mutations in different genes whereas resistance to small molecule drugs is often attributed to mutations in a single target.

Resistance to NaD1 and LL-37 developed slowly compared to itraconazole.

We discovered that resistance to the antifungal peptide's NaD1 and LL-37 developed more slowly in *C. albicans* than resistance to the small molecule azole itraconazole and to a lesser extent (Figure 1). After 20 rounds of selection the MIC of the NaD1-resistant strains was only 40-fold greater than wild type. In contrast LL-37-resistant strains took 13 rounds to reach 40-fold greater MIC than wild type, and itraconazole-resistant strains with a 40-fold greater MIC than wild type were obtained after only 8 rounds of subculturing. The slower development of

resistance to NaD1 compared to itraconazole and LL-37 is likely due to the more complicated multi-step mechanism of action of NaD1. This is consistent with previous studies in *S. cerevisiae* when we observed that development of resistance to NaD1 develops more slowly than resistance to the antifungal caspofungin as well as the cationic peptide BPTI that acts by blocking magnesium uptake into *S. cerevisiae* cells (Bleackley *et al*, 2014; McColl *et al*, 2020).

Genetic characterisation of resistant strains

Whole genome sequencing revealed unique mutations that were linked to either NaD1, LL-37 or itraconazole resistance. The itraconazole-resistant strains which developed resistance the fastest, had the least number of mutations, and the strains with enhanced tolerance to NaD1 had the most mutations consistent with the longer period of subculturing required to generate the tolerance. This supports the hypothesis that an accumulation of mutations over time is responsible for the development of resistance to AMPs compared to single site mutations at hot spots that rapidly decrease efficacy of azoles and echinocandins in drug resistant clinal isolates of fungal pathogens. When comparing the distribution of these mutations, it was important to assess the similarities between LL-37 and NaD1 resistant strains to identify any common resistance mechanisms in AMP resistance. NaD1 and LL-37 had the highest number of mutated genes in common, 13 in total. Four of these genes, *BSD2, ALR1, CTP1*, and *PPZ1,* are involved in cation homeostasis. It has previously been reported that cation transport and homeostasis have a significant role in resistance to cationic antifungal peptides (AFPs) in *C. albicans* (Bleackley *et al.*, 2014; Li *et al*, 2018; McColl *et al.*, 2018). A potential broad-spectrum resistance mechanism to cationic AFPs has been reported for an *agp2*Δ mutant of *S. cerevisiae* whereby resistance was mediated by an accumulation of positive

charges at the cell surface that repelled the positively charged antifungal peptides (Bleackley *et al.*, 2014). Therefore, the mutations in *BSD2, ALR1, CTP1*, and *PPZ1* identified in both the NaD1 and LL-37-resistant strains, indicate this could be a potential broad-spectrum resistance mechanism of *C. albicans*. There were also mutations affecting cell wall genes (*HOC1, WSC1* and *ALG2*). LL-37 and NaD1 both interact with cell wall beta-glucans, therefore the mutations in these cell wall genes could prevent cell wall polysaccharide binding, hindering passage through the fungal cell wall and access to the plasma membrane. Despite the potential for broad spectrum resistance to membrane permeabilising peptides such as LL-37 its interaction with the membrane is more complex (Xhindoli *et al*, 2016), therefore it is likely that LL-37 and other complex AFPs such as NaD1 can likely overcome this barrier due to having multiple surface or intracellular interactions (McColl *et al.*, 2018). Therefore, it is hypothesised that although mutations in genes affecting extracellular cation accumulation would likely inhibit LL-37 interaction, it would only decrease the susceptibility of *C. albicans* to NaD1 and would not completely explain the mechanism of resistance to NaD1. Therefore, we looked further into the mutations responsible for itraconazole, NaD1, LL-37-resistance.

Resistance to itraconazole occurred via "hot-spot" mutations in Erg11.

It has previously been reported that resistance to small molecule drugs such as azoles can be achieved by a single amino acid alteration resulting in the upregulation of Erg11p (Sionov *et al*, 2012). Erg11p is a lanosterol 14α-demethylase which has a key role in the ergosterol synthesis pathway, and is also the target of azole antifungals (Zhang *et al*, 2010). Indeed, our itraconazole-resistant mutants all followed this similar path to resistance, with each acquiring mutations upstream or within protein coding regions of *ERG11* in all of the 11 itraconazole-

353 resistant isolates. The mutations were located at four sites; Y132H, S279F, S405F, and
354 R467K. These mutations have all been reported previously as hot spot mutations that have
355 been observed in clinical isolates of *C. albicans* (Sanglard & Odds, 2002). It has also been
356 reported that an accumulation of multiple mutations in and upstream of *ERG11* can result in
357 more resistance, this would explain why we also identified mutations upstream of *ERG11*
358 (Sanglard & Odds, 2002). We also identified mutations upstream of *ERG26* and *ERG251*,
359 which have not been reported previously, but are also involved in the ergosterol synthesis
360 pathway. Unlike resistance to itraconazole, no hot spots for mutations were detected in any of
361 the NaD1 or LL-37-resistant strains. The impact of these accumulated mutations are
362 discussed below.

363

364 **Resistance to LL-37 has a common theme of cation homeostasis and plasma membrane**
365 **localised proteins.**

366 Whole genome sequencing and SNV characterisation identified mutations unique to LL-37-
367 resistance. Functional characterisation of these genes revealed that there are common themes
368 associated with LL-37 resistance. Several genes had mutations upstream of coding regions
369 that impacted cation transport functions. These were *PDR16, C1_13280C, ALR1,*
370 *C3_04260W, CTP1, C4_02510W, ATM1, CR_04760C, PPZ,1 BDS2* and *CR_08200C*. As
371 mentioned earlier, an example of broad-spectrum resistance to cationic AFPs has been
372 reported in *S. cerevisiae* whereby resistance was mediated by an accumulation of positive
373 charges at the cell surface that repelled positively charged antifungal peptides (Bleackley *et*
374 *al.*, 2014). Therefore, it is likely that *C. albicans* would have a similar mechanism of
375 resistance whereby an extracellular accumulation of the cations impacted by mutations in
376 these genes could repel LL-37 from binding to the negatively charged cell surface.

377

378 The transmembrane transporters *C4_02510W, C6_01400W, C6_04210C, CR_04760C*, and
379 *CR_08200C* are generally part of the major facilitator superfamily MFS or ABC transporter
380 proteins that have a role in *C. albicans* drug resistance (Sanglard *et al*, 1996). Drug efflux is
381 a common mechanism of drug resistance in *C. albicans* and the upregulation of these
382 transporters could result in resistance to LL-37.

383

384 Surprisingly, we did not identify any mutations that impact Xog1p enzyme activity, which is
385 a known cell wall β-1,3-exoglucanase that LL-37 binds to (Tsai *et al.*, 2011). However,
386 mutations were identified upstream of *HOC1* and *ALG2* which may impact
387 mannosyltransferase activity. Upregulation of these genes could result in an increase of
388 mannan accumulation in the cell wall and prevent LL-37 from binding to Xog1p (Hall &
389 Gow, 2013). There were also mutations upstream of *GPR1* which is responsible for triggering
390 the signalling pathway that regulates beta-glucan masking and immune evasion (Ballou *et al*,
391 2016). Therefore, it is hypothesised that increased mannan accumulation in the cell wall and
392 beta-glucan masking could prevent LL-37 from binding to its target, Xog1p.

393 In summary, resistance to LL-37 is achieved via an accumulation of mutations that impact
394 extracellular cation accumulation, increase efflux of LL-37 via transmembrane transporters
395 and mask beta-glucan.

396

397 **_C. albicans_ has a multi-step mechanism of resistance to NaD1**

398 Similarly, to LL-37 resistance, there were no hot-spot regions identified in the NaD1-resistant
399 strains. This is consistent with the complex multi-step mechanism of action that has been

described for NaD1 (Hayes *et al.*, 2014; Payne *et al.*, 2016; Poon *et al.*, 2014; van der Weerden *et al.*, 2010). Functional characterisation was used to identify if there were any cellular functions where multiple genes had mutations. Several genes had mutations upstream of coding regions with metal ion transport functions (*C3_01680C, ECM7, RBT5P, ALR1* and *CTP1*). It has previously been reported that metal ion transport has a significant role in general resistance to antifungals in *C. albicans*. However some of these genes could also have a more specific role in NaD1-resistance (Li *et al.*, 2018). *ECM7* encodes a protein involved in control of calcium homeostasis and the oxidative stress response (Ding *et al*, 2013). It has previously been reported that *C. albicans* mutants lacking *ECM7* accumulate ROS in the presence of H_2O_2, therefore upregulation of *ECM7* could protect cells from ROS production caused by NaD1 (Ding *et al.*, 2013). *RBT5* is a GPI-linked cell wall protein, that binds haemoglobin and internalises it via endocytosis, and is negatively regulated by Hog1p (Weissman & Kornitzer, 2004). Iron plays a significant role in the maintenance of the cell wall architecture, mitochondrial function, filamentous growth, and oxidative response in *C. albicans* (Li *et al.*, 2018). It has been reported by Hayes et al., 2013 that the high-osmolarity glycerol (HOG) stress response pathway functions in fungal protection against NaD1. Another gene with mutations in the NaD1-resistant strains was *XYL2* which is induced upon Hog1 activation, and is a D-xylulose reductase (Enjalbert *et al*, 2006). Identification of genes that are regulated by Hog1, further validates implies the importance of Hog1p in resistance to NaD1. Overall, it is hypothesised that an extracellular accumulation of any of the cationic metals impacted by mutations in these genes could repel NaD1 from being able to bind to the negatively charged cell surface. As well as this these ion transporters, could also play individual roles of protecting *C. albicans* from intracellular stressors caused by NaD1, such as ROS production, cation imbalance or osmotic stress.

There were a group of genes that had mutations that would impact mannosyltransferase activity (*RHD1, SMP3, HOC1 MNT4* and *ALG2*). *C. albicans* cell wall is a two-layered structure. The main core of the cell wall is composed of a β-glucan-chitin skeleton, with chitin located in the inner layer of the cell wall (Garcia-Rubio *et al*, 2020). The outer layer of *C. albicans* cell wall is packed with mannoproteins that are glycosylphosphatidylinositol (GPI)-modified and cross-linked to β-1,6-glucans (Garcia-Rubio *et al.*, 2020). The activity of NaD1 on *Fusarium oxysporum* f. sp. *vasinfectum* hyphae has been reported to be dependent on the presence of the fungal cell wall although the molecular basis of this was not described (van der Weerden *et al.*, 2008). Further studies in *S. cerevisiae* revealed that NaD1 interacts with β-glucan and chitin, the two main polysaccharides in the fungal cell wall and that the rate of transit of NaD1 through the wall appears to decrease when the thickness of the glucan layer is increased (Bleackley *et al*, 2019). However, we did not find mutations that are predicted to impact the biosynthesis or degradation of these polysaccharides. The mutations in the NaD1 resistant mutants would impact the outer mannoprotein layer. The cell wall mannans of *C. albicans* have been reported to mask β-(1,3)-glucan from recognition by Dectin-1 a receptor on phagocytes that has a major role in antifungal immunity (Graus *et al*, 2018). It could therefore be hypothesised that mutations in genes impacting mannan biosynthesis could impact the structure of the mannoprotein and block NaD1 binding and transit through the cell wall. The likely reason we do not see mutations specifically impacting polysaccharides is likely because they are integral for cell wall structure, and mutations in these could decrease cell viability.

There were mutations identified in *RAD53, PSY2* and *PPH3*, these proteins are responsible for recovery from DNA damage (Shi *et al*, 2007). NaD1 induces production of ROS in *C.*

albicans (Hayes *et al*, 2013). Therefore, it is hypothesised that mutations in these genes may help *C. albicans* cells recover from DNA damage caused by the production of ROS.

NaD1 crosses the plasma membrane and enters the cytoplasm, it is speculated that once inside, NaD1 may be an intracellular target, although this target has not been identified. There were mutations in the genes encoding Age3p, Emp46p, Hoc1p, and Usu1p which are involved in trafficking through the Golgi. In a previous study assessing resistance to NaD1 in *S. cerevisiae*, we also identified mutations in genes localised to the Golgi, and thus hypothesised that the Golgi may the NaD1's intracellular target (McColl *et al.*, 2018). However, in *C. albicans* mutants there were also mutations present in *AIP2, ATP16, C1_02290C_A, C3_01680C_A, CR_03860C_A, CR_10830C_A, RAD59,* and *SHE9* which encode mitochondrial proteins. As previously stated, NaD1 causes ROS production in *C. albicans* and mitochondria are both the origin and target of ROS (Terman et al., 2006). Therefore, the mitochondria could be an internal target for NaD1, as mitochondrial dysfunction causes ROS accumulation. The activation of the Hog1 pathway that reduces oxidative stress protects cells from NaD1, therefore mutations that prevent mitochondrial ROS production would also protect the cell from NaD1 (Hayes *et al.*, 2014; Hayes *et al.*, 2013).

In summary, resistance to NaD1 cannot be achieved by a single mutation, this is due to the complex mechanism of action of NaD1. Therefore, resistance to NaD1 occurs via an accumulation of mutations that impact cell well, metal ion transport, DNA damage mediation and the prevention of binding to potential intracellular targets such as the Golgi or mitochondria.

Conclusion

During the last years, the numbers of fungal morbidity and mortality cases caused by *C. albicans* have increased, not only because of improved diagnoses of invasive infections but also because of the development of resistance, limiting efficacy of current antifungals. AMPs have many potential uses in treatment of complex infections, and their mode of action can be exploited for the generation of novel antifungal molecules. The molecular understanding of AMP resistance may reveal novel antifungal targets and aid in the design of new strategies and agents for treatment of drug-resistant fungi. Limited AMP resistance studies have been completed in *C. albicans*. In this paper, we described the development of *C. albicans* tolerance to an antifungal plant defensin NaD1 and the human cathlecidin LL-37. The overall aim was to compare the rate and mechanism of resistance development to a small molecule antifungal of the azole class, itraconazole. This study revealed that resistance to NaD1 and LL-37 develops and had limited effectiveness compared to itraconazole resistance. Increased tolerance to NaD1 and LL-37 developed via the accumulation of multiple mutations over time, and not via a single target site modification as with itraconazole. This study indicates that NaD1 and LL-37, and by extension other antimicrobial peptides, may complement existing clinical antifungals due to their resilience and unique mechanisms of action.

Figure Legends

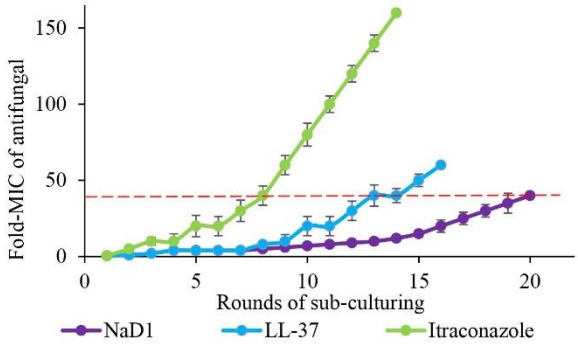
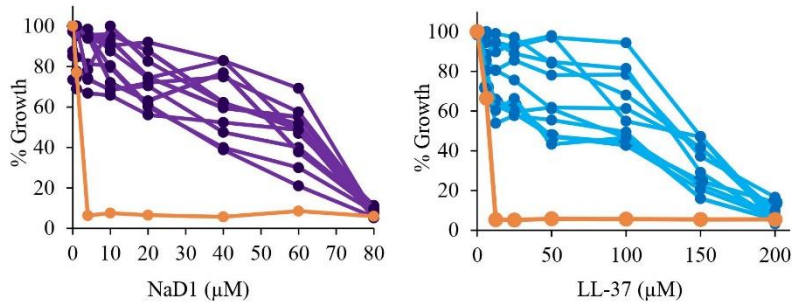


Figure 1. Resistance to NaD1 and LL-37 develops more slowly than resistance to itraconazole.

Development of resistance during sub-culturing in the presence of sub-lethal concentrations of itraconazole, LL-37 or NaD1. The average MICs of eleven independent strains of NaD1, LL-37 and itraconazole-resistant *C. albicans* strains are shown. Red gradient line indicates when each treatment group reached 40-fold resistance. Error bars represent ±standard error of the mean (n = 11).



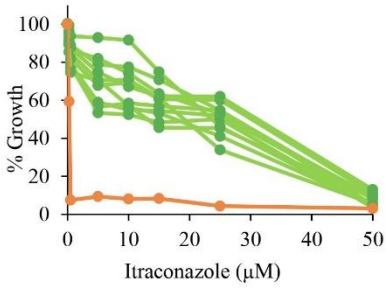
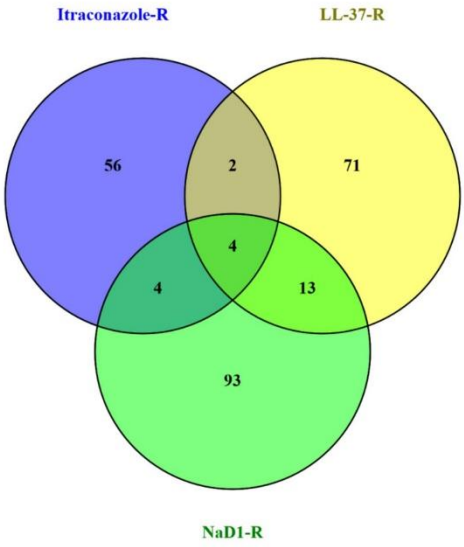


Figure 2. Confirmation of antifungal resistance after removal of selection pressure. Growth of the resistant *C. albicans* strains in the presence of antifungals relative to growth in the absence of any antifungal. **(A)** Growth of the eleven NaD1-resistant lines compared to no treatment control in increasing concentrations of NaD1. **(B)** Effect of LL-37 on the growth of eleven LL-37 resistant lines compared to no treatment control. **(C)** Growth inhibition of eleven itraconazole resistant lines compared to no treatment control.



520

521 **Figure 3.** Similarity of genes impacted by upstream or protein coding mutations across all itraconazole, LL-37

522 and NaD1 resistant strains. Venn diagram created with Jvenn (citation).

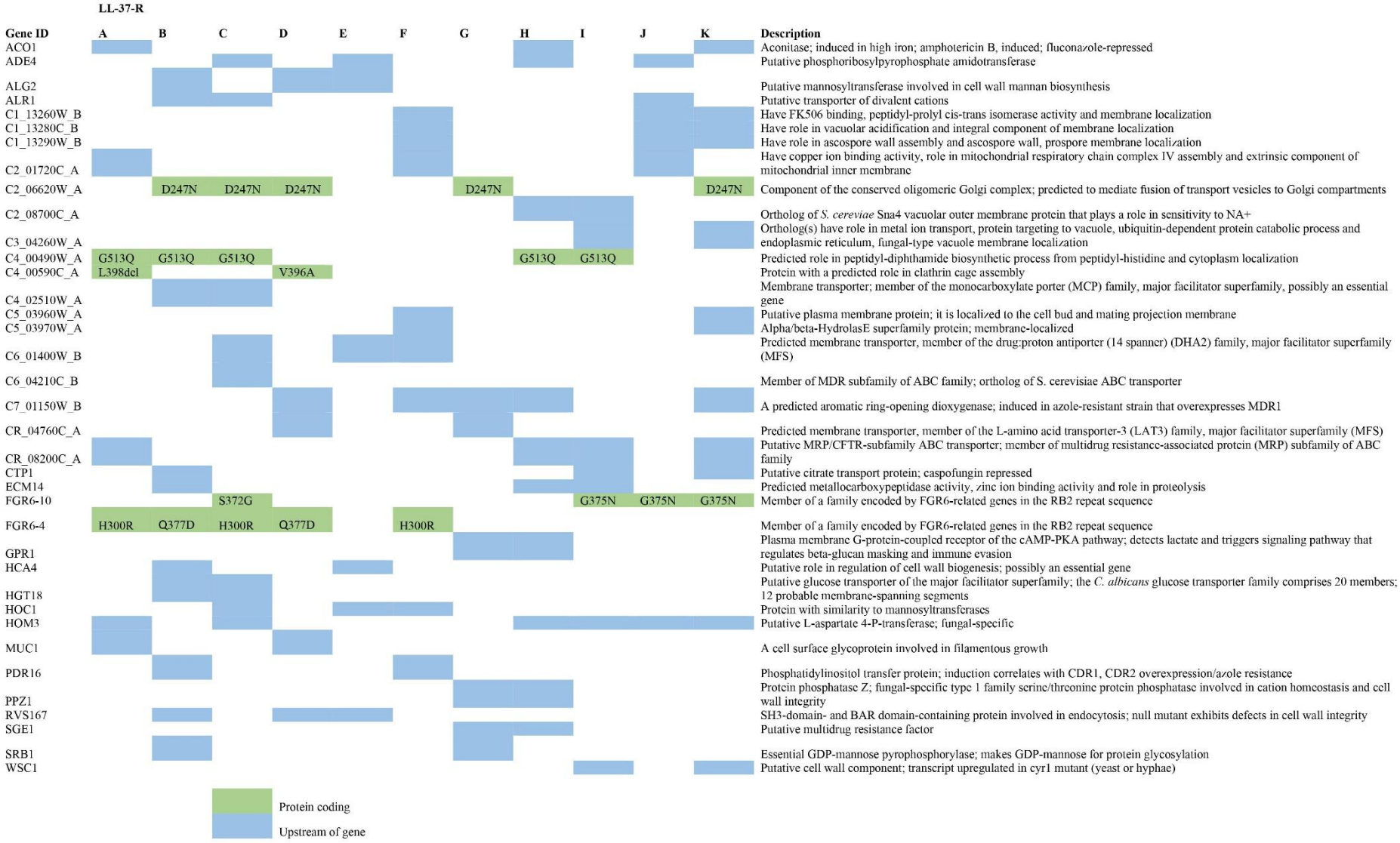


Figure 4. Distribution of variants across LL-37 resistant strains with descriptions of each gene function. Green are mutations impacting coding regions, Blue are mutations impacting upstream of the gene and White is no mutation present in that strain.

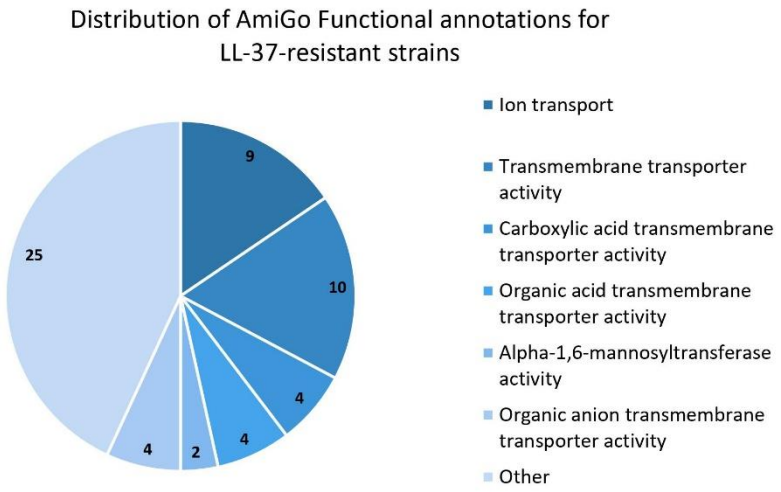


Figure 5. Functional characterisation of LL-37 resistant variants using AmiGO. Characterization revealed groups of genes impacting similar functions in LL-37-resistant strains. The genes are listed in Table 4.

Figure 6. Distribution of variants across NaD1-resistant strains with descriptions of each gene function. Green are mutations impacting coding regions, Blue are mutations impacting upstream of the gene and White is no mutation present in that strain.

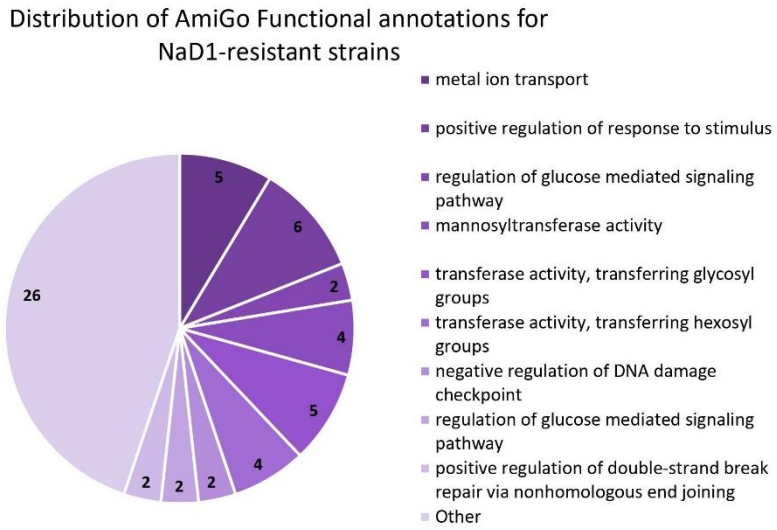


Figure 7. Functional characterisation of NaD1-resistant variants using AmiGO. Characterization revealed groups of genes impacting similar functions in LL-37-resistant strains. The genes are listed in Table 5.

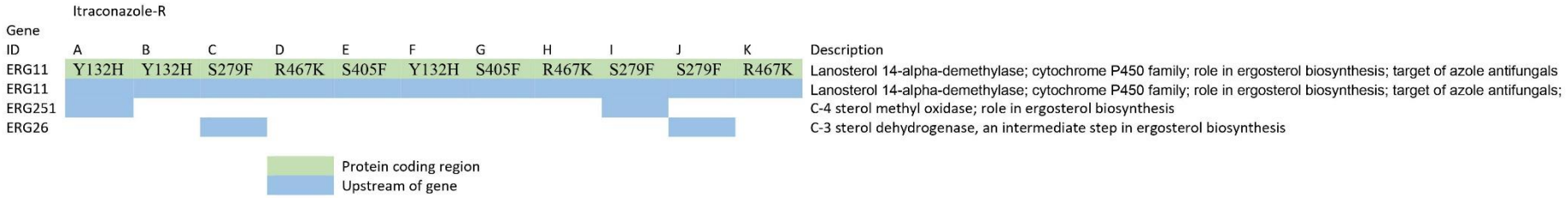


Figure 8. Distribution of variants across Itraconazole resistant strain

Table Legends

Region	Itraconazole-R	LL-37-R	NaD1-R
Intergenic	19	24	36
Upstream	54	70	90
Protein coding	12	20	24
Downstream	49	52	63
Total	134	166	213

Table 1. Table of the distribution of variants after initial variant calling. Highlighted are the Upstream and Protein coding variants that were characterised further.

ID	Description	Mutation
CR_07190W	Ortholog(s) have GTPase activator activity and role in maintenance of actin cytoskeleton polarity	p.Thr833Thr
Hoc1	Protein with similarity to mannosyltransferases	Upstream
Eaf3	Subunit of the NuA4 histone acetyltransferase complex	Upstream
Alr1	Putative transporter of divalent cations	Upstream
C6_01400W	Predicted membrane transporter, member of the drug:proton antiporter (14 spanner) (DHA2) family, major facilitator superfamily (MFS)	
CTP1	Putative citrate transport protein	Upstream
ORF298	Predicted ORF in retrotransposon Tca3	Upstream
PPZ1	Serine/threonine protein phosphatase involved in cation homeostasis and cell wall integrity	Upstream
COX7	Putative cytochrome c oxidase; flucytosine induced; repressed by nitric oxide	Upstream
EMP46	An integral membrane component of ER-derived COPII-coated vesicles	Upstream
WSC1	Putative cell wall component	Upstream
BSD2	have role in metal ion transport, protein targeting to vacuole	Upstream

ALG2	Putative mannosyltransferase involved in cell wall mannan biosynthesis	Upstream
POL1	Putative DNA directed DNA polymerase alpha	Upstream

Table 2. Mutations impacting genes that were identified in multiple treatment groups for LL-37 and NaD1 from Jvenn in Figure 3.

Function	P value	Genes
Metal ion transport	0.03068	C3_01680C, ALR1, C3_04260W, RBT5, ECM7
Positive regulation of response to stimulus	0.04941	PSY2, WSC1, SNF5, CRZ1, ECM7, PPH3
Regulation of glucose mediated signaling pathway	0.06615	PSY2, PPH3
Mannosyltransferase activity	0.00493	RHD1, SMP3, HOC1, ALG2
Transferring glycosyl groups	0.01364	RHD1, SMP3, HOC1, ALG2, MNT4
Transferring hexosyl groups	0.0474	RHD1, SMP3, HOC1, ALG2
Negative regulation of DNA damage checkpoint	0.0332	PSY2, PPH3
Regulation of glucose mediated signaling pathway	0.06615	PSY2, PPH3
Positive regulation of double-strand break repair via nonhomologous end joining	0.0111	PSY2, PPH3

Table 3. Functional characterisation of NaD1-resistant strains using AmiGO.

Function	P value	Genes
Ion transport	0.01368	PDR16, C1_13280C, ALR1, C3_04260W, CTP1, C4_02510W, ATM1, CR_04760C, CR_08200C, BSD2, PPZ1
Transmembrane transporter activity	0.00287	C1_13280C, ALR1, CTP1, C4_02510W, SGE1, C6_01400W, ATM1, HGT18, CR_04760C, CR_08200C
Carboxylic acid transmembrane transporter activity	0.02549	CTP1, C4_02510W, CR_04760C, CR_08200C
Organic acid transmembrane transporter activity	0.02719	CTP1, C4_02510W_, CR_04760C, CR_08200C
Alpha-1,6-mannosyltransferase activity	0.0687	HOC1, ALG2
Organic anion transmembrane transporter activity	0.0643	CTP1, C4_02510W, CR_04760C, CR_08200C

Table 4. Functional characterisation of NaD1-resistant strains using AmiGO

References

Ballou ER, Avelar GM, Childers DS, Mackie J, Bain JM, Wagener J, Kastora SL, Panea MD, Hardison SE, Walker LA *et al* (2016) Lactate signalling regulates fungal β-glucan masking and immune evasion. *Nature microbiology* 2: 16238

Bardou P, Mariette J, Escudié F, Djemiel C, Klopp C (2014) jvenn: an interactive Venn diagram viewer. *BMC Bioinformatics* 15: 293

Bleackley MR, Dawson CS, Payne JAE, Harvey PJ, Rosengren KJ, Quimbar P, Garcia-Ceron D, Lowe R, Bulone V, van der Weerden NL *et al* (2019) The interaction with fungal cell wall polysaccharides determines the salt tolerance of antifungal plant defensins. *Cell Surf* 5: 100026-100026

Bleackley MR, Hayes, B. M., Parisi, K., Saiyed, T., Traven, A., Potter, I. D., van der Weerden, N. L. and Anderson, M. A. (2014) Bovine pancreatic trypsin inhibitor is a new antifungal peptide that inhibits cellular magnesium uptake. *Molecular microbiology* 92: 1188–1197

Bleackley MR, Wiltshire JL, Perrine-Walker F, Vasa S, Burns RL, van der Weerden NL, Anderson MA (2014) Agp2p, the Plasma Membrane Transregulator of Polyamine Uptake, Regulates the Antifungal Activities of the Plant Defensin NaD1 and Other Cationic Peptides. *Antimicrobial Agents and Chemotherapy* 58: 2688-2698

Bongomin F, Gago S, Oladele RO, Denning DW (2017) Global and Multi-National Prevalence of Fungal Diseases—Estimate Precision. *Journal of Fungi* 3

Carbon S, Ireland A, Mungall CJ, Shu S, Marshall B, Lewis S, Ami GOH, Web Presence Working G (2009) AmiGO: online access to ontology and annotation data. *Bioinformatics* 25: 288-289

Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM (2012) A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *Fly* 6: 80-92

de Oliveira Santos GC, Vasconcelos CC, Lopes AJO, de Sousa Cartágenes MdS, Filho AKDB, do Nascimento FRF, Ramos RM, Pires ERRB, de Andrade MS, Rocha FMG *et al* (2018) Candida Infections and Therapeutic Strategies: Mechanisms of Action for Traditional and Alternative Agents. *Frontiers in Microbiology* 9: 1351

den Hertog AL, van Marle J, van Veen HA, Van't Hof W, Bolscher JG, Veerman EC, Nieuw Amerongen AV (2005) Candidacidal effects of two antimicrobial peptides: histatin 5 causes small membrane defects, but LL-37 causes massive disruption of the cell membrane. *The Biochemical journal* 388: 689-695

Ding X, Yu Q, Xu N, Wang Y, Cheng X, Qian K, Zhao Q, Zhang B, Xing L, Li M (2013) Ecm7, a regulator of HACS, functions in calcium homeostasis maintenance, oxidative stress response and hyphal development in Candida albicans. *Fungal genetics and biology : FG & B* 57: 23-32

Enjalbert B, Smith DA, Cornell MJ, Alam I, Nicholls S, Brown AJ, Quinn J (2006) Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen Candida albicans. *Mol Biol Cell* 17: 1018-1032

Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ (2018) Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science (New York, NY)* 360: 739

Garcia-Rubio R, de Oliveira HC, Rivera J, Trevijano-Contador N (2020) The Fungal Cell Wall: Candida, Cryptococcus, and Aspergillus Species. *Frontiers in Microbiology* 10

Gennaro R, Zanetti M (2000) Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. *Biopolymers* 55: 31-49

Ghannoum MA, Rice LB (1999) Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clinical microbiology reviews* 12: 501-517

Graus MS, Wester MJ, Lowman DW, Williams DL, Kruppa MD, Martinez CM, Young JM, Pappas HC, Lidke KA, Neumann AK (2018) Mannan Molecular Substructures Control Nanoscale Glucan Exposure in Candida. *Cell Rep* 24: 2432-2442.e2435

Hall RA, Gow NAR (2013) Mannosylation in Candida albicans: role in cell wall function and immune recognition. *Molecular microbiology* 90: 1147-1161

Hayes BM, Anderson MA, Traven A, van der Weerden NL, Bleackley MR (2014) Activation of stress signalling pathways enhances tolerance of fungi to chemical fungicides and antifungal proteins. *Cellular and molecular life sciences : CMLS* 71: 2651-2666

Hayes BME, Bleackley MR, Wiltshire JL, Anderson MA, Traven A, van der Weerden NL (2013) Identification and Mechanism of Action of the Plant Defensin NaD1 as a New Member of the Antifungal Drug Arsenal against Candida albicans. *Antimicrobial Agents and Chemotherapy* 57: 3667-3675

Lay FT, Brugliera F, Anderson MA (2003) Isolation and Properties of Floral Defensins from Ornamental Tobacco and Petunia. *Plant Physiology* 131: 1283-1293

Lay FT, Mills GD, Poon IKH, Cowieson NP, Kirby N, Baxter AA, van der Weerden NL, Dogovski C, Perugini MA, Anderson MA *et al* (2012) Dimerization of Plant Defensin NaD1 Enhances Its Antifungal Activity. *Journal of Biological Chemistry* 287: 19961-19972

Leeuw Ed, Li C, Zeng P, Li C, Buin MD-d, Lu W-Y, Breukink E, Lu W (2010) Functional interaction of human neutrophil peptide-1 with the cell wall precursor lipid II. *FEBS letters* 584: 1543-1548

Li Y, Sun L, Lu C, Gong Y, Li M, Sun S (2018) Promising Antifungal Targets Against Candida albicans Based on Ion Homeostasis. *Front Cell Infect Microbiol* 8

Magana M, Pushpanathan M, Santos AL, Leanse L, Fernandez M, Ioannidis A, Giulianotti MA, Apidianakis Y, Bradfute S, Ferguson AL *et al* (2020) The value of antimicrobial peptides in the age of resistance. *The Lancet Infectious Diseases* 20: e216-e230

McColl AI, Bleackley MR, Anderson MA, Lowe RGT (2018) Resistance to the Plant Defensin NaD1 Features Modifications to the Cell Wall and Osmo-Regulation Pathways of Yeast. *Frontiers in Microbiology* 9: 1648

McColl AI, Lowe RGT, McKenna JA, Anderson MA, Bleackley MR (2020) Resistance to the antifungal activity of Aprotinin occurs through mutations in genes that function in cation homeostasis. *bioRxiv*: 2020.2006.2022.164863

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M *et al* (2010) The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research* 20: 1297-1303

McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GRS, Thormann A, Flicek P, Cunningham F (2016) The Ensembl Variant Effect Predictor. *Genome Biology* 17: 122

Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ (2020) Antimicrobial host defence peptides: functions and clinical potential. *Nature Reviews Drug Discovery* 19: 311-332

Parisi K, Shafee TMA, Quimbar P, van der Weerden NL, Bleackley MR, Anderson MA (2018) The evolution, function and mechanisms of action for plant defensins. *Seminars in cell & developmental biology*

Payne JA, Bleackley MR, Lee TH, Shafee TM, Poon IK, Hulett MD, Aguilar MI, van der Weerden NL, Anderson MA (2016) The plant defensin NaD1 introduces membrane disorder through a specific interaction with the lipid, phosphatidylinositol 4,5 bisphosphate. *Biochimica et biophysica acta* 1858: 1099-1109

Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A (2017) The global problem of antifungal resistance: prevalence, mechanisms, and management. *The Lancet Infectious diseases* 17: e383-e392

Pfaller MA, Diekema DJ (2010) Epidemiology of invasive mycoses in North America. *Crit Rev Microbiol* 36: 1-53

Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Ellis D, Tullio V, Rodloff A, Fu W, Ling TA (2010) Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of Candida Species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. *Journal of clinical microbiology* 48: 1366-1377

Poon IKH, Baxter AA, Lay FT, Mills GD, Adda CG, Payne JAE, Phan TK, Ryan GF, White JA, Veneer PK *et al* (2014) Phosphoinositide-mediated oligomerization of a defensin induces cell lysis. *eLife* 3: e01808

126 Raheem N, Straus SK (2019) Mechanisms of Action for Antimicrobial Peptides With
127 Antibacterial and Antibiofilm Functions. *Frontiers in Microbiology* 10: 2866

128 Revie NM, Iyer KR, Robbins N, Cowen LE (2018) Antifungal drug resistance: evolution,
129 mechanisms and impact. *Current opinion in microbiology* 45: 70-76

130 Robbins N, Caplan T, Cowen LE (2017) Molecular Evolution of Antifungal Drug Resistance.
131 *Annual Review of Microbiology* 71: 753-775

132 Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP
133 (2011) Integrative Genomics Viewer. *Nature biotechnology* 29: 24-26

134 Sanglard D, Ischer F, Monod M, Bille J (1996) Susceptibilities of Candida albicans multidrug
135 transporter mutants to various antifungal agents and other metabolic inhibitors. *Antimicrobial*
136 *Agents and Chemotherapy* 40: 2300-2305

137 Sanglard D, Odds FC (2002) Resistance of Candida species to antifungal agents: molecular
138 mechanisms and clinical consequences. *The Lancet Infectious Diseases* 2: 73-85

139 Schneider T, Kruse T, Wimmer R, Wiedemann I, Sass V, Pag U, Jansen A, Nielsen AK,
140 Mygind PH, Raventós DS *et al* (2010) Plectasin, a Fungal Defensin, Targets the Bacterial
141 Cell Wall Precursor Lipid II. *Science (New York, NY)* 328: 1168

142 Shahmiri M, Enciso M, Adda CG, Smith BJ, Perugini MA, Mechler A (2016) Membrane
143 Core-Specific Antimicrobial Action of Cathelicidin LL-37 Peptide Switches Between Pore
144 and Nanofibre Formation. *Scientific Reports* 6: 38184

145 Sherry ST, Ward M-H, Kholodov M, Baker J, Phan L, Smigielski EM, Sirotkin K (2001)
146 dbSNP: the NCBI database of genetic variation. *Nucleic Acids Research* 29: 308-311

147 Shi QM, Wang YM, Zheng XD, Lee RT, Wang Y (2007) Critical role of DNA checkpoints in
148 mediating genotoxic-stress-induced filamentous growth in Candida albicans. *Mol Biol Cell*
149 18: 815-826

150 Sionov E, Chang YC, Garraffo HM, Dolan MA, Ghannoum MA, Kwon-Chung KJ (2012)
151 Identification of a Cryptococcus neoformans Cytochrome P450 Lanosterol 14α-Demethylase
152 (Erg11) Residue Critical for Differential Susceptibility between Fluconazole/Voriconazole
153 and Itraconazole/Posaconazole. *Antimicrobial Agents and Chemotherapy* 56: 1162-1169

154 Skrzypek MS, Binkley J, Binkley G, Miyasato SR, Simison M, Sherlock G (2017) The
155 Candida Genome Database (CGD): incorporation of Assembly 22, systematic identifiers and
156 visualization of high throughput sequencing data. *Nucleic acids research* 45: D592-D596

157 Tsai P-W, Yang C-Y, Chang H-T, Lan C-Y (2011) Characterizing the role of cell-wall β-1,3-
158 exoglucanase Xog1p in Candida albicans adhesion by the human antimicrobial peptide LL-
159 37. *PloS one* 6: e21394-e21394

160 Tsai PW, Cheng YL, Hsieh WP, Lan CY (2014) Responses of Candida albicans to the human
161 antimicrobial peptide LL-37. *J Microbiol* 52: 581-589

162 van der Weerden NL, Bleackley MR, Anderson MA (2013) Properties and mechanisms of
163 action of naturally occurring antifungal peptides. *Cellular and Molecular Life Sciences* 70:
164 3545-3570

165 van der Weerden NL, Hancock REW, Anderson MA (2010) Permeabilization of Fungal
166 Hyphae by the Plant Defensin NaD1 Occurs through a Cell Wall-dependent Process. *Journal*
167 *of Biological Chemistry* 285: 37513-37520

168 van der Weerden NL, Lay FT, Anderson MA (2008) The Plant Defensin, NaD1, Enters the
169 Cytoplasm of Fusarium Oxysporum Hyphae. *Journal of Biological Chemistry* 283: 14445-
170 14452

171 Weissman Z, Kornitzer D (2004) A family of Candida cell surface haem-binding proteins
172 involved in haemin and haemoglobin-iron utilization. *Molecular microbiology* 53: 1209-1220

173 Xhindoli D, Pacor S, Benincasa M, Scocchi M, Gennaro R, Tossi A (2016) The human
174 cathelicidin LL-37 — A pore-forming antibacterial peptide and host-cell modulator.
175 *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1858: 546-566

176 Zhang Y-Q, Gamarra S, Garcia-Effron G, Park S, Perlin DS, Rao R (2010) Requirement for
177 Ergosterol in V-ATPase Function Underlies Antifungal Activity of Azole Drugs. *PLoS*
178 *Pathog* 6: e1000939

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Chapter 5

5. Concluding remarks

The studies presented in this thesis were focused on the mechanisms of resistance to antifungal peptides in two yeast species, comparing the findings to the mechanisms of small molecule drug resistance. Assessing resistance to AFPs via laboratory selection provides the opportunity to mimic how resistance may develop to fungal pathogens in a clinical setting, thus enabling the opportunity for clinicians to predict resistance patterns and treatment regimens prior to administration of AFPs to a patient. The development of peptide drugs is ever-increasing, with more than 400 peptidic drugs in clinical development (Basso *et al.*, 2020). Over the last 20 years, a total of 60 peptide drugs have been approved worldwide, with metabolic disorders and cancer as the main targeted therapeutic areas (Lee *et al.*, 2019). Moreover, a number of AMPs are in development for topical antifungal applications, including the treatment of oral and vulvovaginal candidiasis (Mookherjee *et al.*, 2020). This prompted the investigations in this thesis on the rate of development and mechanisms of resistance to AFPs in the two yeast species. This thesis has built on the limited amount of research that has been performed over the last 20 years on resistance development to a small set of AFPs. Prior research has mainly employed resistance selection or deletion screens to elucidate targets and the mechanism of action of AFPs. Genome sequencing methods have improved rapidly over the past decade and have created the opportunity to define all the genetic changes that occur in the pathogen genome during the transition to AFP resistance. In this thesis whole genome sequencing has been used to complete one of the first comprehensive investigations into the genetic determinants of resistance to AFPs via laboratory selection.

5.1 Resistance to AFPs does develop but it is slow and involves mutations in multiple genes.

I discovered that yeast do develop resistance to AFPs such as NaD1, LL-37 and BPTI, but it has limited effectiveness compared to small molecule resistance. Resistance to AFPs developed more slowly and there was a lower fold increase in MIC compared to resistance to the clinical antifungal drugs and it was associated with the fitness defects of slow growth, decreased cell size, and sensitivity to abiotic stressors. The major concern with small molecule drugs that are used in the clinic is the relatively rapid development of resistance, due to single mutations in hot spot regions in the proteins that are the targets for these drugs (Balashov *et al.*, 2006; Flowers *et al.*, 2015). Clinical antifungals generally act by inhibiting single enzymes that are essential for fungal viability. Resistance is acquired relatively rapidly due to the single amino acid changes that block interaction with the antifungal drug without affecting the activity of the enzyme (Prasad *et al.*, 2016). Indeed, we also saw this occur in our caspofungin and itraconazole resistant lines. Resistance to these drugs emerged far more rapidly relative to AFP resistance and was caused by hotspot mutations in the target genes *ERG11* and *FKS1* for itraconazole and caspofungin respectively. In contrast to this, whole genome sequencing of all AFP-resistant isolates revealed that increased tolerance had developed via an accumulation of multiple mutations over time, and not via a single target site modification as occurred with small molecule drug resistance. Therefore, I hypothesise that due to this slow accumulation of mutations over time and the associated fitness defects that resistance to AFPs such as NaD1 and LL-37 will develop relatively slowly, and the resistant fungi will be less viable in a clinical setting. This provides an advantage for AFPs over small molecule drugs.

Indeed, others have also observed this slow rate of resistance development and fitness defects associated with AMPs, particularly in bacterial studies (Leeuw *et al.*, 2010; Magana

et al., 2020; Schneider *et al.*, 2010). Magana and co-workers in their review on antibacterial AMPs hypothesised that the intense competition between bacterial species for nutrients and space within soil, human, and marine microbiota enhances the synthesis and continuous evolution of new AMPs (Magana *et al.*, 2020; Tobias *et al.*, 2017). In order to overcome resistance within these competitive environments, these newly evolved AMPs become increasingly complicated and are able to survive in harsh conditions, making them an ideal new source of AMPs that are resilient to environmental stressors and resistance development (Magana *et al.*, 2020; Teta *et al.*, 2017). It is likely that this is also the case for antifungal peptides, whereby plant or animal hosts are in an arms race with potential pathogens. They continually evolve AFPS with more complex mechanisms of action for protection against potential pathogens which in turn are continually evolving to be less susceptible to those defences. This can in part explain the resilience of AFPs and slow the development of resistance in fungi compared to resistance to small molecule antifungals which have not been produced through a co-evolutionary process.

5.2 Resistance develops more slowly if the mechanism of action involves multiple targets

The hypothesis that resistance will develop more slowly to AFPs that have multiple targets or a complex mechanism of action was further proven in this thesis. Resistance to NaD1 and LL-37 developed more slowly than resistance to BPTI and the small molecule antifungals, which is likely due to its multi-phase mechanism of action which involves interaction with multiple targets (Hayes *et al.*, 2018; Hayes *et al.*, 2013) (Ordonez *et al.*, 2014; Tsai *et al.*, 2011). In contrast, resistance to BPTI developed very quickly, faster than small molecule resistance. This is likely because of the simple mechanism of action of BPTI, whereby it appears to act on a single magnesium transporter. Consequently, any

mutations that increase magnesium uptake would be able to increase resistance to BPTI (Bleackley, 2014). This hypothesis that resistance develops more slowly if the mechanism of action involves multiple targets, provides a theoretical basis for prioritising the clinical development of more complex AMPs over ones with a simpler mechanism of action.

5.3 Resistance to one AFP does not provide resistance to other AFPs

Despite resistance being observed for AFP's, an important discovery I made was that resistance to one AMP does not generate broad spectrum resistance to other AFPs or antifungals. The NaD1 and BPTI-resistant strains were still susceptible to caspofungin, and other AMPs including NaD2, DmAmP1, CP29, and Bac2a. In a clinical setting it is not uncommon that once resistance develops towards one antifungal within a class, that the pathogen will be resistant to the entire antifungal class, and sometimes other classes (Arendrup & Patterson, 2017; Chowdhary *et al.*, 2017). The promising benefit of antifungal peptides, is that their mechanisms of action are extremely diverse, meaning that resistance to one peptide does not necessarily mean resistance to the entire class of peptides. Due to the increased prevalence of super-bugs resistant to multiple antifungal classes, the use of antifungal peptides could create enough therapeutic diversity to overcome this issue.

5.4 Mechanisms of AFP resistance

Whole genome sequencing of all the AFP resistant isolates revealed that resistance to AFPs occurs through a multi-phase mechanism whereby an accumulation of mutations over time impact beta-glucan masking, cation transport, and resistance to ROS and osmotic stress. This adds to the previously reported mechanisms of AFP resistance which employ repulsion, sequestration, removal by efflux pumps and proteolytic degradation. The

findings of my research that contribute to the already established knowledge on AFP resistance will be explored below (Figure 5.1).

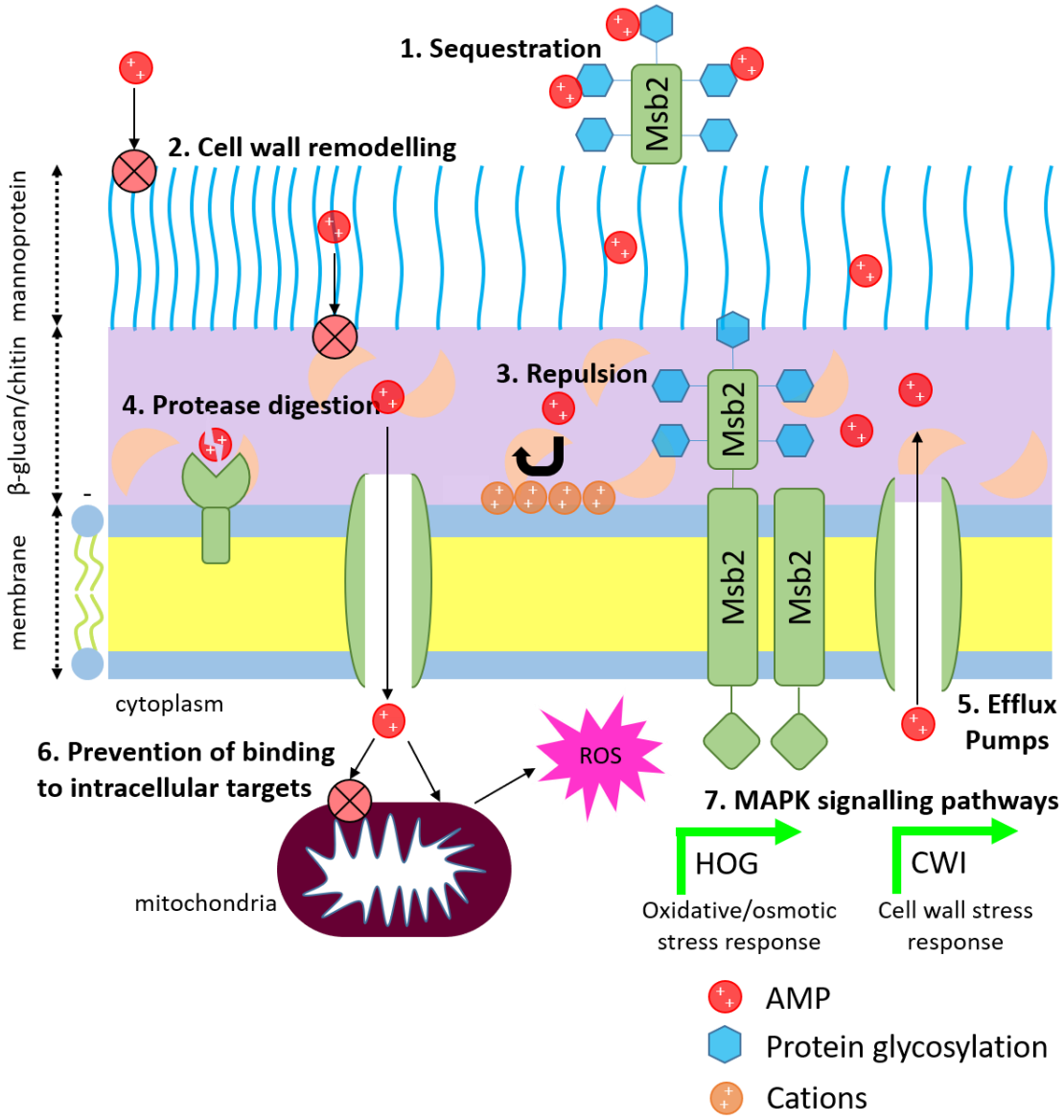


Figure 5.1. Updated mechanism of resistance to AFPs. The mechanisms of resistance are highlighted in bold in the figure and include: **1. Sequestration** by secreted proteins, anionic polysaccharides or mannosylphosphate side chains on glycoproteins. **2. Cell wall remodelling** to prevent binding to cell wall targets and traversing the cell wall. beta glucans. **3. Repulsion** of AFPs by the accumulation of cations on the cell surface**. 4. Protease digestion** using ubiquitin ligases to degrade AFPs. **5. Efflux pumps**, export of AMPs. **6. Prevention of binding to intracellular targets** such as the Golgi or mitochondria. **7. Mitogen-activated protein kinase (MAPK) signalling pathways** in fungi for protection against oxidative, osmotic or cell wall stress.

5.4.1 Repulsion: In both LL-37 and NaD1 resistant strains, I identified plasma membrane transporters that impacted cation and metal transport. I hypothesised that these would result in an accumulation of cations on the cell surface and prevent AFP binding. Indeed, this method of repulsion had already been identified for NaD1 and a range of other AFPs through *S. cerevisiae* deletion screens which identified that deletion of the transport regulator *agp2* led to AFP resistance (Bleackley *et al.*, 2014). Mutations that impact cation transport have the potential to be a broad-spectrum mechanism of resistance to AFPs. To confirm whether there is an accumulation of positive charges on the cell surface, a Sytox green-mediated assay could be used to assess if membrane permeabilization is delayed in the resistant strains compared to the *agp2*Δ mutants and wildtype. Further experimentation would be a binding assay measuring the binding activity of cytochrome *c*, a cationic protein. The levels of cytochrome *c* remaining in the supernatant would be measured after incubation. Wildtype cells would have bound most of the added cytochrome *c*, whereas a negligible amount of cytochrome *c* would be bound to mutants that had increased positive cations on the cell surface.

5.4.2 Sequestration / Cell wall modifications: I identified mutations in genes *CWP2*, *HOC1, WSC1* and *ALG2* which may result in modifications to monosaccharide composition in the cell wall for NaD1 and LL-37 resistant strains. It is hypothesised that modifications to the cell wall would prevent AFPs from being able to traverse the cell wall by either sequestering or repelling the AFP and preventing it from reaching the plasma membrane. An example of sequestration by cell surface glycoproteins has been reported for PAF26 after the observation that deletions in glycosylation genes enhances resistance to this antifungal peptide (Harries et al., 2013). Extracellular concentrations of histatin 5 are also limited by sequestration to the outer layer of mannosylated glycoproteins (Harris *et al.*, 2009) and by the soluble domain of the MbS2 membrane sensor which is a broad range

protectant against AFPs including histatin 5, LL-37, hNP-1 and hBD1 (Szafranski-Schneider *et al.*, 2012). These studies imply that cell wall binding of AFPs has a larger role in their mechanisms of action/resistance than was previously anticipated. However, the content of mannan, chitin and beta-glucans would need examined in the mutants that were generated in this thesis as well as their structural location.

5.4.3 Degradation: Proteases that degrade AFPs have been identified as a mechanism of resistance. Histatin 5 for example is degraded by the extracellular GPI anchored proteases Sap9/10 (Harris *et al.*, 2009). I did not find mutations in Sap9/10, however, mutations in the ubiquitin ligases *TOM1* and *RSP5* were identified in NaD1-resistant strains. These ligases have a role in protein degradation and could therefore be responsible for degrading AFP's upon entering the cytoplasm. However, due to the rigid scaffold and the 4 disulphide bonds of plant defensins, they are usually resistant to protease activity therefore it is unlikely that these mutations would have a large contribution to resistance (Parisi *et al.*, 2020). Unlike plant defensins, LL-37 is more susceptible to proteolytic degradation (McCrudden *et al.*, 2014), however I did not identify any proteases to be associated with increased resistance to LL-37.

5.4.4 Protection from ROS and osmotic stress: The mechanism of action of many AFPs leads to ROS production (Basso *et al.*, 2020). Therefore, decreasing ROS production will increase resistance to these AFPs. This protective mechanism normally occurs by activation of the HOG pathway that protects against both ROS and osmotic stress (Hayes *et al.*, 2014). Indeed, I identified several mutations that impact osmotic stress and ROS reduction in the NaD1-resistant isolates. Although there were no mutations that directly impacted Hog1, there were mutations that impacted genes that are regulated by Hog1 including the glucose transporter *FPS1*, D-xylulose reductase *XYL2*, candadylisin *ECE1*, and GPI linked cell wall protein *RBT5*, (Hayes *et al.*, 2014). These mutations likely protect the cells from osmotic stress and ROS production, which is a known mechanism of AFP directed cell killing. In

addition to this, there were mutations that impacted mitochondrial function, and would likely result in protection from ROS production. This coincides with the previously reported petite mutants (with deletion of the mitochondrial genome) that were more resistant to NaD1 and had reduced ROS production (Hayes *et al.*, 2013). The importance of mitochondrial function for NaD1's mechanism of action was also reported after screens of the yeast non-essential gene library (Parisi *et al.*, 2019). I did not identify any mutations in the genes reported in that paper.

5.4.5 Efflux pumps: The use of efflux pumps to remove AFPs has been reported in the human salivary peptide Histatin 5 whereby resistance was partially mediated by the Flu1 efflux pump (Li *et al.*, 2013). I also identified the use of efflux pumps to enhance resistance to the human AMP, LL-37. Resistance was associated with mutations in the ABC transporters CR_08200C_A and C6_04210C_B and the major facilitator superfamily transporters C4_02510W_A, CR_04760C_A, and HGT18. Due to the number of transporters identified and the partial resistance observed with Histatin 5 resistance, it is likely that each of these transporters also only induces partial resistance to LL-37.

Other mechanisms of resistance identified were mutations that may impact Golgi and mitochondria function. Others have reported that AFPS enter cells and are likely to have intracellular targets (Parisi *et al.*, 2018; Seyfi *et al.*, 2020; van der Weerden *et al.*, 2013), but very few of these targets have been elucidated. Based on this speculation, the intracellular targets of NaD1 could be the Golgi or mitochondria and consequently resistance may be associated with decreased binding to intracellular targets. Mutations in plasma membrane transporters that result in the intracellular accumulation of ions and other substances could also protect from cell death. For example, mutations in *FPS1* could result in the intracellular accumulation of glycerol therefore protecting NaD1 from osmotic stress,

or mutations in the transporters sky1 and ptk2 could result in protection from magnesium efflux caused by BPTI.

Some of the resistance mechanisms that were revealed in this study are related to those reported previously but there were differences in the genes responsible. This is probably because the limited AFP resistance studies that have been conducted to date have focused on human AFPs such as lactoferrin, hNP-1, hBD1-3 and histatins, which have different mechanisms of action and would likely elicit different resistance responses compared to plant defensin such as NaD1. The early research on the mechanism of action of AMPs was largely focused on the interaction with bacteria and led to the hypothesis that the main mechanism of action was membrane permeabilization. Other than the repulsion of AFPs mentioned above which would prevent plasma membrane binding, the findings from these studies in conjunction with recent evidence from other researchers indicates that this is not the main mechanism of action of AFPs. Overall, the results identified in this study add to the already identified mechanisms of resistance to AMPs as well as identifying novel mechanisms of resistance that will help in designing therapeutic strategies for future AMPs.

5.5 Benefits of laboratory selection

It was hypothesised that the mutations that arise from our method of laboratory selection for resistance would be more like those that would occur in the clinic as opposed to mutations created by chemical mutagenesis or identified in knockout screens. Indeed, this was the case for our caspofungin and itraconazole-resistant strains, which both had mutations that have been reported previously in *C. albicans* clinical isolates (Balashov *et al.*, 2006; Flowers *et al.*, 2015). This confirms that investigating the potential for

resistance development prior to clinical use will provide valuable insight into the mechanisms of resistance, and how the antifungal agent should be administered.

5.6 Limitations of these studies and further experiments

Despite the advantages of laboratory selection for resistance, there are still limitations to *in-vitro* experimentation. Although we have done an in-silico estimation of the contribution each mutation would have to AFP resistance, these would still need to be confirmed with experimental validation. Complementation experiments should be conducted with the wild type genes to determine whether they abrogate the resistance phenotype to provide strong evidence that these genes are the main resistance determinants. Further characterization of the single-gene deletion strains or the developed resistant-strains would provide more support that certain genes directly impact the resistant phenotype. For example, it could be assessed whether the resistant or knockout strains have altered cell walls or glycoproteins with a higher mannan content or have a higher/lower intracellular concentration of ions compared to wildtype.

Another limitation of this research is that it was done in an *in-vitro* environment, and thus has not provided any information on the influence of host during an infection response. In addition, the viability of the mutated strains in a host setting was not checked. It is important to note that the experiments done with the pathogen *C. albicans* in this thesis employed a disabled yeast strain (auxotrophic for uridine, histidine and arginine) for safety reasons and thus the drug resistant strains could not be assessed for virulence in a host model. The selection for resistance could be repeated by co-culturing the fungal pathogen with human innate immune cells, or in a mammalian model with an AMP over time, but it would not be ethical to develop a drug resistant strain that could be

transmitted. Therefore, future work will be reliant on *in-vitro* experiments to assess resistance.

There are other methods that can be used to evolve and assess laboratory selection for resistance. In the clinic, a person may not get the maximum dose required for complete killing, usually due to co-morbidity, missing doses or not completing the script. It will be interesting to assess whether the clinical isolates of *C. albicans* already display resistance to AFP's, in particular the human AFPs such as histatin, LL-37 and the human defensins, which are essential components of the innate immune system. These clinical isolates could also be sequenced to assess if the resistance genes I have identified are already mutated in these strains.

In these experiments, we assessed a single isolate from a culture of resistant strains. It is important to consider that in a host there will be population of *C. albicans* which will have genetic variation. Therefore, another way to assess resistance is to sequence the entire population of cells after subculture in the presence of the AFP and examine the read depth and read count to determine the number of times the same mutation has arisen in the population. It is likely that some mutations will be more dominant but other mutations may contribute to partial resistance and affect medication efficacy.

Due to the increased prevalence of antifungal disease and resistance, understanding the mechanisms of resistance to our new arsenal of clinical drugs is essential. The studies in this thesis provide a method and introduction to understanding how fungi develop resistance to AFP's. An in depth understanding of how resistance develops to these peptides will provide invaluable information for anyone intending to use these peptides in a clinical setting. The studies in this thesis have highlighted the complexity of AMP resistance compared to small molecule drug resistance and have provided information on

the mechanisms of action and resistance to AMPs that may not have been elucidated otherwise. Overall, these studies indicate that NaD1, BPTI and LL-37, and by extension other AMPs, may complement existing clinical antifungals. Therefore, development of AFPs as therapeutics should be seriously considered to reduce the burden of antifungal resistance.

References

Abdi M, Mirkalantari S, Amirmozafari N (2019) Bacterial resistance to antimicrobial peptides. *Journal of Peptide Science* 25: 3210

Aerts AM, Bammens L, Govaert G, Carmona-Gutierrez D, Madeo F, Cammue B, Thevissen K (2011) The antifungal plant defensin HsAFP1 from Heuchera sanguinea induces apoptosis in Candida albicans. *Frontiers in microbiology* 2: 47

Aerts AM, François IE, Meert EM, Li QT, Cammue BP, Thevissen K (2007) The antifungal activity of RsAFP2, a plant defensin from raphanus sativus, involves the induction of reactive oxygen species in *Candida albicans*. *Journal of molecular microbiology and biotechnology* 13: 243-247

Amerikova M, Pencheva El-Tibi I, Maslarska V, Bozhanov S, Tachkov K (2019) Antimicrobial activity, mechanism of action, and methods for stabilisation of defensins as new therapeutic agents. *Biotechnology & Biotechnological Equipment* 33: 671-682

Andersson DI, Hughes D, Kubicek-Sutherland JZ (2016) Mechanisms and consequences of bacterial resistance to antimicrobial peptides. *Drug Resistance Updates* 26: 43-57

Aoki W, Ueda M (2013) Characterization of antimicrobial peptides toward the development of novel antibiotics. *Pharmaceuticals (Basel)* 6: 1055-1081

Arendrup MC, Patterson TF (2017) Multidrug-resistant *Candida*: Epidemiology, molecular mechanisms, and treatment. *The Journal of Infectious Diseases* 216: 445-451

Ashrafi N, Lapthorn C, Pullen FS, Naclerio F, Nielsen Birthe V (2017) High performance liquid chromatography coupled to electrospray ionisation mass spectrometry method for the detection of salivary human neutrophil alpha defensins HNP1, HNP2, HNP3 and HNP4. *Analytical Methods* 9: 6482-6490

Avery SV, Singleton I, Magan N, Goldman GH (2019) The fungal threat to global food security. *Fungal Biology* 123: 555-557

Bagnat M, Keränen S, Shevchenko A, Shevchenko A, Simons K (2000) Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. *Proceedings of the National Academy of Sciences* 97: 3254

Balashov SV, Park S, Perlin DS (2006) Assessing Resistance to the Echinocandin Antifungal Drug Caspofungin in *Candida albicans* by Profiling Mutations in FKS1. *Antimicrobial Agents and Chemotherapy* 50: 2058-2063

Band VI, Weiss DS (2015) Mechanisms of antimicrobial peptide resistance in Gram-negative bacteria. *Antibiotics (Basel, Switzerland)* 4: 18-41

Barbosa Pelegrini P, del Sarto RP, Silva ON, Franco OL, Grossi-de-Sa MF (2011) Antibacterial peptides from plants: What they are and how they probably work. *Biochemistry Research International* 2011: 250349

Basso V, Tran DQ, Ouellette AJ, Selsted ME (2020) Host defense peptides as templates for antifungal drug development. *Journal of Fungi* 6: 241

Baxter A, Richter V, Lay F, Poon I, Adda C, Veneer P, Phan TK, Bleackley M, Anderson M, Kvansakul M *et al* (2015) The tomato defensin TPP3 binds phosphatidylinositol (4,5)-bisphosphate via a conserved dimeric cationic grip conformation to mediate cell lysis. *Molecular and cellular biology* 35: 1964-1978

Beardsley J, Halliday CL, Chen SCA, Sorrell TC (2018) Responding to the emergence of antifungal drug resistance: perspectives from the bench and the bedside. *Future microbiology* 13: 1175-1191

Bechinger B, Lohner K (2006) Detergent-like actions of linear amphipathic cationic antimicrobial peptides. *Biochimica et biophysica acta* 1758: 1529-1539

Benincasa M, Scocchi M, Pacor S, Tossi A, Nobili D, Basaglia G, Busetti M, Gennaro R (2006) Fungicidal activity of five cathelicidin peptides against clinically isolated yeasts. *Journal of Antimicrobial Chemotherapy* 58: 950-959

Berger S, El Chazli Y, Babu AF, Coste AT (2017) Azole resistance in *Aspergillus fumigatus*: A consequence of antifungal use in agriculture? *Frontiers in Microbiology* 8: 1024

Bleackley MR, Dawson CS, Payne JAE, Harvey PJ, Rosengren KJ, Quimbar P, Garcia-Ceron D, Lowe R, Bulone V, van der Weerden NL *et al* (2019) The interaction with fungal cell wall polysaccharides determines the salt tolerance of antifungal plant defensins. *Cell surface* 5: 100026-100026

Bleackley MR, Hayes, B. M., Parisi, K., Saiyed, T., Traven, A., Potter, I. D., van der Weerden, N. L. and Anderson, M. A. (2014) Bovine pancreatic trypsin inhibitor is a new antifungal peptide that inhibits cellular magnesium uptake. *Molecular microbiology* 92: 1188–1197

Bleackley MR, Payne JAE, Hayes BME, Durek T, Craik DJ, Shafee TMA, Poon IKH, Hulett MD, van der Weerden NL, Anderson MA (2016) *Nicotiana alata* defensin chimeras reveal differences in the mechanism of fungal and tumor cell killing and an enhanced antifungal variant. *Antimicrobial Agents and Chemotherapy* 60: 6302-6312

Bleackley MR, Wiltshire JL, Perrine-Walker F, Vasa S, Burns RL, van der Weerden NL, Anderson MA (2014) Agp2p, the plasma membrane transregulator of polyamine uptake, regulates the antifungal activities of the plant defensin NaD1 and other cationic peptides. *Antimicrobial agents and chemotherapy* 58: 2688-2698

Bondaryk M, Staniszewska M, Zielińska P, Urbańczyk-Lipkowska Z (2017) Natural antimicrobial peptides as inspiration for design of a new generation of antifungal compounds. *Journal of fungi* 3: 47

Brown AJP, Cowen LE, di Pietro A, Quinn J (2017) Stress adaptation. *Microbiology Spectrum* 5: 2165

Casadevall A (2017) Don't forget the fungi when considering global catastrophic biorisks. *Health Security* 15: 341-342

Castanheira M, Deshpande LM, Messer SA, Rhomberg PR, Pfaller MA (2020) Analysis of global antifungal surveillance results reveals predominance of Erg11 Y132F alteration among azole-resistant *Candida parapsilosis* and *Candida tropicalis* and country-specific isolate dissemination. *International Journal of Antimicrobial Agents* 55: 105799

Chaabane F, Graf A, Jequier L, Coste AT (2019) Review on antifungal resistance mechanisms in the emerging pathogen *Candida auris*. *Frontiers in Microbiology* 10: 2788

Chan DI, Prenner EJ, Vogel HJ (2006) Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action. *Biochimica et biophysica acta* 1758: 1184-1202

Chen L, Xu YH, Zhou C, Zhao J, Li CY, Wang R (2010) Overexpression of CDR1 and CDR2 genes plays an important role in fluconazole resistance in *Candida albicans* with G487T and T916C mutations. *The Journal of international medical research* 38: 536-545

Chen SCA, Sorrell TC (2007) Antifungal agents. *Medical Journal of Australia* 187: 404-409

Chowdhary A, Sharma C, Meis JF (2017) *Candida auris*: A rapidly emerging cause of hospital-acquired multidrug-resistant fungal infections globally. *PLOS Pathogens* 13: 1006290

Ciociola T, Giovati L, Conti S, Magliani W, Santinoli C, Polonelli L (2016) Natural and synthetic peptides with antifungal activity. *Future medicinal chemistry* 8: 1413-1433

Coleman JJ, Mylonakis E (2009) Efflux in fungi: la pièce de résistance. *PLoS Pathogens* 5: 1000486

Cools TL, Struyfs C, Bruno PAC, Thevissen K (2017) Antifungal plant defensins: increased insight in their mode of action as a basis for their use to combat fungal infections. *Future microbiology* 12: 441–454

Cowen LE, Sanglard D, Howard SJ, Rogers PD, Perlin DS (2014) Mechanisms of antifungal drug resistance. *Cold Spring Harbor Perspectives in Medicine* 5: 19752-19752

Cowen LE, Singh SD, Köhler JR, Collins C, Zaas AK, Schell WA, Aziz H, Mylonakis E, Perfect JR, Whitesell L *et al* (2009) Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. *Proceedings of the National Academy of Sciences of the United States of America* 106: 2818-2823

Cowen LE, Steinbach WJ (2008) Stress, drugs, and evolution: the role of cellular signaling in fungal drug resistance. *Eukaryotic cell* 7: 747-764

de Medeiros LN, Domitrovic T, de Andrade PC, Faria J, Bergter EB, Weissmüller G, Kurtenbach E (2014) Psd1 binding affinity toward fungal membrane components as assessed by SPR: The role of glucosylceramide in fungal recognition and entry. *Biopolymers* 102: 456-464

den Hertog AL, van Marle J, van Veen HA, Van't Hof W, Bolscher JG, Veerman EC, Nieuw Amerongen AV (2005) Candidacidal effects of two antimicrobial peptides: histatin 5 causes small membrane defects, but LL-37 causes massive disruption of the cell membrane. *The Biochemical journal* 388: 689-695

Dong J, Vylkova S, Li XS, Edgerton M (2003) Calcium blocks fungicidal activity of human salivary histatin 5 through disruption of binding with *Candida albicans*. *Journal of dental research* 82: 748-752

Dracatos PM, van der Weerden NL, Carroll KT, Johnson ED, Plummer KM, Anderson MA (2014) Inhibition of cereal rust fungi by both class I and II defensins derived from the flowers of *Nicotiana alata*. *Molecular Plant Pathology* 15: 67-79

El-Mounadi K, Islam KT, Hernández-Ortiz P, Read ND, Shah DM (2016) Antifungal mechanisms of a plant defensin MtDef4 are not conserved between the ascomycete fungi *Neurospora crassa* and *Fusarium graminearum*. *Molecular microbiology* 100: 542-559

Ellis D (2002) Amphotericin B: spectrum and resistance. *Journal of Antimicrobial Chemotherapy* 49: 7-10

Ferket KKA, Levery SB, Park C, Cammue BPA, Thevissen K (2003) Isolation and characterization of *Neurospora crassa* mutants resistant to antifungal plant defensins. *Fungal Genetics and Biology* 40: 176-185

Fisher MC, Gurr SJ, Cuomo CA, Blehert DS, Jin H, Stukenbrock EH, Stajich JE, Kahmann R, Boone C, Denning DW *et al* (2020) Threats posed by the fungal kingdom to humans, wildlife, and agriculture. *mBio* 11: 449-420

Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ (2018) Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science (New York, NY)* 360: 739

Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, Gurr SJ (2012) Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484: 186-194

Fitzgerald-Hughes DH, Coleman DC, O'Connell BC (2007) Differentially expressed proteins in derivatives of *Candida albicans* displaying a stable histatin 3-resistant phenotype. *Antimicrobial agents and chemotherapy* 51: 2793-2800

Fitzgerald DH, Coleman DC, O'Connell BC (2003) Binding, internalisation and degradation of histatin 3 in histatin-resistant derivatives of *Candida albicans*. *FEMS Microbiology Letters* 220: 247-253

Flowers SA, Colón B, Whaley SG, Schuler MA, Rogers PD (2015) Contribution of clinically derived mutations in ERG11 to azole resistance in *Candida albicans*. *Antimicrobial Agents and Chemotherapy* 59: 450

Fones HN, Bebber DP, Chaloner TM, Kay WT, Steinberg G, Gurr SJ (2020) Threats to global food security from emerging fungal and oomycete crop pathogens. *Nature Food* 1: 332-342

Fox JL (2013) Antimicrobial peptides stage a comeback. *Nat Biotechnol* 31: 379-382

Fuentefria AM, Pippi B, Dalla Lana DF, Donato KK, de Andrade SF (2018) Antifungals discovery: an insight into new strategies to combat antifungal resistance. *Letters in Applied Microbiology* 66: 2-13

Gao GH, Liu W, Dai JX, Wang JF, Hu Z, Zhang Y, Wang DC (2001) Solution structure of PAFP-S: a new knottin-type antifungal peptide from the seeds of *Phytolacca americana*. *Biochemistry* 40: 10973-10978

Ghannoum MA, Rice LB (1999) Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clinical microbiology reviews* 12: 501-517

Gifford JL, Hunter HN, Vogel HJ (2005) Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cellular and molecular life sciences* 62: 2588-2598

Gracy J, Chiche L (2011) Structure and modeling of knottins, a promising molecular scaffold for drug discovery. *Current Pharmaceutical Design* 17: 4337-4350

Hale J, Hancock R (2008) Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. *Expert review of anti-infective therapy* 5: 951-959

Halliday CL, Slavin MA, Chen SCA (2017) Antifungal susceptibility testing of *Candida* and *Cryptococcus Species* and mechanisms of resistance: Implications for clinical laboratories. *Current Fungal Infection Reports* 11: 124-133

Hancock RE, Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature Biotechnology* 24: 1551-1557

Harries E, Carmona L, Muñoz A, Ibeas JI, Read ND, Gandía M, Marcos JF (2013) Genes involved in protein glycosylation determine the activity and cell internalization of the antifungal peptide PAF26 in Saccharomyces cerevisiae. *Fungal genetics and biology* 58: 105-115

Harris M, Mora-Montes HM, Gow NAR, Coote PJ (2009) Loss of mannosylphosphate from *Candida albicans* cell wall proteins results in enhanced resistance to the inhibitory effect of a cationic antimicrobial peptide via reduced peptide binding to the cell surface. *Microbiology (Reading)* 155: 1058-1070

Hawkins NJ, Fraaije BA (2016) Predicting resistance by mutagenesis: Lessons from 45 years of MBC resistance. *Frontiers in microbiology* 7: 1814-1814

Hayes BM, Anderson MA, Traven A, van der Weerden NL, Bleackley MR (2014) Activation of stress signalling pathways enhances tolerance of fungi to chemical fungicides and antifungal proteins. *Cellular and molecular life sciences* 71: 2651-2666

Hayes BME, Bleackley MR, Anderson MA, van der Weerden NL (2018) The plant defensin NaD1 enters the cytoplasm of *Candida Albicans* via endocytosis. *Journal of fungi* 4: 20

Hayes BME, Bleackley MR, Wiltshire JL, Anderson MA, Traven A, van der Weerden NL (2013) Identification and mechanism of action of the plant defensin NaD1 as a new member of the antifungal drug arsenal against *Candida albicans*. *Antimicrobial Agents and Chemotherapy* 57: 3667-3675

Heilmann CJ, Schneider S, Barker KS, Rogers PD, Morschhäuser J (2010) An A643T mutation in the transcription factor Upc2p causes constitutive ERG11 upregulation and increased fluconazole resistance in *Candida albicans*. *Antimicrobial Agents and Chemotherapy* 54: 353-359

Helmerhorst EJ, Breeuwer P, van't Hof W, Walgreen-Weterings E, Oomen LC, Veerman EC, Amerongen AVN, Abee T (1999) The cellular target of histatin 5 on Candida albicans is the energized mitochondrion. *Journal of Biological Chemistry* 274: 7286-7291

Henzler-Wildman KA, Martinez GV, Brown MF, Ramamoorthy A (2004) Perturbation of the hydrophobic core of lipid bilayers by the human antimicrobial peptide LL-37. *Biochemistry* 43: 8459-8469

Ho CH, Piotrowski J, Dixon SJ, Baryshnikova A, Costanzo M, Boone C (2011) Combining functional genomics and chemical biology to identify targets of bioactive compounds. *Current opinion in chemical biology* 15: 66-78

Hokken MWJ, Zwaan BJ, Melchers WJG, Verweij PE (2019) Facilitators of adaptation and antifungal resistance mechanisms in clinically relevant fungi. *Fungal Genetics and Biology* 132: 103254

Hollmann A, Martinez M, Maturana P, Semorile LC, Maffia PC (2018) Antimicrobial Peptides: Interaction with model and biological membranes and synergism with chemical antibiotics. *Frontiers in Chemistry* 6: 204

Hsu C-H, Chen C, Jou M-L, Lee AY-L, Lin Y-C, Yu Y-P, Huang W-T, Wu S-H (2005) Structural and DNA-binding studies on the bovine antimicrobial peptide, indolicidin: evidence for multiple conformations involved in binding to membranes and DNA. *Nucleic acids research* 33: 4053-4064

Hull CM, Parker JE, Bader O, Weig M, Gross U, Warrilow AG, Kelly DE, Kelly SL (2012) Facultative sterol uptake in an ergosterol-deficient clinical isolate of *Candida glabrata* harboring a missense mutation in ERG11 and exhibiting cross-resistance to azoles and amphotericin B. *Antimicrobial Agents and Chemotherapy* 56: 4223-4232

Jang WS, Bajwa JS, Sun JN, Edgerton M (2010) Salivary histatin 5 internalization by translocation, but not endocytosis, is required for fungicidal activity in *Candida albicans*. *Molecular microbiology* 77: 354-370

Järvå M, Lay F, Phan TK, Humble C, Poon I, Bleackley M, Anderson M, Hulett M, Kvansakul M (2018a) X-ray structure of a carpet-like antimicrobial defensin–phospholipid membrane disruption complex. *Nature Communications* 9: 1962

Järvå M, Phan TK, Lay F, Caria S, Kvansakul M, Hulett M (2018b) Human -defensin 2 kills *Candida albicans* through phosphatidylinositol 4,5-bisphosphate–mediated membrane permeabilization. *Science Advances* 4: 979

Joo H-S, Fu C-I, Otto M (2016) Bacterial strategies of resistance to antimicrobial peptides. *Philosophical Transactions of the Royal Society B: Biological* 71: 20150292

Kai-Larsen Y, Agerberth B (2008) The role of the multifunctional peptide LL-37 in host defense. *Frontiers in Bioscience* 13: 3760-3767

Kang X, Dong F, Shi C, Liu S, Sun J, Chen J, Li H, Xu H, Lao X, Zheng H (2019) DRAMP 2.0, an updated data repository of antimicrobial peptides. *Science Data* 6: 148

Komatsu T, Salih E, Helmerhorst EJ, Offner GD, Oppenheim FG (2011) Influence of Histatin 5 on *Candida albicans* mitochondrial protein expression assessed by quantitative mass spectrometry. *Journal of Proteome Research* 10: 646-655

Kosikowska P, Lesner A (2016) Antimicrobial peptides (AMPs) as drug candidates: a patent review (2003-2015). *Expert Opinion on Therapeutic Patents* 26: 689-702

Kovaleva V, Bukhteeva I, Kit OY, Nesmelova IV (2020) Plant Defensins from a structural perspective. *International Journal of Molecular Sciences* 21: 5307

Krishnakumari V, Rangaraj N, Nagaraj R (2009) Antifungal activities of human beta-defensins HBD-1 to HBD-3 and their C-terminal analogs Phd1 to Phd3. *Antimicrobial Agents and Chemotherapy* 53: 256-260

Kumar P, Kizhakkedathu JN, Straus SK (2018) Antimicrobial peptides: Diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo. *Biomolecules* 8: 4

Kumar R, Chadha S, Saraswat D, Bajwa JS, Li RA, Conti HR, Edgerton M (2011) Histatin 5 uptake by *Candida albicans* utilizes polyamine transporters Dur3 and Dur31 proteins. *The Journal of biological chemistry* 286: 43748-43758

Lamoth F, Alexander BD, Juvvadi PR, Steinbach WJ (2015) Antifungal activity of compounds targeting the Hsp90-calcineurin pathway against various mould species. *Journal of Antimicrobial Chemotherapy* 70: 1408-1411

Lamoth F, Juvvadi PR, Gehrke C, Steinbach WJ (2013) In vitro activity of calcineurin and heat shock protein 90 Inhibitors against *Aspergillus fumigatus* azole- and echinocandin-resistant strains. *Antimicrobial Agents and Chemotherapy* 57: 1035-1039

Lay FT, Brugliera F, Anderson MA (2003) Isolation and properties of floral defensins from ornamental tobacco and petunia. *Plant Physiology* 131: 1283-1293

Lay FT, Mills GD, Poon IKH, Cowieson NP, Kirby N, Baxter AA, van der Weerden NL, Dogovski C, Perugini MA, Anderson MA *et al* (2012) Dimerization of plant defensin NaD1 enhances its antifungal activity. *Journal of Biological Chemistry* 287: 19961-19972

Lee AC, Harris JL, Khanna KK, Hong JH (2019) A comprehensive review on current advances in peptide drug development and design. *International Journal of Molecular Sciences* 20: 2383

Lee DG, Kim DH, Park Y, Kim HK, Kim HN, Shin YK, Choi CH, Hahm KS (2001) Fungicidal effect of antimicrobial peptide, PMAP-23, isolated from porcine myeloid

against *Candida albicans*. *Biochemical and biophysical research communications* 282: 570-574

Lee DG, Kim PI, Park Y, Park SC, Woo ER, Hahm KS (2002) Antifungal mechanism of SMAP-29 (1-18) isolated from sheep myeloid mRNA against *Trichosporon beigelii*. *Biochemical and biophysical research communications* 295: 591-596

Lee H, Lee DG (2018) Novel Approaches for efficient antifungal drug action. *Journal of Microbiology and Biotechnology* 28: 1771-1781

Lee Y, Puumala E, Robbins N, Cowen LE (2020) Antifungal drug resistance: Molecular mechanisms in *Candida albicans* and beyond. *Chemical Reviews*

Leeuw Ed, Li C, Zeng P, Li C, Buin MD-d, Lu W-Y, Breukink E, Lu W (2010) Functional interaction of human neutrophil peptide-1 with the cell wall precursor lipid II. *FEBS letters* 584: 1543-1548

Li M, Cha DJ, Lai Y, Villaruz AE, Sturdevant DE, Otto M (2007) The antimicrobial peptide-sensing system aps of *Staphylococcus aureus*. *Molecular microbiology* 66: 1136-1147

Li R, Kumar R, Tati S, Puri S, Edgerton M (2013) *Candida albicans* Flu1-Mediated Efflux of Salivary Histatin 5 Reduces Its Cytosolic Concentration and Fungicidal Activity. *Antimicrobial Agents and Chemotherapy* 57: 1832

Li XS, Sun JN, Okamoto-Shibayama K, Edgerton M (2006) *Candida albicans* cell wall ssa proteins bind and facilitate import of salivary histatin 5 required for toxicity. *The Journal of biological chemistry* 281: 22453-22463

Lis M, Bhatt S, Schoenly NE, Lee AY, Nislow C, Bobek LA (2013) Chemical genomic screening of a Saccharomyces cerevisiae genomewide mutant collection reveals genes

required for defense against four antimicrobial peptides derived from proteins found in human saliva. *Antimicrobial agents and chemotherapy* 57: 840-847

Lis M, Bobek LA (2008) Proteomic and metabolic characterization of a *Candida albicans* mutant resistant to the antimicrobial peptide MUC7 12-mer. *FEMS Immunology & Medical Microbiology* 54: 80-91

Lis M, Fuss JR, Bobek LA (2009) Exploring the mode of action of antimicrobial peptide MUC7 12-mer by fitness profiling of Saccharomyces cerevisiae genomewide mutant collection. *Antimicrobial agents and chemotherapy* 53: 3762-3769

Magana M, Pushpanathan M, Santos AL, Leanse L, Fernandez M, Ioannidis A, Giulianotti MA, Apidianakis Y, Bradfute S, Ferguson AL *et al* (2020) The value of antimicrobial peptides in the age of resistance. *The Lancet Infectious Diseases* 20: 216-230

Mahlapuu M, Håkansson J, Ringstad L, Björn C (2016) Antimicrobial peptides: An emerging category of therapeutic agents. *Frontiers in Cellular and Infection Microbiology* 6: 194-194

Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FCS, Odds FC, Vanden Bossche H (1999) Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. *Microbiology (Reading, England)* 145: 2701-2713

Martin SW, Konopka JB (2004) Lipid raft polarization contributes to hyphal growth in *Candida albicans*. *Eukaryotic cell* 3: 675-684

Mattila J-P, Sabatini K, Kinnunen PKJ (2008) Oxidized phospholipids as potential molecular targets for antimicrobial peptides. *Biochimica et biophysica acta* 1778: 2041-2050

McCrudden M, McLean D, Zhou M, Shaw J, Linden G, Irwin C, Lundy F (2014) The host defence peptide LL-37 is susceptible to proteolytic degradation by wound fluid isolated from foot ulcers of diabetic patients. *International Journal of Peptide Research and Therapeutics* 20: 457–464

Mello EO, Ribeiro SF, Carvalho AO, Santos IS, Da Cunha M, Santa-Catarina C, Gomes VM (2011) Antifungal activity of PvD1 defensin involves plasma membrane permeabilization, inhibition of medium acidification, and induction of ROS in fungi cells. *Current microbiology* 62: 1209-1217

Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ (2020) Antimicrobial host defence peptides: functions and clinical potential. *Nature Reviews Drug Discovery* 19: 311-332

Muñoz A, Chu M, Marris PI, Sagaram US, Kaur J, Shah DM, Read ND (2014) Specific domains of plant defensins differentially disrupt colony initiation, cell fusion and calcium homeostasis in *Neurospora crassa*. *Molecular microbiology* 92: 1357-1374

Munoz A, López-García B, Marcos JF (2006) Studies on the mode of action of the antifungal hexapeptide PAF26. *Antimicrobial agents and chemotherapy* 50: 3847-3855

Nguyen LT, Haney EF, Vogel HJ (2011) The expanding scope of antimicrobial peptide structures and their modes of action. *Trends in biotechnology* 29: 464-472

O'Meara TR, Robbins N, Cowen LE (2017) The Hsp90 chaperone network modulates *Candida* virulence traits. *Trends in microbiology* 25: 809-819

O'Shaughnessy EM, Lyman CA, Walsh TJ (2009) Amphotericin B: Polyene resistance mechanisms. In: *Antimicrobial Drug Resistance: Mechanisms of Drug Resistance,* Mayers D.L. (ed.) pp. 295-305. Humana Press: Totowa, NJ

Ordonez SR, Amarullah IH, Wubbolts RW, Veldhuizen EJA, Haagsman HP (2014) Fungicidal mechanisms of cathelicidins LL-37 and CATH-2 revealed by live-cell imaging. *Antimicrobial Agents and Chemotherapy* 58: 2240

Parisi K, Doyle S, Lee E, Lowe R, Weerden N, Anderson M, Bleackley M (2019) Screening the *Saccharomyces cerevisiae* nonessential gene deletion library reveals diverse mechanisms of action for antifungal plant defensins. *Antimicrobial Agents and Chemotherapy* 63: 1097

Parisi K, Poon S, Renda RF, Sahota G, English J, Yalpani N, Bleackley MR, Anderson MA, van der Weerden NL (2020) Improving the digestibility of plant defensins to meet regulatory requirements for transgene products in crop protection. *Frontiers in Plant Science* 11: 1227

Parisi K, Shafee TMA, Quimbar P, van der Weerden NL, Bleackley MR, Anderson MA (2018) The evolution, function and mechanisms of action for plant defensins. *Seminars in cell & developmental biology* 88: 107-118

Park K, Oh D, Shin SY, Hahm KS, Kim Y (2002) Structural studies of porcine myeloid antibacterial peptide PMAP-23 and its analogues in DPC micelles by NMR spectroscopy. *Biochemical and biophysical research communications* 290: 204-212

Pasupuleti M, Schmidtchen A, Malmsten M (2012) Antimicrobial peptides: key components of the innate immune system. *Critical reviews in biotechnology* 32: 143-171

Pathirana RU, Friedman J, Norris HL, Salvatori O, McCall AD, Kay J, Edgerton M (2018) Fluconazole-resistant *Candida auris* is susceptible to salivary Histatin 5 killing and to intrinsic host defenses. *Antimicrobial Agents and Chemotherapy* 62: 1872

Payne JA, Bleackley MR, Lee TH, Shafee TM, Poon IK, Hulett MD, Aguilar MI, van der Weerden NL, Anderson MA (2016) The plant defensin NaD1 introduces membrane disorder through a specific interaction with the lipid, phosphatidylinositol 4,5 bisphosphate. *Biochimica et biophysica acta* 1858: 1099-1109

Pea F, Lewis RE (2018) Overview of antifungal dosing in invasive candidiasis. *Journal of Antimicrobial Chemotherapy* 73: i33-i43

Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A (2017) The global problem of antifungal resistance: prevalence, mechanisms, and management. *The Lancet Infectious diseases* 17: 383-392

Pianalto KM, Alspaugh JA (2016) New horizons in antifungal therapy. *Journal of fungi* 2

Pierce SE, Davis RW, Nislow C, Giaever G (2007) Genome-wide analysis of barcoded Saccharomyces cerevisiae gene-deletion mutants in pooled cultures. *Nature protocols* 2: 2958

Pinto E, Monteiro C, Maia M, Faria MA, Lopes V, Lameiras C, Pinheiro D (2018) *Aspergillus Species* and antifungals susceptibility in clinical setting in the north of Portugal: *Cryptic species* and emerging azoles resistance in *A. fumigatus*. *Frontiers in Microbiology* 9: 1656

Poon IKH, Baxter AA, Lay FT, Mills GD, Adda CG, Payne JAE, Phan TK, Ryan GF, White JA, Veneer PK *et al* (2014) Phosphoinositide-mediated oligomerization of a defensin induces cell lysis. *eLife* 3: 01808

Posteraro B, Sanguinetti M, Sanglard D, La Sorda M, Boccia S, Romano L, Morace G, Fadda G (2003) Identification and characterization of a *Cryptococcus neoformans* ATP binding cassette (ABC) transporter-encoding gene, CnAFR1, involved in the resistance to fluconazole. *Molecular microbiology* 47: 357-371

Prasad R, Shah A, Rawal M (2016) Antifungals: Mechanism of action and drug resistance. *Advances in experimental medicine and biology* 892: 327-349

Puri S, Edgerton M (2014) How does it kill?: understanding the candidacidal mechanism of salivary histatin 5. *Eukaryotic cell* 13: 958-964

Raheem N, Straus SK (2019) Mechanisms of action for antimicrobial peptides with antibacterial and antibiofilm functions. *Frontiers in Microbiology* 10: 2866

Ramamoorthy V, Cahoon EB, Li J, Thokala M, Minto RE, Shah DM (2007) Glucosylceramide synthase is essential for alfalfa defensin-mediated growth inhibition but not for pathogenicity of *Fusarium graminearum*. *Molecular microbiology* 66: 771-786

Rautenbach M, Troskie AM, Vosloo JA (2016a) Antifungal peptides: To be or not to be membrane active. *Biochimie* 130: 132-145

Rautenbach M, Troskie AM, Vosloo JA, Dathe ME (2016b) Antifungal membranolytic activity of the tyrocidines against filamentous plant fungi. *Biochimie* 130: 122-131

Rees DC, Lipscomb WN (1982) Refined crystal structure of the potato inhibitor complex of carboxypeptidase A at 2.5 A resolution. *Journal of molecular biology* 160: 475-498

Rego A, Trindade D, Chaves SR, Manon S, Costa V, Sousa MJ, Côrte-Real M (2014) The yeast model system as a tool towards the understanding of apoptosis regulation by sphingolipids. *FEMS yeast research* 14: 160-178

Revie NM, Iyer KR, Robbins N, Cowen LE (2018) Antifungal drug resistance: evolution, mechanisms and impact. *Current opinion in microbiology* 45: 70-76

Robbins N, Caplan T, Cowen LE (2017a) Molecular Evolution of Antifungal Drug Resistance. *Annual Review of Microbiology* 71: 753-775

Rosana Y, Yasmon A, Lestari DC (2015) Overexpression and mutation as a genetic mechanism of fluconazole resistance in *Candida albicans* isolated from human immunodeficiency virus patients in Indonesia. *Journal of Medical Microbiology* 64: 1046-1052

Roy H (2009) Tuning the properties of the bacterial membrane with aminoacylated phosphatidylglycerol. *IUBMB Life* 61: 940-953

Sagaram US, El-Mounadi K, Buchko GW, Berg HR, Kaur J, Pandurangi RS, Smith TJ, Shah DM (2013) Structural and functional studies of a phosphatidic acid-binding antifungal plant defensin MtDef4: Identification of an RGFRRR motif governing fungal cell entry. *PLOS ONE* 8: 82485

Sagaram US, Pandurangi R, Kaur J, Smith TJ, Shah DM (2011) Structure-activity determinants in antifungal plant defensins MsDef1 and MtDef4 with different modes of action against *Fusarium graminearum*. *PLOS ONE* 6: 18550

Schneider T, Kruse T, Wimmer R, Wiedemann I, Sass V, Pag U, Jansen A, Nielsen AK, Mygind PH, Raventós DS *et al* (2010) Plectasin, a fungal defensin, targets the bacterial cell wall precursor Lipid II. *Science (New York, NY)* 328: 1168

Schuber F (1989) Influence of polyamines on membrane functions. *Biochemical Journal* 260: 1

Sengupta D, Leontiadou H, Mark AE, Marrink S-J (2008) Toroidal pores formed by antimicrobial peptides show significant disorder. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1778: 2308-2317

Sevcsik E, Pabst G, Jilek A, Lohner K (2007) How lipids influence the mode of action of membrane-active peptides. *Biochimica et biophysica acta* 1768: 2586-2595

Seyfi R, Kahaki FA, Ebrahimi T, Montazersaheb S, Eyvazi S, Babaeipour V, Tarhriz V (2020) Antimicrobial peptides (AMPs): Roles, functions and mechanism of action. *International Journal of Peptide Research and Therapeutics* 26: 1451-1463

Shafee T, Anderson MA (2019) A quantitative map of protein sequence space for the cis-defensin superfamily. *Bioinformatics* 35: 743-752

Shafee TMA, Lay FT, Hulett MD, Anderson MA (2016) The defensins consist of two independent, convergent protein superfamilies. *Molecular Biology and Evolution* 33: 2345-2356

Shafee TMA, Lay FT, Phan TK, Anderson MA, Hulett MD (2017) Convergent evolution of defensin sequence, structure and function. *Cellular and Molecular Life Sciences* 74: 663-682

Shoemaker DD, Lashkari DA, Morris D, Mittmann M, Davis RW (1996) Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar–coding strategy. *Nature genetics* 14: 450

Sierotzki H, Parisi S, Steinfeld U, Tenzer I, Poirey S, Gisi U (2000) Mode of resistance to respiration inhibitors at the cytochrome bc1 enzyme complex of *Mycosphaerella fijiensis* field isolates. *Pest Management Science* 56: 833-841

Singh A, Prasad R (2011) Comparative lipidomics of azole sensitive and resistant clinical isolates of *Candida albicans* reveals unexpected diversity in molecular lipid imprints. *PLoS One* 6: 19266

Singh SD, Robbins N, Zaas AK, Schell WA, Perfect JR, Cowen LE (2009) Hsp90 governs echinocandin resistance in the pathogenic yeast *Candida albicans* via calcineurin. *PLoS Pathogens* 5: 1000532

Situ H, Wei G, Smith CJ, Mashhoon S, Bobek LA (2003) Human salivary MUC7 mucin peptides: effect of size, charge and cysteine residues on antifungal activity. *The Biochemical journal* 375: 175-182

Skerlavaj B, Benincasa M, Risso A, Zanetti M, Gennaro R (1999) SMAP-29: a potent antibacterial and antifungal peptide from sheep leukocytes. *FEBS letters* 463: 58-62

Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IS, Denning DW (2002) Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate. *Fungal Genetics and Biology* 36: 199-206

Spelbrink RG, Dilmac N, Allen A, Smith TJ, Shah DM, Hockerman GH (2004) Differential antifungal and calcium channel-blocking activity among structurally related plant defensins. *Plant Physiology* 135: 2055

Sun JN, Li W, Jang WS, Nayyar N, Sutton MD, Edgerton M (2008) Uptake of the antifungal cationic peptide Histatin 5 by *Candida albicans* Ssa2p requires binding to non-conventional sites within the ATPase domain. *Molecular microbiology* 70: 1246-1260

Swidergall M, Ernst JF (2014) Interplay between *Candida albicans* and the antimicrobial peptide armory. *Eukaryotic cell* 13: 950-957

Szafranski-Schneider E, Swidergall M, Cottier F, Tielker D, Román E, Pla J, Ernst JF (2012) Msb2 shedding protects *Candida albicans* against antimicrobial peptides. *PLoS pathogens* 8: 1002501-1002501

Taipale M, Jarosz DF, Lindquist S (2010) HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nature reviews Molecular cell biology* 11: 515-528

Takahashi D, Shukla SK, Prakash O, Zhang G (2010) Structural determinants of host defense peptides for antimicrobial activity and target cell selectivity. *Biochimie* 92: 1236-1241

Takeshita N, Higashitsuji Y, Konzack S, Fischer R (2008) Apical sterol-rich membranes are essential for localizing cell end markers that determine growth directionality in the filamentous fungus *Aspergillus nidulans*. *Molecular Biology Cell* 19: 339-351

Teta R, Marteinsson VT, Longeon A, Klonowski AM, Groben R, Bourguet-Kondracki M-L, Costantino V, Mangoni A (2017) Thermoactinoamide A, an antibiotic lipophilic cyclopeptide from the Icelandic thermophilic bacterium *Thermoactinomyces vulgaris*. *Journal of Natural Products* 80: 2530-2535

Thevissen K, Cammue BP, Lemaire K, Winderickx J, Dickson RC, Lester RL, Ferket KK, Van Even F, Parret AH, Broekaert WF (2000a) A gene encoding a sphingolipid biosynthesis enzyme determines the sensitivity of *Saccharomyces cerevisiae* to an antifungal plant defensin from dahlia (Dahlia merckii). *Proceedings of the National Academy of Sciences of the United States of America* 97: 9531-9536

Thevissen K, de Mello Tavares P, Xu D, Blankenship J, Vandenbosch D, Idkowiak-Baldys J, Govaert G, Bink A, Rozental S, de Groot PW *et al* (2012) The plant defensin RsAFP2 induces cell wall stress, septin mislocalization and accumulation of ceramides in *Candida albicans*. *Molecular microbiology* 84: 166-180

Thevissen K, Francois IE, Takemoto JY, Ferket KK, Meert EM, Cammue BP (2003) DmAMP1, an antifungal plant defensin from dahlia (Dahlia merckii), interacts with sphingolipids from *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* 226: 169-173

Thevissen K, Osborn RW, Acland DP, Broekaert WF (1997) Specific, high affinity binding sites for an antifungal plant defensin on Neurospora crassa hyphae and microsomal membranes. *Journal of Biological Chemistry* 272: 32176-32181

Thevissen K, Osborn RW, Acland DP, Broekaert WF (2000b) Specific Binding Sites for an Antifungal Plant Defensin from Dahlia (Dahlia merckii) on Fungal Cells Are Required for Antifungal Activity. *Molecular Plant-Microbe Interactions* 13: 54-61

Thevissen K, Warnecke DC, François IE, Leipelt M, Heinz E, Ott C, Zähringer U, Thomma BP, Ferket KK, Cammue BP (2004) Defensins from insects and plants interact with fungal glucosylceramides. *The Journal of biological chemistry* 279: 3900-3905

Tobias NJ, Wolff H, Djahanschiri B, Grundmann F, Kronenwerth M, Shi Y-M, Simonyi S, Grün P, Shapiro-Ilan D, Pidot SJ *et al* (2017) Natural product diversity associated with the nematode symbionts Photorhabdus and Xenorhabdus. *Nature microbiology* 2: 1676-1685

Troskie AM, Rautenbach M, Delattin N, Vosloo JA, Dathe M, Cammue BP, Thevissen K (2014) Synergistic activity of the tyrocidines, antimicrobial cyclodecapeptides from *Bacillus aneurinolyticus*, with amphotericin B and caspofungin against *Candida albicans* biofilms. *Antimicrobial Agents and Chemotherapy* 58: 3697-3707

Tsai P-W, Cheng Y-L, Hsieh W-P, Lan C-Y (2014a) Responses of *Candida albicans* to the human antimicrobial peptide LL-37. *Journal of Microbiology* 52: 581-589

Tsai P-W, Yang C-Y, Chang H-T, Lan C-Y (2011) Characterizing the role of cell-wall β-1,3-exoglucanase Xog1p in *Candida albicans* adhesion by the human antimicrobial peptide LL-37. *PloS one* 6: 21394-21394

Tsai PW, Cheng YL, Hsieh WP, Lan CY (2014b) Responses of *Candida albicans* to the human antimicrobial peptide LL-37. *Journal of Microbiology* 52: 581-589

Tsao S, Rahkhoodaee F, Raymond M (2009) Relative contributions of the *Candida albicans* ABC transporters Cdr1p and Cdr2p to clinical azole resistance. *Antimicrobial agents and chemotherapy* 53: 1344-1352

Tzeng YL, Stephens DS (2015) Antimicrobial peptide resistance in *Neisseria meningitidis*. *Biochimica et biophysica acta* 1848: 3026-3031

van der Weerden NL, Bleackley MR, Anderson MA (2013) Properties and mechanisms of action of naturally occurring antifungal peptides. *Cellular and Molecular Life Sciences* 70: 3545-3570

van der Weerden NL, Hancock REW, Anderson MA (2010) Permeabilization of fungal hyphae by the plant defensin NaD1 occurs through a cell wall-dependent process. *Journal of Biological Chemistry* 285: 37513-37520

van der Weerden NL, Lay FT, Anderson MA (2008) The plant defensin, NaD1, enters the cytoplasm of *Fusarium oxysporum* hyphae. *Journal of Biological Chemistry* 283: 14445-14452

Vandamme D, Landuyt B, Luyten W, Schoofs L (2012) A comprehensive summary of LL-37, the factotum human cathelicidin peptide. *Cell Immunology* 280: 22-35

Vincent BM, Lancaster AK, Scherz-Shouval R, Whitesell L, Lindquist S (2013) Fitness trade-offs restrict the evolution of resistance to amphotericin B. *PLoS biology* 11: 1001692

Wang G (2014) Human antimicrobial peptides and proteins. *Pharmaceuticals (Basel)* 7: 545-594

Wiederhold NP (2017) Antifungal resistance: current trends and future strategies to combat. *Infect Drug Resist* 10: 249-259

Yeaman MR, Büttner S, Thevissen K (2018) Regulated cell death as a therapeutic target for novel antifungal peptides and biologics. *Oxidative Medicine and Cellular Longevity* 2018: 5473817

Yeaman MR, Yount NY (2003) Mechanisms of antimicrobial peptide action and resistance. *Pharmacological reviews* 55: 27-55

Zhang L-j, Gallo RL (2016) Antimicrobial peptides. *Current Biology* 26: 14-19

Zoran T, Sartori B, Sappl L, Aigner M, Sánchez-Reus F, Rezusta A, Chowdhary A, Taj-Aldeen SJ, Arendrup MC, Oliveri S *et al* (2018) Azole-resistance in *Aspergillus terreus* and related Species: An emerging problem or a rare phenomenon? *Frontiers in Microbiology* 9: 516

Appendix

Manuscript:

McColl AI, Bleackley MR, Parisi K, Anderson MA, (2020) The Mechanisms of Resistance to Antifungal Peptides.

The development of resistance to clinical antifungals

Fungal diseases are estimated to cause over 1.6 million deaths annually and over one billion people globally suffer from fungal diseases (Fisher *et al*, 2020). However most estimates of the incidence of fungal disease are conservative because public health surveillance of these diseases is generally not compulsory (Casadevall, 2017). Fungal diseases are also a major threat to food security because damage to crop plants causes major losses in yield and food quality (Avery *et al*, 2019; Fisher *et al*, 2012; Fones *et al*, 2020). Today, crop-destroying fungi account for perennial yield losses of ~20% worldwide, with a further 10% loss postharvest (Fisher *et al.*, 2020).

Currently, only a limited number antifungal drugs have been approved for use in humans (Pianalto & Alspaugh, 2016). These antifungals are generally grouped into three classes based on their site of action. They are the azoles, which inhibit ergosterol synthesis (the main fungal sterol); polyenes, which interact with fungal membrane sterols and echinochandins, which affect the cell wall by inhibiting (1,3)-β-D-glucan synthases (Chen & Sorrell, 2007). Unfortunately new species of multidrug-resistant pathogenic fungi are emerging and are of great concern in the clinical setting (Fisher *et al*, 2018). Resistance evolves via natural selection through random mutation, or by application of an evolutionary stress on a population (Revie *et al*, 2018). Pathogens that acquire a beneficial mutation that allows them to survive the stress will live on to reproduce, whereas the bulk of the population will have their growth retarded or will be killed. The organisms with the beneficial mutation then pass this trait to their offspring generating a fully resistant generation. A pathogen with a range of resistance genes conferring resistance to more than one drug, is called multi-drug resistant or, informally, a superbug. Examples of this are clinical isolates of *Candida auris* which have acquired resistance to all the major classes of antifungals and isolates of *Aspergillus fumigatus* which are resistant to all primary azole treatments (Perlin *et al*, 2017; Pinto *et al*, 2018). Factors

contributing towards the development of resistance include incorrect diagnosis, unnecessary prescriptions, and improper use by patients (e.g., when dosages are too low and don't lead to rapid killing, or when treatment courses are not long enough or used for long periods prophylactically) (Pea & Lewis, 2018). The use of fungicides in agriculture for prevention and treatment of fungal diseases in crops can also contribute to resistance in people exposed to those fungicides (Berger *et al*, 2017). Most human pathogens also have environmental niches, implying that the agricultural use of fungicides with similarities to medically-approved drugs imposes the concrete risk of fostering drug resistance via environmental exposure imparting selective pressures that benefit resistant strains (Berger *et al.*, 2017; Wiederhold, 2017) . Emergence of antifungal resistance can endanger the already limited treatments options, with calamitous effects for treatment outcomes (Perlin *et al.*, 2017). New antifungal compounds with different mechanisms of action are needed to overcome the problem of growing antifungal resistance. Novel antifungal agents should have broad-spectrum activity, target specificity, low toxicity, a diverse mode of action and no antagonistic effects with other medications (Aoki & Ueda, 2013; Ciociola *et al*, 2016). Although new drugs may not fulfil all these criteria, these properties should be used as guidelines in drug discovery (Wang, 2014). Recently, there has been an increased interest in antimicrobial peptides as a promising approach for discovery and development of novel antifungal agents (Mookherjee *et al*, 2020). In this context, peptides have favourable properties, such as moderate immunogenicity as described below, strong antimicrobial activity, high specificity and affinity for targets, distinct mechanisms of action, good organ and tissue penetration and broad-spectrum activity (Aoki & Ueda, 2013; Ciociola *et al.*, 2016). However, before a new antifungal agent is adopted, we need to understand how resistance may develop prior to clinical use. This will help in determining which peptides should be used in combination therapy with

existing antifungal drugs and in prioritising development of antifungal peptides with a lower propensity for resistance.

Mechanisms of Resistance to clinical fungicides

Resistance to current antifungals can develop in multiple ways and is broadly characterized by the mechanism by which resistance occurs. These mechanisms include drug target alteration or overexpression, upregulation of multidrug transporters, and activation of stress responses (Table 1) (Cowen *et al*, 2014; Ghannoum & Rice, 1999; Mookherjee *et al.*, 2020).

Drug target alteration/overexpression

The most common way for resistance to occur is through the alteration of a protein target site that the antifungals bind to (Hokken *et al*, 2019). When fungi grow, mutations in their genome can occur due to replication error. These mutations can alter the amino acid sequence of the target site, therefore changing its structure and reducing the likelihood of binding by a fungicide (Sierotzki *et al*, 2000). A common mechanism of azole resistance in *C. albicans* involves amino acid substitutions in the drug target, Erg11, which leads to lower drug-binding affinity (Revie *et al.*, 2018). Over 140 amino acid substitutions in Erg11 have been associated with azole resistance, with the majority of these substitutions clustered into hot-spot regions (Arendrup & Patterson, 2017; Castanheira *et al*, 2020; Halliday *et al*, 2017; Marichal *et al*, 1999). Azole resistance has also been identified in Cryptococcus and Aspergillus species with mutations identified in the Lanosterol 14-α Sterol Demethylase Gene *CYP51A* (Zoran et al, 2018).

Overexpression of *ERG11* is also common in azole-resistant clinical isolates of *C. albicans* and *A. fumigatus* and contributes directly to increased target abundance,

ultimately lowering drug susceptibility (Pinto *et al.*, 2018; Robbins *et al*, 2017b). In *C. albicans*, the transcriptional activator, Upc2, is a crucial regulator of many ergosterol biosynthesis genes, including *ERG11*. Gain-of-function mutations in *UPC2* cause the constitutive overexpression of ergosterol biosynthesis genes, a higher ergosterol content and a reduction in fluconazole susceptibility (Heilmann *et al*, 2010).

Resistance to polyenes such as amphotericin B is uncommon. However, in the rare incidence that it does occur, it is mediated by alterations in enzymes that reduce drug-binding affinity or deplete ergosterol from the membrane (Ellis, 2002; O'Shaughnessy *et al*, 2009). In *C. albicans*, reduced amphotericin B susceptibility can occur through mutations in several ergosterol biosynthesis enzymes, including *ERG2*, *ERG3*, *ERG5*, and *ERG11* (Ellis, 2002; O'Shaughnessy *et al.*, 2009). Likewise, for *C. glabrata*, mutations in *ERG2*, *ERG6*, and *ERG11* have been documented in polyene-resistant clinical isolates (Hull *et al*, 2012).

Another less commonly used class of antifungal is flucytosine, which is a nucleoside analogue that inhibits nucleic acid synthesis. In all *Candida* species, flucytosine resistance has been linked to mutations in *FUR1,* a gene involved in 5-FC metabolism (Chaabane *et al*, 2019).

Echinocandin resistance is primarily mediated by mutations in the *FKS* genes which encode the catalytic subunit of 1,3-β-glucan synthase, which is necessary for production of 1,3-β-D-glucan, an essential component of all fungal cell walls. In *C. albicans*, mutations that confer echinocandin resistance occur at hot-spot regions in the essential gene, *FKS1* (Balashov *et al*, 2006). More recently, sequencing of 38 *C. auris* strains led to the discovery of an additional hot-spot mutation in *FKS1* that leads to a S639F amino acid substitution that is correlated with pan-echinocandin resistance (Chaabane *et al.*, 2019).

Upregulation of multidrug transporters

Another prominent mechanism of resistance is drug efflux. The main class of efflux pumps implicated in azole drug resistance is the ATP-binding cassette (ABC) superfamily. ABC transporters possess two transmembrane-spanning domains and two cytoplasmic nucleotide-binding domains (NBDs) (Coleman & Mylonakis, 2009). The NBD drives the movement of substrates across the fungal membrane via ATP hydrolysis (Coleman & Mylonakis, 2009). In *C. albicans*, overexpression of two homologous ABC transporters, Cdr1p and Cdr2p, has been frequently implicated in azole resistance (Tsao *et al*, 2009). The second class of efflux pumps implicated in azole resistance is the major facilitator (MF) superfamily. Like the ABC superfamily, MF transporters also possess transmembrane-spanning helices but use the proton gradient generated across the plasma membrane to drive MF-mediated translocation (Coleman & Mylonakis, 2009). Fluconazole resistance has been linked to Mdr1 (multidrug resistance 1) overexpression (Rosana *et al*, 2015). In *C. neoformans* and *A. fumigatus*, the ABC transporters responsible for azole efflux are Afr1 and AtrF, respectively (Posteraro *et al*, 2003; Slaven *et al*, 2002)

Activation of stress responses

The diverse and dynamic niches that fungal pathogens inhabit are subject to a variety of environmental fluctuations, including temperature, pH, and nutrient levels which are capable of perturbing cellular homeostasis and imposing significant stress on the fungal cell (Brown *et al*, 2017). Antifungal agents represent a chemical stressor these pathogens must recognize, respond to, and adapt to in order to survive (Cowen & Steinbach, 2008; Robbins *et al*, 2017a). Consequently, fungal pathogens have evolved broad stress-response circuitry that enables them to thrive in the presence of diverse cellular insults (Hayes *et al*, 2014b). A global cellular regulator that governs stress responses in diverse

fungal pathogens is the essential molecular chaperone, heat shock protein 90 (Hsp90) (O'Meara *et al*, 2017). Hsp90 is highly abundant, and its function is tightly coupled to environmental perturbations. It interacts with over 20 co-chaperones that facilitate the recognition of specific client proteins, which are enriched in kinases, signal transducers, and transcription factors, many of which serve as hubs in regulatory networks (Taipale *et al*, 2010). Thus, Hsp90 regulates basal tolerance and resistance to azole, echinocandin and polyene antifungals through multiple signalling cascades (Cowen *et al.*, 2014; Cowen *et al*, 2009; Singh *et al*, 2009; Vincent *et al*, 2013).

Mode of resistance	Antifungal class	Detailed mechanism	Isolates	Reference
Drug target alteration /overexpression	Azoles (eg. Itraconazole)	Mutation in *ERG11* GOF mutation in *UPC2* Mutations in *CYP51A*	*Candida parapsilosis* and *C. tropicalis C. albicans, C. glabrata* and more recently *C. auris Cryptococcus species*, and *Aspergillus species*	(Arendrup & Patterson, 2017; Castanheira *et al.*, 2020; Halliday *et al.*, 2017; Heilmann *et al.*, 2010; Marichal *et al.*, 1999; Revie *et al.*, 2018; Zoran *et al.*, 2018)
	Echinochandins (eg. Caspofungin)	Mutation in *FKS1*	*C. albicans, C. auris,* and *C. glabrata*	(Balashov *et al.*, 2006; Chaabane *et al.*, 2019).
	Polyenes	LOF mutation in *ERG* genes	*Candida species, Fusarium species, Scedosporium apiospermum*, and *Sporothrix schenckii*	(Ellis, 2002; Hull *et al.*, 2012; O'Shaughnessy *et al.*, 2009)
Efflux pump overexpression	Azoles	Overexpression of *CDR1, CDR2,* Mdr-like pumps, *MDR1–4*, and ABC transporters *AFR1* and *ATRF*	*C. albicans, C. neoformans,* and *A. fumigatus*	(Chen *et al*, 2010; Posteraro *et al.*, 2003; Rosana *et al.*, 2015; Slaven *et al.*, 2002; Tsao *et al.*, 2009)
Cellular stress response pathways	Azoles Echinocandins Polyenes	Inhibition of Hsp90 and downstream signalling cascades	*Candida* and *Aspergillus species*	(Cowen *et al.*, 2014; Cowen *et al.*, 2009; Lamoth *et al*, 2015; Lamoth *et al*, 2013; Singh *et al.*, 2009; Vincent *et al.*, 2013)

Table 1.1. Summary of mechanisms of resistance to small molecule drugs and species in which resistance has been identified.

In depth reviews have been completed on the disadvantages of clinical drug resistance (Cowen *et al.*, 2014; Lee *et al*, 2020; Robbins *et al.*, 2017a). The rapidly increasing resistance toward conventional antifungals that is presented in these reviews has led to the conclusion that without urgent action previously effective therapeutic strategies for fungal diseases are not sustainable. Due to the limited number of available antifungals, and the similarities in their activity spectrum as well as mode of action, intensive nonclinical and clinical research needs to be conducted to identify new antifungal therapies. Interestingly, the antimicrobial peptides (AMPs) are gaining more attention as novel drug candidates (Kosikowska & Lesner, 2016; Mookherjee *et al.*, 2020). AMPs have been found in virtually all organisms and they display remarkable structural and functional diversity, which make them especially interesting compounds for the development of novel therapeutics.

Introduction to Antimicrobial Peptides

Antimicrobial peptides (AMP's) are naturally occurring molecules that provide a first line of defence against pathogenic microorganisms. AMP's exhibit a broad range of inhibitory activity, protecting their host by either directly killing or retarding the growth of pathogens, or by activating the immune system, wound healing or the inflammatory response in higher eukaryotes (Mookherjee *et al.*, 2020). Plants, mammals and insects all release AMP's in response to infections from bacteria or fungi. Microorganisms also release AMP's to kill competing microorganisms (Magana *et al*, 2020; Tobias *et al*, 2017). AMP's can be divided into several different classes with different mechanisms of action and different microbial targets including yeast, fungi, bacteria, and parasites (Zhang & Gallo, 2016). Peptides have promising properties for clinical development, such as moderate immunogenicity, strong antimicrobial activity, high specificity and affinity for targets, distinct mechanisms of action, good organ and tissue penetration and broad-spectrum activity (Aoki & Ueda, 2013; Ciociola

167 *et al.*, 2016). Against a background of rapidly increasing resistance development to
168 conventional antimicrobials all over the world, efforts to bring AMPs into clinical use are
169 accelerating. There are encouraging examples of AMPs already introduced into the market,
170 and many AMPs have been or are currently being tested in clinical trials (Fox, 2013;
171 Mahlapuu *et al*, 2016b; Mookherjee *et al.*, 2020), which provides a reason for investigation
172 into the mechanisms of action and resistance of the most promising candidates.
173

174 **Structure and diversity of AMP's**
175

176 AMP's are typically 10- 50 amino acids in length, are generally positively charged and often
177 contain hydrophobic residues, forming unique amphipathic structures (Pasupuleti *et al*,
178 2012). The amphipathicity of AMPs facilitates their interactions with microbial membranes
179 while their cationic nature makes them ideal for targeting the negatively charged surface of
180 bacteria and fungi (Hancock & Sahl, 2006). AMP's can be classified based on their
181 secondary structure, these include α-helical and β-sheet peptides as well as peptides with an
182 extended/random-coil structure (Nguyen *et al*, 2011; Takahashi *et al*, 2010; van der Weerden
183 *et al*, 2013) (Figure 1). α-helical peptides are unstructured in aqueous solutions, but when
184 they encounter a biological membrane, they form an amphipathic helical structure (Pasupuleti
185 *et al.*, 2012; Yeaman & Yount, 2003). The most studied examples of α-helical peptides are
186 the human cathelicidin LL-37, and the histidine-rich peptides from human saliva, histatin 3
187 and 5 (Dong *et al*, 2003; Fitzgerald *et al*, 2003; Vandamme *et al*, 2012). β-sheet peptides
188 usually have two or more β-strands that are stabilized with inter-strand disulfide bonds. Due
189 to their rigid structure, these peptides do not undergo drastic conformational changes in
190 aqueous solution or when interacting with biological membranes (Nguyen *et al.*, 2011). The
191 knottin-type peptides such as PAFP-S, from *Phytolacca americana,* are an example of β-
192 sheet peptides, which as the name suggests, have a cystine-stabilized, "knotted" topology,

193 defined by two parallel disulfide bonds threaded by a third disulfide bond (Gao *et al*, 2001;
194 Gracy & Chiche, 2011). α-defensins such as human HNP4 (Ashrafi *et al*, 2017) have 6
195 conserved cysteine residues which form 3 disulfide bonds in the 3 stranded β-sheet fold.
196 There are also examples of more complex peptides, that contain a combination of α-helical
197 and β-sheet structures (Kovaleva *et al*, 2020; Rees & Lipscomb, 1982). They include
198 vertebrate β-defensins and defensins from insects, invertebrates and molluscs. This group of
199 defensins includes human HBD2 (Krishnakumari *et al*, 2009) . β-defensins have 6 conserved
200 cysteines forming 3 disulfide bonds and differ from α-defensins in the connectivity of
201 disulfide bonds and the addition of a short α helix. Despite their extensive structural and
202 functional similarities the vertebrate β-defensins and defensins from insects, invertebrates and
203 molluscs actually consist of two evolutionarily independent superfamilies (trans and cis-
204 defensins) having arisen by convergent evolution (Shafee & Anderson, 2019; Shafee *et al*,
205 2016; Shafee *et al*, 2017). Most *trans*-defensins occur in vertebrates (fish, reptiles, birds and
206 mammals) (Shafee *et al.*, 2016). The larger superfamily is named the *cis*-defensins and is
207 dominated by plant defensins (Shafee & Anderson, 2019). Plant defensins have very different
208 sequences and mechanisms of action but have a common fold consisting of a triple-stranded,
209 anti-parallel β-sheet connected to an α-helix by three disulfide bonds forming a cystine-
210 stabilized αβ motif (Parisi *et al*, 2018; van der Weerden *et al.*, 2013). A fourth disulfide bond
211 joins the N- and C-termini creating an extremely stable protein (van der Weerden *et al.*,
212 2013).
213 Lastly, there are a small number of AMP's that lack a secondary structure and often contain a
214 high content of arginine, proline, tryptophan, and/or histidine residues (Nguyen *et al.*, 2011;
215 Takahashi *et al.*, 2010), that have an extended/random coil structure. A well characterized
216 peptide from this class is indolicidin a 13 amino acid cationic peptide from bovine

neutrophils whereby it is globular and amphipathic in solution and changes to a wedge like shape on interaction with a membrane (Hsu *et al*, 2005).

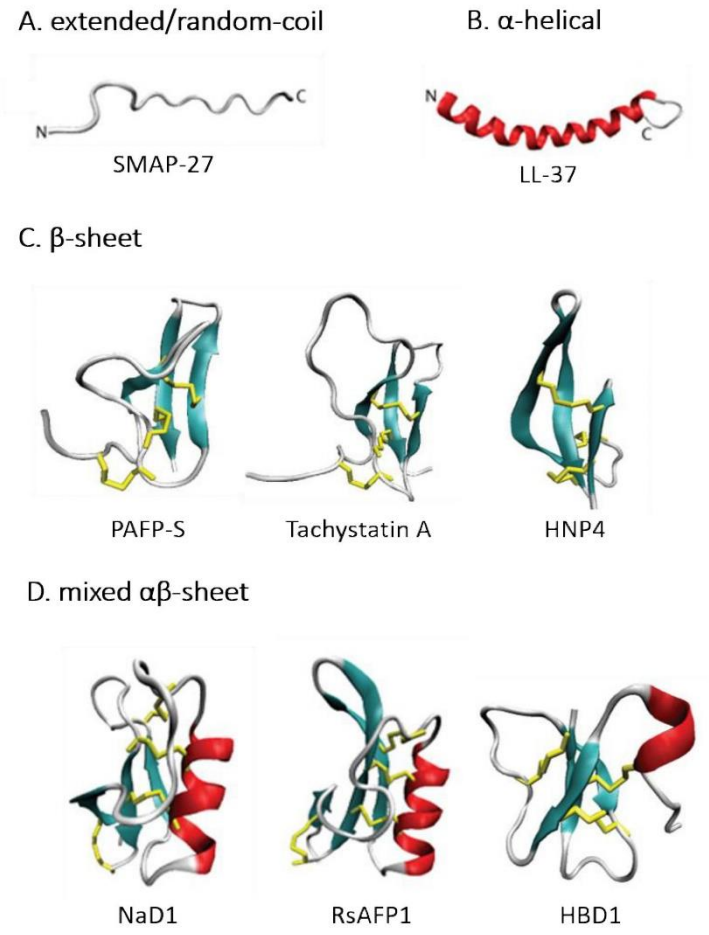


Figure 1.1. Four structural classes of antimicrobial peptides. (A) extended coil structure of SMAP-27 (PDB code 1FRY); **(B)** α -helical structure of human cathelicidin LL-37 (PDB code 2K6O); **(C)** β –sheeted PAFP-S (PDB code 1DKC), tachystatin A (PDB code 1CIX) and HNP4

224 (PDB code 1ZMM); **(D)** mixed αβ structures of the plant defensins NaD1 (PDB code 1MR4) and
225 RsAFP1 (PDB code 2N2R), and the human beta defensin HBD1 (PDB code 1IJU).
226
227 AMP's are universal; they are produced by organisms in all kingdoms of life. According to
228 the DRAMP (Data repository of antimicrobial peptides) (Last updated on 2020-9-14), there
229 are currently 20592 entries, 5877 of which are general AMPs (containing natural and
230 synthetic AMPs), 14739 patent AMPs and 76 AMPs in drug development (preclinical or
231 clinical stage) (Kang *et al*, 2019). AMP's can be constitutively expressed or induced after
232 exposure to stress or invading pathogens. In mammals, AMP's have been isolated from
233 exposed tissue areas such as the skin, eyes, ears, mouth and urinary and intestinal tracts. An
234 example, is the α-helical peptide, human cathelicidin LL-37 and lactoferrin from human tears
235 (Tsai *et al*, 2014b; Wang, 2014) and the histidine-rich peptides from human saliva, histatin 3
236 and 5 (Dong *et al.*, 2003; Fitzgerald *et al.*, 2003; Vandamme *et al.*, 2012). In humans, AMP's
237 have other roles which is why they are also called Host Defence Peptides. These functions
238 are immune modulation, apoptosis, and wound healing (Mookherjee *et al.*, 2020). Microbes
239 also produce AMP's, usually to limit the growth of competing microorganisms such as other
240 bacteria or fungi. An example of a microbial AMP is polymyxin B from *Bacillus polymyxa*,
241 which kills gram negative bacteria, and vancomycin which is a non-cationic peptide that kills
242 gram positive bacteria. Both of these AMPs have been developed into FDA approved
243 antibiotics (Mahlapuu *et al.*, 2016b). Antimicrobial peptides are also produced by plants and
244 insects as a first line of defence against potentially damaging pathogens, for example, the
245 plant defensin NaD1, from *Nicotiana alata,* is active against both filamentous fungi and yeast
246 (Hayes *et al*, 2013; van der Weerden *et al*, 2008). In this thesis I will be focusing on peptides
247 that have antifungal properties known as Antifungal peptides (AFPs).
248

Mechanism of action of Antimicrobial peptides

The modes of action of antimicrobial peptides are diverse and can be difficult to categorise (Amerikova *et al*, 2019; Raheem & Straus, 2019; van der Weerden *et al.*, 2013). Membrane disruptive mechanisms involve cell membrane penetration or the combination of AMPs with the membrane component of the microorganism. The characteristics of their amino acid composition, amphipathicity, and electric charge allow these peptides to induce membrane permeabilsation or disruption, leading to membrane depolarisation, loss of vital organelles and other cellular components, and cell lysis and death (Barbosa Pelegrini *et al*, 2011; Bondaryk *et al*, 2017; Rautenbach *et al*, 2016a). However, these peptides can also act via membrane non-disruptive mechanisms, by interacting with intracellular targets after crossing the plasma membrane or disrupting key cellular processes (Rautenbach *et al.*, 2016a).

Membrane-disruptive mechanisms

Antifungal peptides are able to inhibit fungal growth by the disruption of cellular membrane integrity resulting in the leakage of ions and other molecules (Yeaman & Yount, 2003), together with a general loss of membrane functionality. Various models and modes of action have been proposed for the disruption of membrane integrity via pore formation, particularly in bacteria (Seyfi *et al*, 2020). These models are discussed in a number of reviews on the mode of action of antimicrobial peptides (Bechinger & Lohner, 2006; Hollmann *et al*, 2018; Nguyen *et al.*, 2011; Sengupta *et al*, 2008; Seyfi *et al.*, 2020) and therefore will not be discussed in detail in this review. Most of these models of membrane permeabilisation and pore formation are based on studies with bacterial membranes (Mattila *et al*, 2008). However, some of these modes of action may not be valid for fungi as the structure of fungal membranes differs significantly from bacterial membranes and fungal membranes have unique components such as the sterol ergosterol and fungal specific sphingolipids (Nguyen *et al.*, 2011; Sevcsik *et al*, 2007; Singh & Prasad, 2011). Other modes of membrane

permeabilisation include binding with membrane lipids and fungal specific membrane components (Cools *et al*, 2017), changes in membrane potential due to peptide accumulation in outer leaflet, or association with small anions leading to electroporation (Chan *et al*, 2006) and dissipated membrane potential (Gifford *et al*, 2005).

Examples of AMP's that act by membrane permeabilization are the cathelicidins from sheep (SMAP-29) and cow (BMAP-27, BMAP-28) which form amphipathic α-helices in a hydrophobic environment. They have C-terminal hydrophobic domains, with strong membrane permeabilization activities (Benincasa *et al*, 2006; Skerlavaj *et al*, 1999). SMAP-29 concentrates on the plasma membrane of treated cells and causes propidium iodide uptake provided the cells are metabolically active (Lee *et al*, 2002). In a hydrophobic environment, PMAP-23 (from pigs) forms two short α-helices joined by a flexible region (Park *et al*, 2002). This peptide binds to the plasma membrane of treated cells and is active against *C. albicans* protoplasts indicating interaction with the cell wall is not required for inhibitory activity (Lee *et al*, 2001). The β-hairpin peptide protegrin and the extended, tryptophan-rich peptide indolicidin (both from pigs), also exhibit candidacidal activity through membrane permeabilization (Benincasa *et al.*, 2006).

So far, only one cathelicidin, LL-37, has been identified in humans and will be one of the peptides investigated further in his thesis (Kai-Larsen & Agerberth, 2008). It forms an amphipathic α-helix and binds to the cell wall and plasma membrane of treated cells (den Hertog *et al*, 2005). It disrupts the *C. albicans* cell membrane completely and allows leakage of small nucleotides such as ATP and proteins of up to 40 kDa into the medium (den Hertog *et al.*, 2005). Insertion of LL-37 into membranes is equally dependent on hydrophobic interactions between the peptide and acyl chains of the membrane lipids as it is on electrostatic interactions with lipid head groups (Henzler-Wildman *et al*, 2004). LL-37 is

301 known to associate with cell wall components of *C. albicans*, (Burton and Steel, 2009) such
302 as Xog1p and inhibit cell adhesion (Tsai *et al*, 2014a; Tsai *et al*, 2011). Analysis by live-cell
303 imaging showed that LL-37 peptides kill *C. albicans* rapidly. (Ordonez *et al*, 2014). The
304 strong membrane binding of LL-37 during the killing process led to the suggestion that direct
305 membrane permeabilization is the main mechanism of action. However, small amounts of
306 LL-37 peptides were detected intracellularly, even before PI influx and vacuolar expansion
307 took place. Therefore the authors hypothesised that a relatively high concentration of peptides
308 is required at the membrane for the peptides to traverse the cell boundaries (Ordonez *et al.*,
309 2014).
310
311 Extensive research on plant defensin–target interactions revealed two groups of fungal lipid
312 targets, namely sphingolipids and phospholipids (PLs) (Cools *et al.*, 2017). Lipid rafts
313 containing glycosphingolipids (GSLs) and ergosterol are present at the growth tip of budding
314 yeast and hyphae (including filamentous fungal hyphae), making this a vulnerable target
315 (Martin & Konopka, 2004; Takeshita *et al*, 2008). The binding of a peptide to GSLs could
316 have a major influence on cell function as these lipids participate in signal transduction, and
317 protein delivery of glycerophosphatidylinositol (GPI)-anchored membrane proteins in the
318 lipid rafts (Aerts *et al*, 2007; Bagnat *et al*, 2000; Thevissen *et al*, 2004). The GPI anchoring
319 on the outer membrane leaflet results in presentation of the protein on the surface of the cell.
320 The antifungal plant defensin from *Dahlia merckii*, *Dm*AMP1, interacts with the fungal
321 specific sphingolipid $M(IP)_2C$ from *S. cerevisiae*, leading to a biphasic permeabilisation of
322 the fungal membrane (Thevissen *et al*, 2003b). Furthermore the presence of another fungal
323 specific lipid, ergosterol, together with $M(IP)_2C$, enhanced *Dm*AMP1 activity (Thevissen *et*
324 *al.*, 2003b). The plant defensins NaD1 and TTP3, as well as human β-defensin 2 bind to the
325 membrane phospholipid phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) directly causing

membrane permeabilisation in tumor cells and potentially in fungal hyphae (Baxter *et al*, 2015; Järvå *et al*, 2018b; Payne *et al*, 2016a; Poon *et al*, 2014). Furthermore, these researchers have high-resolution structural evidence for the formation of a carpet-like configuration by NaD1 during the initial stages of membrane encounter with target phospholipids (Järvå *et al*, 2018a).

While most AMPs interact with and influence the integrity of microbial membranes, it is not fully understood whether membrane permeabilization is always a lethal event or whether the membrane is the only site of action (Cools *et al.*, 2017) Indeed in some cases, loss of membrane integrity may occur after cell death has been induced by another mechanism.

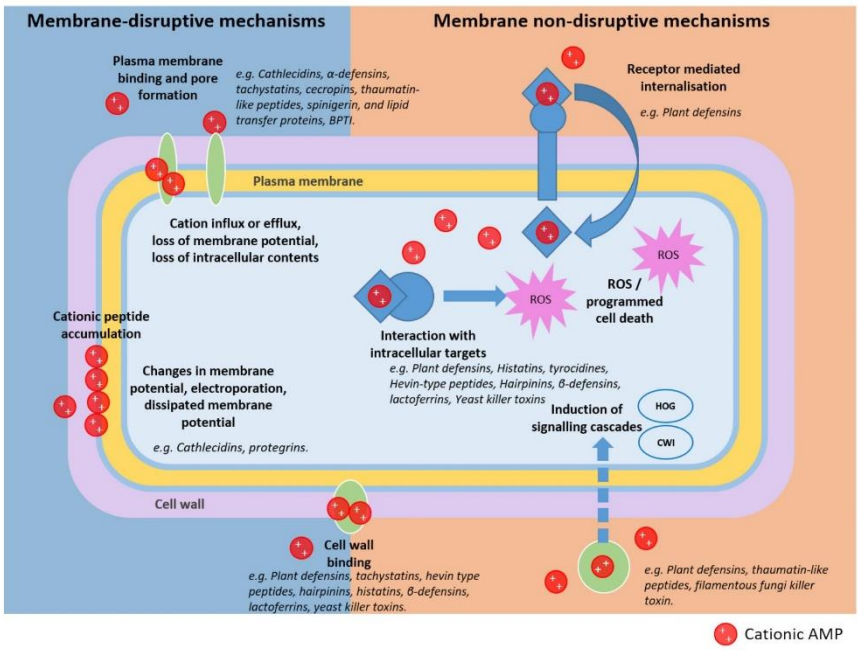


Figure 1.2. Mechanism of action of antifungal peptides. The mechanism of action of AFPs can be categorised into membrane disruptive and membrane non-disruptive mechanisms. Membrane disruptive mechanisms include plasma membrane binding and cell wall binding leading to membrane permeabilization and loss of intracellular contents, or cationic peptide accumulation on the plasma

membrane leading to change in membrane potential. Membrane non-disruptive mechanisms include entering the cell via binding to cell wall polysaccharides or receptor mediated internalisation, once inside the cytoplasm they bind to intracellular targets which can cause ROS production or programmed cell death. A result of cationic peptide interaction with fungal cells is the induction of signalling cascades such as the HOG and CWI pathway. This an updated version of van der Weerden's 2013 figure (van der Weerden *et al.*, 2013).

Membrane-nondisruptive mechanisms

As stated earlier, the most studied mechanism of action of cationic antimicrobial peptides has been the interaction with, and disruption of, the cytoplasmic membrane. More recently a number of cationic antimicrobial peptides have been reported to act on internal targets, either as their major mechanism of action following their translocation across the membrane or as an additive effect, combined with (often incomplete) membrane disruption.

Another peptide that will be investigated in this thesis is bovine pancreatic trypsin inhibitor BPTI also known as aprotinin which inhibits growth of *Saccharomyces cerevisiae* and the human pathogen *Candida albicans*. Bleackley and co-workers (2014) revealed by screening of the yeast heterozygous essential deletion collection that the magnesium transporter Alr1p was the likely BPTI target. This was confirmed when BPTI treatment of wild type cells resulted in a lowering of cellular Mg^{2+} levels and the observation thatpopulations treated with BPTI had fewer cells in the S -phase of the cell cycle and a corresponding increase of cells in the G_0/G_1 and G_2 phases. Furthermore the same patterns of cell cycle arrest obtained with BPTI were also obtained with the magnesium channel inhibitor hexamine (III) cobalt chloride (Bleackley, 2014). Analysis of the growth inhibition of *C. albicans* revealed that BPTI acts via the same mechanism in the two yeast species (Bleackley, 2014).

366 It has been proposed more recently that cationic antimicrobial peptides have a 'multitarget'-
367 mechanism of action (Hale & Hancock, 2008; Kumar *et al*, 2018), whereby they are able to
368 interact with multiple anionic targets. Certainly, there are many examples of cationic
369 antimicrobial peptides with more than one non-membranous target site within a fungal cell
370 (Lee & Lee, 2018; Puri & Edgerton, 2014; van der Weerden *et al.*, 2013; Yeaman *et al*,
371 2018).
372
373 NaD1 is one of the best characterized AMPs with a multi-site mechanism of action, which
374 makes it an advantageous peptide to study in this thesis. NaD1 a plant defensin from the
375 ornamental tobacco *Nicotiana alata* is active against several pathogenic fungi (Dracatos *et al*,
376 2014; Lay *et al*, 2003; van der Weerden *et al.*, 2008) and functions to protect the floral tissues
377 against damage from potential fungal pathogens (Lay *et al*, 2012). Treatment of fungal cells
378 with NaD1 led to damage of the inner leaflet of the cell membrane and cell death within 10
379 min of exposure to NaD1 (Hayes *et al.*, 2014b; Payne *et al*, 2016b; van der Weerden *et al*,
380 2010). The mechanism of cell death is complicated and involves interactions with multiple
381 fungal components (Parisi *et al.*, 2018). Firstly, NaD1 requires the presence of the cell wall
382 polysaccharide 1,3-β-glucan to initiate its specific and lethal effect on fungal cells (Bleackley
383 *et al*, 2019; van der Weerden *et al.*, 2008). As mentioned earlier, NaDI forms dimers that
384 bind tightly to phosphatidylinositol 4, 5-bisphosphate ($PI(4,5)P_2$) which is located on the
385 inner leaflet of the plasma membrane (Poon *et al.*, 2014). However interaction with $PI(4,5)P_2$
386 may not be essential for the antifungal mechanism because NaD1 variants that do not bind
387 $PI(4,5)P_2$ still kill fungal cells (Bleackley *et al*, 2016). In *C. albicans,* NaD1 accumulates on
388 the surface of the plasma membrane and traverses via endocytosis (Hayes *et al*, 2018). Once
389 NaD1 enters the cytoplasm it interacts with intracellular targets leading to the production of
390 ROS, permeabilisation of the plasma membrane, granulation of the cytoplasm and cell death

391 (Hayes *et al.*, 2018; Hayes *et al.*, 2013; van der Weerden *et al.*, 2010; van der Weerden *et al.*,
392 2008). Activation of the HOG1 pathway in response to NaD1 mediated osmotic and
393 oxidative stress along with other stressors has also been reported to be protective at sub-lethal
394 concentrations of NaD1 (Hayes *et al.*, 2014b).
395
396 The histatins are a well characterised group of histidine-rich peptides from human saliva that
397 are active against several *Candida* species (den Hertog *et al.*, 2005; Pathirana *et al*, 2018).
398 Histatin 5, a cleavage product of histatin 3, is the most potent of these molecules and as such,
399 the most well studied. The heat shock protein Ssa2p, a 70-kDa cell wall protein in *C.*
400 *albicans*, is the binding site for histatin 5 (Li *et al*, 2006; Sun *et al*, 2008). The presence of the
401 Ssa2p is required for susceptibility of *C. albicans* to histatin 5 and internalization of histatin 5
402 into cells (Li *et al.*, 2006; Sun *et al.*, 2008). Uptake of histatin 5 into *C. albicans* cells is
403 dependent on the presence of two polyamine transporters, Dur3 and Dur31, which usually
404 function in spermidine uptake (Kumar *et al*, 2011). Internalization must occur by
405 translocation, not endocytosis, for histatin to act as an antifungal molecule against *C.*
406 *albicans* (Jang *et al*, 2010). Upon internalization, histatin 5 travels to the mitochondria
407 providing respiration is underway and causes a loss of mitochondrial membrane integrity
408 (Komatsu *et al*, 2011; Puri & Edgerton, 2014)

409 MtDef4 a 47 amino acid protein from the model legume *Medicago truncatula* is active
410 against the filamentous fungal pathogen, *F. graminearum* and *N. crassa* (Ramamoorthy et al,
411 2007; Sagaram et al, 2011). Sagaram and coworkers discovered that the loop 5 sequence
412 (RGFRRR) binds to phosphatidic acid (PA), and is essential for MtDef4 entry into fungal
413 cells (Sagaram *et al*, 2013). MtDef4 also disrupts Ca^{2+} signalling and/or homeostasis leading
414 to inhibition of hyphal growth and fusion (Muñoz *et al*, 2014; Spelbrink *et al*, 2004). Once in
415 the cytoplasm MtDef4 interferes with internal cellular targets of *F. graminearum* that are still

416 unknown (Sagaram *et al.*, 2013; Sagaram *et al.*, 2011). In a later study, it was discovered that
417 membrane permeabilization is required for the antifungal activity of MtDef4 against *F.*
418 *graminearum* but not against *N. crassa*. MtDef4 appears to translocate into *F. graminearum*
419 autonomously using a partially energy-dependent pathway (El-Mounadi *et al*, 2016). By
420 contrast, internalization of MtDef4 in *N. crassa* is energy-dependent and involves
421 endocytosis (El-Mounadi *et al.*, 2016). This led to the suggestion that the plasma membrane
422 localized phospholipase D, involved in the biosynthesis of PA, is needed for entry of this
423 defensin into *N. crassa*, but not into *F. graminearum* (El-Mounadi *et al.*, 2016).

424

425 ROS production is a component of the antifungal activity of a number of peptides (Basso *et*
426 *al*, 2020), including the tyrocidines which induced the generation of ROS in *C. albicans*
427 (Troskie *et al*, 2014). However, ROS formation was not essential for the activity of the
428 tyrocidines, as their antifungal activity was surprisingly significantly higher in the presence
429 of the antioxidant, ascorbic acid (Troskie *et al.*, 2014). As the tyrocidines induce rapid
430 permeabilisation of fungal membranes, ROS are probably a consequence of osmotic stress
431 and/or binding to GlcCer-ergosterol rich lipid rafts (Rautenbach *et al*, 2016b). GlcCer-
432 dependent activity of the plant defensin *Pv*D1 directly induces the formation of ROS in *C.*
433 *albicans* and *F. oxysporum*, as well as nitric oxide in *C. albicans* (de Medeiros *et al*, 2014;
434 Mattila *et al.*, 2008). Furthermore, *Pv*D1 activity on *C. albicans* leads to disorganisation in
435 the cytoplasm and plasma membrane. The ROS formation by *Pv*D1 action likely enhances
436 the delayed *Pv*D1 membrane permeabilisation, as well as induction of programmed cell death
437 in *C. albicans* and *F. oxysporum* (Mello *et al*, 2011). *Rs*AFP2 a plant defensin from radish
438 inhibits *C. albicans* by binding to GlcCer, inducing endogenous ROS, which explains the
439 *Rs*AFP2-induced biphasic membrane leakage (Aerts *et al.*, 2007; Thevissen *et al*, 2012).

Following initial interaction with GlcCer, downstream signalling pathways lead to the induction of ROS, membrane permeabilisation and programmed cell death (Rego *et al*, 2014). Therefore suppression of ROS production would increase fungal resistance to these antifungal peptides. This was indeed the case with *Rs*AFP2 as the presence of ascorbic acid led to a significant loss in fungicidal activity, linking the involvement of ROS induction to *Rs*AFP2's activity against *C. albicans* (Aerts *et al.*, 2007).

Because of their broad spectrum of activity, AMPs are considered promising alternatives to conventional antimicrobial agents. Therefore, understanding the mechanism of action of AMP's is a crucial step in understanding the mechanisms of resistance, and in turn assessing their potential for clinical use.

Mechanism of resistance to antimicrobial peptides

Some researchers have reported that development of microbial resistance to antimicrobial peptides (AMPs) is unlikely to occur (Mahlapuu *et al*, 2016a). This has led to the hypothesis that AMPs have larger interactive surfaces with the targets in the pathogen than can be achieved with small molecule drugs (Tsomaia, 2015) and hence single amino acid substitutions are unlikely to lead to rapid resistance. Furthermore, AMPs have complicated mechanisms of action and often interact with more than a single target in the fungus so that multiple mutations are needed for full resistance. However, some bacteria and fungi have developed mechanisms to resist the toxic effects of AMPs, explaining why commensal organisms and pathogens survive the arsenal of AMPs presented by epithelial and mucosal surfaces and neutrophils.

Mechanisms of resistance to antibacterial peptides

In order to understand the potential for resistance to develop to AMPs in fungi, it is important to understand the mechanisms that have already been identified in bacteria (Abdi *et al*, 2019; Band & Weiss, 2015; Joo *et al*, 2016). Common AMP resistance mechanisms in bacteria are repulsion, sequestration, removal and degradation (Figure 3).

Repulsion. Gram-positive and gram-negative bacteria incorporate positive charges into their normally negatively charged cell surfaces (either the membrane or cell wall) to decrease interaction with the cationic AMPs. The modifications vary between bacterial species, but common targets are the most abundant negatively charged molecules on the outer surface such as teichoic acids on gram +ve bacteria and lipopolysaccharide (LPS) on gram -ve bacteria. AMP resistance is enhanced by the formation of lysyl-phosphatidyl glycerol (L-PG) by the enzyme multi peptide resistance factor, Mprf, which is present in many gram +ve and gram -ve bacteria (Roy, 2009). Bacteria have specific sensors that activate AMP resistance mechanisms when AMPs are present. The regulatory systems have been most extensively studied in the gram -ve bacterium *Salmonella typhimurium* where the PmrAB, Pho PQ and Rcs regulatory systems mediate many of the LPS modifications that increase surface charge (Andersson *et al*, 2016). The best studied regulatory system in gram +ve bacteria is the antimicrobial peptide sensing system of *Staphylococcus epidermidis* which induces D-alanylation of teichoic acids, incorporation of lysyl-phospatidylglycerol into the bacterial membrane coupled with an increase in lysine biosynthesis and activation of the VraFG AMP efflux pump (Li *et al*, 2007).

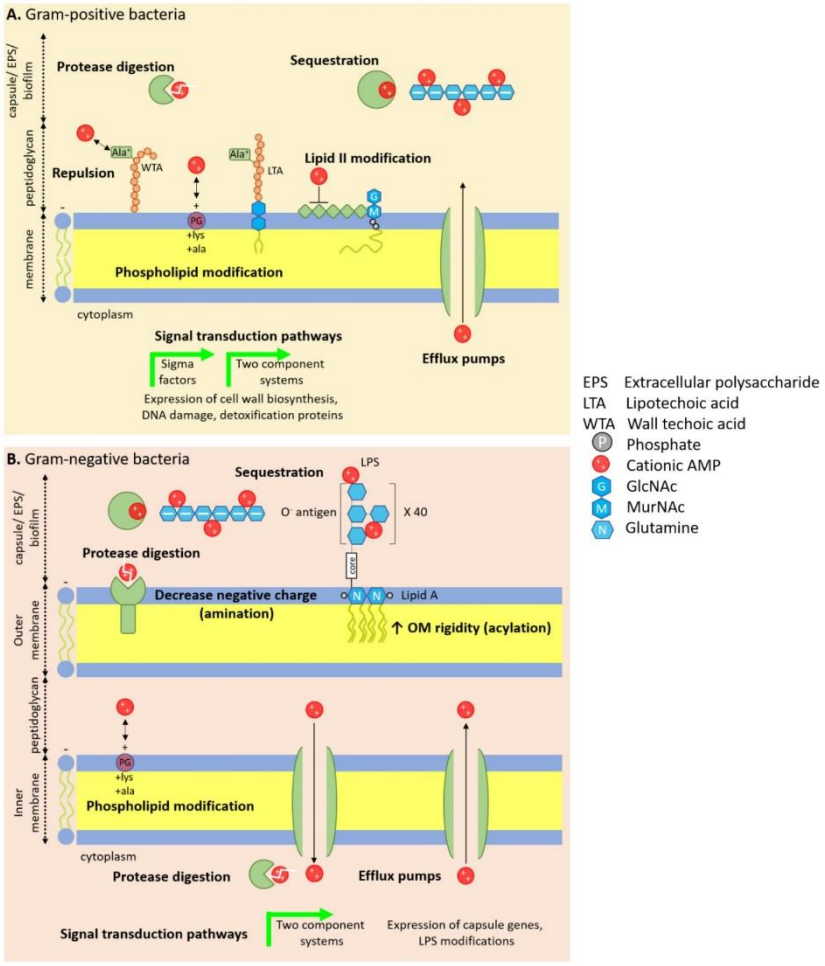
Sequestration. This involves secretion of proteins or anionic extracellular polysaccharides into the cell walls, capsules or biofilms that bind the AMPs and restrict access to the cell membrane. Examples are the anionic capsular

polysaccharides of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*, the O-antigen of lipopolysaccharide on gram -ve bacteria and polysaccharide intercellular adhesin (PIA also known as poly-N-acetyl glucosamine) which is produced by a variety of bacteria including Staphylococcal species and *E.coli* and is crucial for biofilm formation. Alginate, a negatively charged polymer secreted by *Pseudomonas aeruginosa* during biofilm formation, also sequesters AMPs. The intrinsic resistance of biofilms to AMPs is likely to limit the therapeutic potential of AMPs for treatment of biofilm infections.

Removal. AMPs that have inserted into the membrane or entered the cytoplasm can be removed by efflux pumps. The resistance/nodulation/cell division (RND) efflux pumps are present in many gm-ve bacteria. For example, the MtrCDE pump of *Neisseria gonorrhoeae* enhances tolerance to LL-37 and protegrin (Tzeng & Stephens, 2015).

Degradation. Proteolytic degradation by extracellular proteases, outer membrane proteases or by cytosolic proteases after uptake by transporters. Examples are PgtE in *S. typhimurium, S.aureus* aureolysin and OmT in *E.coli*. The linear AMPs such as LL-37 are more susceptible to degradation than AMPs such as the defensins that have compact scaffolds held together by several disulphide bonds.

Additional resistance mechanisms are modification of the pentapeptide on Lipid II, a prominent AMP target and alteration of the rigidity of the membrane by acylation of Lipid A.



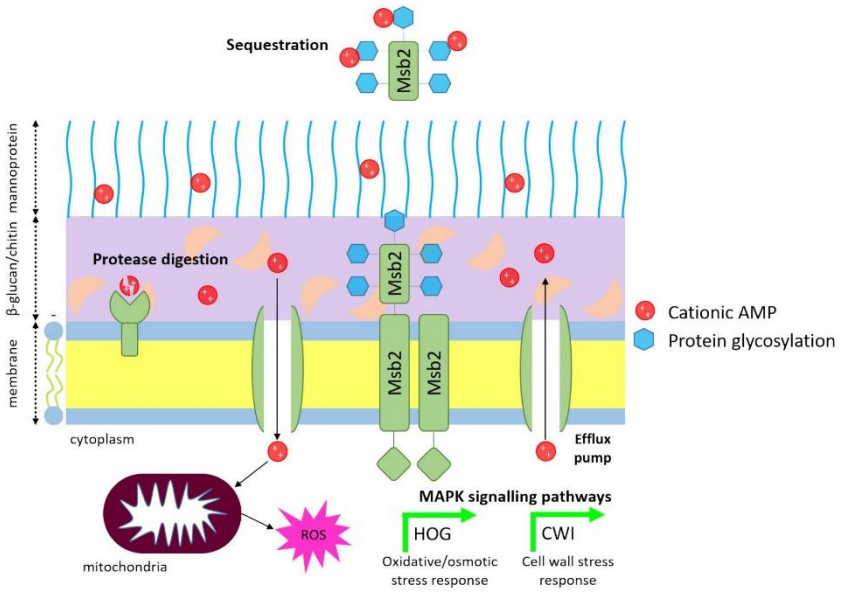
510

511 **Figure 1.3. Mechanisms of resistance to Antibacterial peptides**. (A) Gram +(ve) bacteria. **(B)** Gram
512 –(ve) bacteria. The mechanisms of resistance are highlighted in bold in the figure and include: **Protease**
513 **digestion**: degradation by secreted proteases, outer membrane (OM) proteases or cytosolic proteases.
514 **Sequestration** by secreted proteins, anionic polysaccharides or O antigen. **Electrostatic Repulsion,** by
515 alanylated lipoteichoic acid (LTA), wall teichoic acid (WTA) or aminoacylated phospatidylglycerol
516 (PG). **Lipid II modification**, blocking of antimicrobial peptides (AMP) binding by altering the
517 pentapeptide on lipid II. **Efflux pumps**, export of AMPs by efflux pumps. **Signal transduction**

518 **pathway** activation that induce expression of genes that reinforce the wall or detoxify products of AMP
519 activity. **Lipid A modification** by amine compounds, enhanced membrane rigidity by lipid A acylation.
520 This figure is an adaptation from (Mookherjee *et al.*, 2020).
521

522 ## Mechanisms of resistance to Antifungal peptides

523 Studies on the mechanisms that fungi employ to enhance tolerance to AFPs are limited and
524 have mainly focused on Candida species which also employ repulsion, sequestration, removal
525 by efflux pumps and proteolytic degradation for protection against the human AFPs LL-37,
526 histatin 5, hNP-1, hBD1-3 lactoferrin and hGAPDH (Swidergall & Ernst, 2014) (Figure 4).
527 Histatin 5 for example is degraded by the extracellular GPI anchored proteases Sap9/10.
528 Extracellular concentrations of histatin 5 are also limited by sequestration to the outer layer
529 of mannosylated glycoproteins (Harris *et al*, 2009) and by the soluble domain of the MbS2
530 membrane sensor which is a broad range protectant against AFPs including histatin 5, LL-37,
531 hNP-1 and hBD1 (Szafranski-Schneider *et al*, 2012). After traversing the fungal cell wall
532 Histatin 5 gains access to the plasma membrane and enters cells via a polyamine transporter.
533 After reaching threshold levels Histatin 5 stimulates production of reactive oxygen species
534 and ATP efflux. The Flu1 efflux pump reduces the cytoplasmic concentration of Histatin 5
535 but it is not upregulated in response to AFP exposure and is not sufficient for complete
536 resistance (Li *et al*, 2013). The cell responds instead by activation of the High Osmolarity
537 Glycerol (Hog1) MAP kinase pathway which induces transcription of genes that protect
538 against oxidative and osmotic stress. The Hog1 and Mkc1 cell wall integrity (CWI) pathways
539 protect Candida against a variety of AFPs including histatin 5 and hβD2. These pathways are
540 also active in filamentous fungi for example the plant pathogen *Fusarium graminearum*
541 which can survive exposure to plant defensins by inducing transcription of genes that protect
542 against oxidative stress and reinforce the fungal cell (Hayes *et al.*, 2014b).



543

544 **Figure 1.4**. **Mechanism of resistance to Antifungal peptides.** The mechanisms of resistance are
545 highlighted in bold in the figure and include: **Sequestration** by secreted proteins, anionic
546 polysaccharides or mannosylphosphate side chains on glycoproteins. **Efflux pumps**, export of AMPs.
547 **Signal transduction pathway** activation that induce expression of genes that reinforce the wall or
548 detoxify products of AMP activity. **Mitogen-activated protein kinase (MAPK) signalling pathways**
549 in fungi for protection against oxidative, osmotic or cell wall stress. This figure is an adaptation from
550 (Mookherjee *et al.*, 2020).

551

552 Most AMP resistance studies have been conducted in bacteria and in an *in vitro* setting where
553 there is a high selection for resistance development. These studies are essential to gain more
554 information regarding the ability of microbial pathogens to develop AFP resistance in
555 advance of their practical application. Moreover, development and characterization of
556 resistant microorganisms in the laboratory can provide a way to advance our knowledge

557 regarding the mechanisms of action of antimicrobial peptides. Thus far, there are few reports
558 describing successful attempts at obtaining such mutants in fungi.
559
560 **Determining AFP resistance mechanisms via Yeast deletion screens**
561
562 The development of chemical genomic assays with *S. cerevisiae* mutant libraries has
563 facilitated identification of the mechanism of action of many known drugs and novel small
564 molecules. The complete genome sequence of the yeast *S. cerevisiae* was published over 20
565 years ago and has become an essential tool for small molecule discovery and characterisation
566 (Ho *et al*, 2011). The genome sequence enabled construction of an almost complete set of
567 haploid non-essential gene deletion mutants as well as a set of heterozygous deletion mutants
568 in the essential genes (Pierce *et al*, 2007). This was accomplished using homologous
569 recombination to replace each gene with a *Kan*MX dominant drug resistant marker. An
570 important benefit of this deletion strategy is that amplification of the *Kan*MX cassette to
571 generate the deletion construct introduced two unique 20 nucleotide sequences or barcodes
572 for each gene deletion, which can be used to identify each deletion mutant (Giaever *et al*,
573 2002; Ho *et al.*, 2011; Winzeler *et al*, 1999). These libraries have been used to identify genes
574 that affect the relative fitness of *S. cerevisiae* upon exposure to chemical drugs or selective
575 growth conditions. Initially these collections were screened in 96 well microtitre plates but
576 the development of high density colony arrays and next generation barcode sequencing have
577 substantially improved the throughput and decreased the amount of drug needed to perform
578 these screens (Ho *et al.*, 2011; Shoemaker *et al*, 1996).
579 There are many examples in the literature where the mechanisms of action and/or interacting
580 partners of biological molecules for antimicrobial peptides have been deciphered through
581 screens of the non-essential gene deletion library of *S .cerevisiae* (Ho *et al*, 2009; Ho *et al.*,

582 2011; Lum *et al*, 2004; Parsons *et al*, 2006; Piotrowski *et al*, 2015; Xu *et al*, 2007). Fitness
583 profiling of chemical genomic screens is also used to identify deletion strains with enhanced
584 resistance to a particular drug. Altering the amount of activity of a gene product via mutation,
585 overexpression, down regulation or deletion can modify the cellular response to a chemical
586 (Kemmer *et al*, 2009). Drug efflux, drug permeability and stress response pathways are also
587 resistance traits (Shiver *et al*, 2016). These modifications indicate fungal viability, stress
588 survival and resistance to anti-microbial peptides which aid in deciphering the mechanism of
589 action for a drug, but they can also provide a resource for resistance in a pathogen to an
590 inhibitor.
591 Most researchers screen the library for gene deletions that enhance sensitivity to their drug of
592 interest. The identification of deletion strains that are hypersensitive to sub-inhibitory levels
593 of a drug is often used for discovery of genes/pathways that protect yeast in the presence of
594 the drug. For example, Hayes and colleagues (2014) discovered that yeast switch on the
595 Hog1 stress pathway for protection against the oxidative stress that occurs during exposure to
596 sub-inhibitory doses of NaD1. This plant defensin activates production of reactive oxygen
597 species as part of its mechanism of action and thus deletion of Hog1 renders yeast more
598 sensitive to NaD1 (Hayes *et al*, 2014a). In another example, a screen of the library with sub-
599 inhibitory concentrations of caspofungin led to the discovery that activation of the protein
600 kinase C cell integrity pathway provides protection against sub-lethal amounts of caspofungin
601 (Reinoso-Martín *et al*, 2003).
602 Library screens are scoured for strains with enhanced resistance to assist in determining the
603 mechanism of action of an AMP but also to explore how strains may develop AMP
604 resistance. A subsequent screen of the *S. cerevisiae* library with levels of caspofungin that
605 were above the minimal inhibitory concentration (MIC) led to the identification of another 9
606 gene deletions that enhanced resistance to caspofungin. These genes were involved in cell

607 wall and membrane function, ergosterol biosynthesis, vacuole function and transcription
608 (Markovich *et al*, 2004). A separate screen of the *S. cerevisiae* deletion library determined
609 that cells without the *agp2* gene had enhanced resistance to NaD1 (Bleackley *et al*, 2014).
610 Cells with a deletion of *agp2* accumulate positively charged molecules at the surface
611 repelling cationic antifungal peptides (Schuber, 1989). In a screen of the *S. cerevisiae* non-
612 essential deletion collection, mutants with increased tolerance to PAF26, a synthetic cationic
613 antifungal hexapeptide were identified with deletions in protein glycosylation genes,
614 indicating the importance of the glycosylation pathway in the mechanism of antifungal
615 activity (Harries *et al*, 2013). This complemented previous studies with PAF26 interaction
616 with the outer layers of the fungal cell before translocation into the cell where PAF26 exerts
617 its killing (Munoz *et al*, 2006). All of the strains identified in the screens with various AMPS
618 have deletions in non-essential genes highlighting genes that could be involved in resistance
619 to several AMPs.
620 The mechanisms of action for antimicrobial peptides (AMPs) are complex and unlike
621 commercially available antifungals, which most often act through specific interactions with
622 microbial proteins, lipids or carbohydrates, AMPs generally act through a more versatile
623 approach that involves multiple cellular targets. Yeast screens have played a major role in
624 defining these complex mechanisms. The mechanism of action for the AMP NbD6 involves
625 binding to the cell surface, triggering of ROS production that may signal autophagy
626 activating vacuolar fusion, the vacuole is subsequently disrupted, and cell death occurs
627 (Parisi *et al*, 2019a). These steps were identified via a screen of the *S. cerevisiae* non-
628 essential gene deletion library and then confirmed by confocal microscopy.
629 Barseq screening allowed for yeast deletion screens to be performed on a pooled collection of
630 deletions. This decreased the amount of time and resources that were required to perform
631 these screens and made it much easier to perform multiple screens in parallel. Parallel yeast

632 deletion screens on AFPs have been done using human salivary peptides and plant defensins.
633 Genes with deletions associated with the RIM101 pathway were sensitive to the MUC7
634 human salivary AMP, a 12 mer, in a sensitivity screen (Lis *et al*, 2013; Lis *et al*, 2009).
635 RIM101 functions in protecting fungi against human salivary AMPs. In addition, the direct
636 selection technique with the AMP MUC7, whereby a pool is repeatedly treated with MUC7,
637 identified one clone with elevated survival rates. This clone had a deletion in the *MIS1* gene
638 encoding mitochondrial C_1-tetrahydrofolate synthase (Lis *et al.*, 2009). Another
639 mitochondrial mutant displayed enhanced resistance to MUC7 in a separate screen (Lis *et al.*,
640 2013). This mutation decreases energy production, a condition known to protect against
641 AMPs (Helmerhorst *et al*, 1999). Strains with deletions in ATP transport and export had
642 enhanced resistance to plant defensins, NbD6, SBI6, DmAMP1 and NaD1, suggesting that
643 altering energy levels during cellular metabolism protects the pathogen from several plant
644 defensins with different mechanism of action (Parisi *et al.*, 2019a). Mitochondrial function
645 was identified in a yeast screen for the mechanism of action for HsAFP1. This screen result,
646 together with previous data, revealed that HsAFP1 binds to the cell wall and plasma
647 membrane, penetrates the cytoplasm to target the mitochondria, activates ROS production
648 and cell death occurs (Aerts *et al*, 2011; Parisi *et al*, 2019b; Thevissen *et al*, 1997).
649
650 Screening of yeast deletion libraries have largely focused on determining the mechanisms of
651 action for selected AMPs but screening also has the potential to identify likely routes of
652 resistance. Collating data of the results of strains with increased tolerance to AMPs from
653 multiple screens could create an expanded chemical genomics data set to reveal new
654 determinants of antibiotic and antifungal resistance. Full gene deletions, in the case of the
655 non-essential gene collection, are often associated with fitness defects. These defects and the
656 genetic mechanisms for resistance in the clinic or field will be different but these screens will

give insight into the potential biological mechanisms of resistance. Furthermore, the collated data resource could provide valuable information into the effects of drugs on cells as well as targets or pathways that could evolve resistance to multiple AMPs.

Laboratory based evolution of resistance to antifungal peptides

The yeast deletion screens discussed above focused on loss of gene function. Although this method has been very productive for describing the mode of action of AFPs, it does not provide enough insight into whether mutations of these targets would provide robust resistance in the clinic. Clinical antifungal resistance is often not associated with a total loss of function, but with changes to enzyme active sites or upregulation of drug efflux pumps and/or other genes that counter act the effect of the antifungal (Cowen et al., 2014). Therefore, in order to understand the potential for clinical resistance risk in advance, there needs to be a more comprehensive approach to studying the development of resistance. One tool for the prediction of resistance risk is laboratory selection for resistance.

Laboratory selection for AFP resistance via chemical mutagenesis

Chemical mutagenesis involves producing drug resistant strains by randomly introducing DNA mutations by the introduction of a mutagen (usually UV or chemicals such as ethylmethanesulphonate (EMS) (Hawkins & Fraaije, 2016). Mutagenesis enables resistance to emerge more rapidly in the laboratory than in the field, but may produce mutations that would not emerge under field conditions (Hawkins & Fraaije, 2016). An example of this is the laboratory selection of resistance to the antifungal peptide MUC7. MUC7 is a 12 amino acid cationic peptide from the N-terminal portion of human mucin MUC7, that has potent antifungal properties (Situ *et al*, 2003). MUC7-resistant mutants of *C. albicans* were developed by mutagenesis with EMS (Lis & Bobek, 2008). One colony, exhibiting the highest level of

682 MUC7 resistance in a killing assay, was characterized further. This colony, designated mutant
683 #37 had a 6-fold increase in MIC compared to the parental strain. The mutant exhibited lowered
684 ATP content along with a relatively lower rate of oxygen consumption, as well as an inability
685 to grow on nonfermentable carbon sources. The authors suggested that the resistance is likely
686 to be associated with changes in metabolic regulation, rather than the lack of functional
687 mitochondria, as determined by rhodamine 123 staining (Lis & Bobek, 2008). Therefore, it is
688 possible that the lowered metabolism of the mutant #37 led to its resistance to the MUC7 12-
689 mer. (Situ *et al.*, 2003). However, the lowered level of metabolism and inability to grow on
690 fermentable carbon sources is also likely to decrease the survival of mutant #37 within a viable
691 host. Another example of a mutation leading to resistance that is associated with decreased
692 fitness is the mutants of *Neurospora crassa* that were generated by chemical mutagenesis using
693 EMS and selected for resistance towards the radish defensin RsAFP2 (Ferket *et al*, 2003).
694 These mutants grew about 10-fold slower than the *N. crassa* wildtype and had lost the ability
695 to form ascospores (Ferket *et al.*, 2003). Characterisation of the lipids in the membranes of the
696 two *N. crassa* mutants revealed structurally different glucosylceramides, novel
697 glycosphingolipids, and an altered level of steryl glucosides (Ferket *et al.*, 2003). Subsequent
698 work revealed that the sphingolipid GlcCer in the fungal plasma membrane and cell wall , is
699 essential for the activity of RsAFP2 (Aerts *et al.*, 2007; Thevissen *et al.*, 2012; Thevissen *et al*,
700 2003a; Thevissen *et al.*, 2004)
701

702 **Serial passaging to generate AFP resistance**

703 As stated previously, mutagenesis is a way to speed up the development of resistant mutants,
704 although it also produces mutants that are less viable and thus unlikely to survive in a clinical
705 setting. Another tool for analysis of resistance in fungi is to use selection for resistance in the
706 laboratory without the introduction of a mutagen. This method involves sub-culturing, or

707 passaging, the parental fungal strain at sub-inhibitory and/or increasing concentrations of AMP
708 until resistance reaches a certain threshold, usually at least 4-fold more resistant than wildtype.
709 Although there will be a reduced number of mutations, it is considered more likely that these
710 mutations could occur in a clinical setting (Beardsley *et al*, 2018).
711
712 One of the first attempts at laboratory selection for AFP resistance was used by Thevissen et
713 al., 2000, to determine the mechanism of action of the dahlia defensin DmAMP1. They used
714 selection in liquid medium containing 5 μM Dm-AMP1 to isolate two independent Dm-AMP1-
715 resistant *S. cerevisiae* strains (DM1 and DM2) (Thevissen *et al*, 2000b). Wild-type yeast was
716 sensitive to Dm-AMP1 at 2 μM, whereas the Dm-AMP1-resistant yeast mutants were resistant
717 up to 40 μM Dm-AMP1. A complementation approach followed that consisted of the
718 introduction of clones from a genomic library from the DmAMP1-sensitive wild-type yeast
719 into the DmAMP1-resistant yeast mutant and screening for restored sensitivity to
720 DmAMP1(Thevissen *et al*, 2000a). The gene that restored DmAMP1 sensitivity to the resistant
721 mutant was *IPT1*, a gene encoding an enzyme involved in the last step of the synthesis of the
722 sphingolipid mannose-(inositol-phosphate)$_2$-ceramide. This revealed DmAMP1's mechanism
723 of action, which is specific interaction with the sphingolipid M(IP)$_2$C from *S. cerevisiae*,
724 leading to a biphasic permeabilisation of the fungal membrane (Thevissen *et al.*, 2003b).
725
726 The human protein histatin has a complex mechanism of action that involves binding,
727 internalisation, intracellular targeting and release of ATP (Pathirana *et al.*, 2018; Puri &
728 Edgerton, 2014). Resistant *C. albicans* mutants have been developed by sequential exposure
729 to increasing concentrations of histatin 3 (Fitzgerald *et al.*, 2003). Proteomic analysis was used
730 to identify alterations between the histatin-resistant strains and the histatin-susceptible parent
731 to identify other potential targets (Fitzgerald-Hughes *et al*, 2007). This proteomic analysis

732 revealed that 59 proteins were differentially expressed compared to the parental strain. Proteins
733 that were absent from the wildtype but present in the histatin-resistant strains, included
734 isocitrate lyase (Icl1p), fructose biphosphate aldolase (Fba1p), pyruvate decarboxylase
735 (Pdc2p), and ketol-acid reductoisomerase (Ilv5p) which impact metabolic pathways. The
736 resistant strains also decreased rates of oxygen consumption and histatin 3-mediated ATP
737 release compared with wildtype. The authors concluded that these data support an important
738 role for metabolic pathways in the histatin resistance mechanism and suggest that there may be
739 several intracellular targets for histatin 3 in *C. albicans* (Fitzgerald-Hughes *et al.*, 2007).
740
741 A more recent study by McColl et al 2018 used the prototypic plant defensin NaD1 in serial
742 passages with the model fungus *S. cerevisiae* to examine the development of resistance to
743 plant antifungal peptides and compare this to caspofungin resistance (McColl *et al*, 2018).
744 The yeast strains did develop tolerance to NaD1, but it occurred more slowly than to the
745 clinically used antifungal caspofungin. The MIC of NaD1-resistant strains was only 10-fold
746 greater than wild type, which was less than the equivalent caspofungin-resistant strains (20-
747 fold greater than wild type). Further characterization of the strains with increased tolerance to
748 NaD1 also revealed growth defects and changes in tolerance to abiotic stressors. Sequencing
749 the genomes of the NaD1-resistant strains with increased tolerance failed to identify any
750 'hotspot' mutations associated with increased tolerance to NaD1 and led to the identification
751 of 12 genes that are potentially involved in resistance. The functional diversity of these genes
752 revealed that the mechanism of NaD1 is likely to involve more than a single protein target.
753 This contrasts with resistance to caspofungin, which was acheived by a single amino acid
754 alteration in the target β-glucan synthase, Fks1p. The NaD1-resistant strains acquired
755 mutations related to the protection from osmotic stress, alteration of the cell wall, solute
756 transport, signaling, and cation homeostasis One of the mutations was identified across

multiple strains. This mutation was in *FPS1* and revealed that one mechanism of resistance to NaD1 that involves the osmotic stress response pathway. One possible mechanism for *FPS1*-mediated NaD1 resistance is that *FPS1* mutants accumulate high intracellular concentrations of glycerol, which stabilizes lipid bilayers and protects the cellular organelles that are targeted by NaD1. The authors suggest that it would be more difficult to generate resistance to antimicrobial peptides such as NaD1 compared to small molecule antifungals, as resistance to NaD1 developed slowly via an accumulation of single nucleotide mutations and also incurred a general fitness penalty (McColl *et al.*, 2018).

In a similar study by McColl et al, the bovine pancreatic trypsin inhibitor BPTI was serial passaged with the model organism *S. cerevisiae* (McColl et al, 2020). When compared to the previous study (McColl *et al.*, 2018), resistance to BPTI developed more quickly than resistance to the plant defensin NaD1 and the clinical antifungal, caspofungin.
Full genome sequencing of resistant lines revealed that resistance to BPTI developed as the result of a deleterious mutation in either the *ptk2* or *sky1* genes. This revealed that cation homeostasis and transport functions were particularly affected in *S. cerevisiae* after exposure to BPTI. This was consistent with prior studies demonstrating that BPTI interacts with the transporter Alr1p, blocking magnesium uptake into *S. cerevisiae* (Bleackley et al., 2014). Surprisingly, there were not any mutations in the *alr1* gene in the BPTI-resistant strains. *ALR1* is an essential gene and it is possible that mutations that would block BPTI binding are also deleterious to the essential function of the gene product in cellular Mg2+ uptake and therefore are not viable or have a serious fitness penalty. Therefore, the mutations that were observed in the BPTI resistant strains probably decrease release of magnesium and other cations from the cell, protecting the yeast from the limiting intracellular magnesium levels that arise when BPTI blocks the magnesium transporter Alr1p. Rapid development of

782 resistance to the antifungal activity of BPTI would seem to contradict the idea that AMPs are
783 superior to small molecule drugs. However, it is likely to be a reflection of the superiority of
784 fungicidal molecules as antifungals compared to fungistatic molecules. BPTI is a fungistatic
785 peptide, that is, it inhibits fungal growth but does not actively kill the fungus (Bleackley et
786 al., 2014) leaving a larger pool of living cells to develop mutations that confer resistance.
787 Both NaD1 and caspofungin are fungicidal molecules. Fungicidal drugs are usually the
788 preferred choice of treatment in the clinic because they act quickly and kill almost all cells.
789 The authors also suggest that because the resistance that was developed to either NaD1 or
790 BPTI could be reverted by the addition of very low concentrations of the partner peptide in
791 synergy assays indicates that if AMPs were developed for clinical use any increase in
792 pathogen tolerance may be easy to combat through the use of combinatorial therapies that
793 inactivate the stress response related resistance mechanisms.
794

795 # Conclusion

796

797 Only a handful of studies have been completed thus far on laboratory selection for resistance
798 to AFPs. These studies have also focused on determining the mechanism of action of AFPs
799 instead of predicting the mechanisms of resistance for future clinical use. As well as this, some
800 of these studies are up to 20 years old and have relied mostly on phenotype characterisation
801 instead of whole genome sequencing. Due to the prevalence of resistance to all the major
802 classes of clinical antifungals, and the understanding that fungal pathogens also have the
803 potential to develop resistance to AFPs, it is important to further investigate the mechanisms
804 of fungal resistance to a range of AFPs.

805

806 # References

807 Abdi M, Mirkalantari S, Amirmozafari N (2019) Bacterial resistance to antimicrobial
808 peptides. *Journal of Peptide Science* 25: e3210

809 Aerts AM, Bammens L, Govaert G, Carmona-Gutierrez D, Madeo F, Cammue B, Thevissen
810 K (2011) The antifungal plant defensin HsAFP1 from Heuchera sanguinea induces apoptosis
811 in Candida albicans. *Frontiers in microbiology* 2: 47

812 Aerts AM, François IE, Meert EM, Li QT, Cammue BP, Thevissen K (2007) The antifungal
813 activity of RsAFP2, a plant defensin from raphanus sativus, involves the induction of reactive
814 oxygen species in Candida albicans. *Journal of molecular microbiology and biotechnology*
815 13: 243-247

816 Amerikova M, Pencheva El-Tibi I, Maslarska V, Bozhanov S, Tachkov K (2019)
817 Antimicrobial activity, mechanism of action, and methods for stabilisation of defensins as
818 new therapeutic agents. *Biotechnology & Biotechnological Equipment* 33: 671-682

819 Andersson DI, Hughes D, Kubicek-Sutherland JZ (2016) Mechanisms and consequences of
820 bacterial resistance to antimicrobial peptides. *Drug Resistance Updates* 26: 43-57

821 Aoki W, Ueda M (2013) Characterization of Antimicrobial Peptides toward the Development
822 of Novel Antibiotics. *Pharmaceuticals (Basel)* 6: 1055-1081

823 Arendrup MC, Patterson TF (2017) Multidrug-Resistant Candida: Epidemiology, Molecular
824 Mechanisms, and Treatment. *The Journal of Infectious Diseases* 216: S445-S451

825 Ashrafi N, Lapthorn C, Pullen FS, Naclerio F, Nielsen Birthe V (2017) High performance
826 liquid chromatography coupled to electrospray ionisation mass spectrometry method for the
827 detection of salivary human neutrophil alpha defensins HNP1, HNP2, HNP3 and HNP4.
828 *Analytical Methods* 9: 6482-6490

829 Avery SV, Singleton I, Magan N, Goldman GH (2019) The fungal threat to global food
830 security. *Fungal Biology* 123: 555-557

831 Bagnat M, Keränen S, Shevchenko A, Shevchenko A, Simons K (2000) Lipid rafts function
832 in biosynthetic delivery of proteins to the cell surface in yeast. *Proceedings of the National*
833 *Academy of Sciences* 97: 3254

834 Balashov SV, Park S, Perlin DS (2006) Assessing Resistance to the Echinocandin Antifungal
835 Drug Caspofungin in Candida albicans by Profiling Mutations in FKS1. *Antimicrobial Agents*
836 *and Chemotherapy* 50: 2058-2063

837 Band VI, Weiss DS (2015) Mechanisms of Antimicrobial Peptide Resistance in Gram-
838 Negative Bacteria. *Antibiotics (Basel, Switzerland)* 4: 18-41

839 Barbosa Pelegrini P, del Sarto RP, Silva ON, Franco OL, Grossi-de-Sa MF (2011)
840 Antibacterial Peptides from Plants: What They Are and How They Probably Work.
841 *Biochemistry Research International* 2011: 250349

842 Basso V, Tran DQ, Ouellette AJ, Selsted ME (2020) Host Defense Peptides as Templates for
843 Antifungal Drug Development. *Journal of Fungi* 6

844 Baxter A, Richter V, Lay F, Poon I, Adda C, Veneer P, Phan TK, Bleackley M, Anderson M,
845 Kvansakul M *et al* (2015) The Tomato Defensin TPP3 Binds Phosphatidylinositol (4,5)-
846 Bisphosphate via a Conserved Dimeric Cationic Grip Conformation To Mediate Cell Lysis.
847 *Molecular and cellular biology* 35: 1964-1978

848 Beardsley J, Halliday CL, Chen SCA, Sorrell TC (2018) Responding to the emergence of
849 antifungal drug resistance: perspectives from the bench and the bedside. *Future microbiology*
850 13: 1175-1191

851 Bechinger B, Lohner K (2006) Detergent-like actions of linear amphipathic cationic
852 antimicrobial peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1758: 1529-
853 1539

854 Benincasa M, Scocchi M, Pacor S, Tossi A, Nobili D, Basaglia G, Busetti M, Gennaro R
855 (2006) Fungicidal activity of five cathelicidin peptides against clinically isolated yeasts. *The*
856 *Journal of antimicrobial chemotherapy* 58: 950-959

857 Berger S, El Chazli Y, Babu AF, Coste AT (2017) Azole Resistance in Aspergillus
858 fumigatus: A Consequence of Antifungal Use in Agriculture? *Front Microbiol* 8: 1024

859 Bleackley MR, Dawson CS, Payne JAE, Harvey PJ, Rosengren KJ, Quimbar P, Garcia-Ceron
860 D, Lowe R, Bulone V, van der Weerden NL *et al* (2019) The interaction with fungal cell wall

polysaccharides determines the salt tolerance of antifungal plant defensins. *Cell Surf* 5: 100026-100026

Bleackley MR, Hayes, B. M., Parisi, K., Saiyed, T., Traven, A., Potter, I. D., van der Weerden, N. L. and Anderson, M. A. (2014) Bovine pancreatic trypsin inhibitor is a new antifungal peptide that inhibits cellular magnesium uptake. *Molecular microbiology* 92: 1188–1197

Bleackley MR, Payne JAE, Hayes BME, Durek T, Craik DJ, Shafee TMA, Poon IKH, Hulett MD, van der Weerden NL, Anderson MA (2016) Nicotiana alata Defensin Chimeras Reveal Differences in the Mechanism of Fungal and Tumor Cell Killing and an Enhanced Antifungal Variant. *Antimicrobial Agents and Chemotherapy* 60: 6302-6312

Bleackley MR, Wiltshire JL, Perrine-Walker F, Vasa S, Burns RL, van der Weerden NL, Anderson MA (2014) Agp2p, the plasma membrane transregulator of polyamine uptake, regulates the antifungal activities of the plant defensin NaD1 and other cationic peptides. *Antimicrobial agents and chemotherapy* 58: 2688-2698

Bondaryk M, Staniszewska M, Zielińska P, Urbańczyk-Lipkowska Z (2017) Natural Antimicrobial Peptides as Inspiration for Design of a New Generation Antifungal Compounds. *Journal of fungi (Basel, Switzerland)* 3

Brown AJP, Cowen LE, di Pietro A, Quinn J (2017) Stress Adaptation. *Microbiol Spectr* 5: 10.1128/microbiolspec.FUNK-0048-2016

Casadevall A (2017) Don't Forget the Fungi When Considering Global Catastrophic Biorisks. *Health Secur* 15: 341-342

Castanheira M, Deshpande LM, Messer SA, Rhomberg PR, Pfaller MA (2020) Analysis of global antifungal surveillance results reveals predominance of Erg11 Y132F alteration among azole-resistant Candida parapsilosis and Candida tropicalis and country-specific isolate dissemination. *International Journal of Antimicrobial Agents* 55: 105799

Chaabane F, Graf A, Jequier L, Coste AT (2019) Review on Antifungal Resistance Mechanisms in the Emerging Pathogen Candida auris. *Frontiers in Microbiology* 10

888 Chan DI, Prenner EJ, Vogel HJ (2006) Tryptophan- and arginine-rich antimicrobial peptides:
889 Structures and mechanisms of action. *Biochimica et Biophysica Acta (BBA) - Biomembranes*
890 1758: 1184-1202

891 Chen L, Xu YH, Zhou C, Zhao J, Li CY, Wang R (2010) Overexpression of CDR1 and
892 CDR2 Genes Plays an Important Role in Fluconazole Resistance in Candida albicans with
893 G487T and T916C Mutations. *The Journal of international medical research* 38: 536-545

894 Chen SCA, Sorrell TC (2007) Antifungal agents. *Medical Journal of Australia* 187: 404-409

895 Ciociola T, Giovati L, Conti S, Magliani W, Santinoli C, Polonelli L (2016) Natural and
896 synthetic peptides with antifungal activity. *Future medicinal chemistry* 8: 1413-1433

897 Coleman JJ, Mylonakis E (2009) Efflux in fungi: la pièce de résistance. *PLoS Pathog* 5:
898 e1000486

899 Cools TL, Struyfs C, Bruno PAC, Thevissen K (2017) Antifungal plant defensins: increased
900 insight in their mode of action as a basis for their use to combat fungal infections. *Future*
901 *microbiology* 12: 441-441–454

902 Cowen LE, Sanglard D, Howard SJ, Rogers PD, Perlin DS (2014) Mechanisms of Antifungal
903 Drug Resistance. *Cold Spring Harb Perspect Med* 5: a019752-a019752

904 Cowen LE, Singh SD, Köhler JR, Collins C, Zaas AK, Schell WA, Aziz H, Mylonakis E,
905 Perfect JR, Whitesell L *et al* (2009) Harnessing Hsp90 function as a powerful, broadly
906 effective therapeutic strategy for fungal infectious disease. *Proc Natl Acad Sci U S A* 106:
907 2818-2823

908 Cowen LE, Steinbach WJ (2008) Stress, drugs, and evolution: the role of cellular signaling in
909 fungal drug resistance. *Eukaryot Cell* 7: 747-764

910 de Medeiros LN, Domitrovic T, de Andrade PC, Faria J, Bergter EB, Weissmüller G,
911 Kurtenbach E (2014) Psd1 binding affinity toward fungal membrane components as assessed
912 by SPR: The role of glucosylceramide in fungal recognition and entry. *Biopolymers* 102:
913 456-464

914 den Hertog AL, van Marle J, van Veen HA, Van't Hof W, Bolscher JG, Veerman EC, Nieuw
915 Amerongen AV (2005) Candidacidal effects of two antimicrobial peptides: histatin 5 causes

916 small membrane defects, but LL-37 causes massive disruption of the cell membrane. *The*
917 *Biochemical journal* 388: 689-695

918 Dong J, Vylkova S, Li XS, Edgerton M (2003) Calcium blocks fungicidal activity of human
919 salivary histatin 5 through disruption of binding with Candida albicans. *Journal of dental*
920 *research* 82: 748-752

921 Dracatos PM, van der Weerden NL, Carroll KT, Johnson ED, Plummer KM, Anderson MA
922 (2014) Inhibition of cereal rust fungi by both class I and II defensins derived from the flowers
923 of Nicotiana alata. *Molecular Plant Pathology* 15: 67-79

924 El-Mounadi K, Islam KT, Hernández-Ortiz P, Read ND, Shah DM (2016) Antifungal
925 mechanisms of a plant defensin MtDef4 are not conserved between the ascomycete fungi
926 Neurospora crassa and Fusarium graminearum. *Molecular microbiology* 100: 542-559

927 Ellis D (2002) Amphotericin B: spectrum and resistance. *The Journal of antimicrobial*
928 *chemotherapy* 49 Suppl 1: 7-10

929 Ferket KKA, Levery SB, Park C, Cammue BPA, Thevissen K (2003) Isolation and
930 characterization of Neurospora crassa mutants resistant to antifungal plant defensins. *Fungal*
931 *Genetics and Biology* 40: 176-185

932 Fisher MC, Gurr SJ, Cuomo CA, Blehert DS, Jin H, Stukenbrock EH, Stajich JE, Kahmann
933 R, Boone C, Denning DW *et al* (2020) Threats Posed by the Fungal Kingdom to Humans,
934 Wildlife, and Agriculture. *mBio* 11: e00449-00420

935 Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ (2018) Worldwide emergence of resistance to
936 antifungal drugs challenges human health and food security. *Science (New York, NY)* 360:
937 739

938 Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, Gurr SJ (2012)
939 Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484: 186-194

940 Fitzgerald-Hughes DH, Coleman DC, O'Connell BC (2007) Differentially expressed proteins
941 in derivatives of Candida albicans displaying a stable histatin 3-resistant phenotype.
942 *Antimicrobial agents and chemotherapy* 51: 2793-2800

943 Fitzgerald DH, Coleman DC, O'Connell BC (2003) Binding, internalisation and degradation
944 of histatin 3 in histatin-resistant derivatives of Candida albicans. *FEMS Microbiology Letters*
945 220: 247-253

946 Fones HN, Bebber DP, Chaloner TM, Kay WT, Steinberg G, Gurr SJ (2020) Threats to
947 global food security from emerging fungal and oomycete crop pathogens. *Nature Food* 1:
948 332-342

949 Fox JL (2013) Antimicrobial peptides stage a comeback. *Nat Biotechnol* 31: 379-382

950 Gao GH, Liu W, Dai JX, Wang JF, Hu Z, Zhang Y, Wang DC (2001) Solution structure of
951 PAFP-S: a new knottin-type antifungal peptide from the seeds of Phytolacca americana.
952 *Biochemistry* 40: 10973-10978

953 Ghannoum MA, Rice LB (1999) Antifungal agents: mode of action, mechanisms of
954 resistance, and correlation of these mechanisms with bacterial resistance. *Clinical*
955 *microbiology reviews* 12: 501-517

956 Giaever G, Chu AM, Ni L, Connelly C, Riles L, Veronneau S, Dow S, Lucau-Danila A,
957 Anderson K, Andre B (2002) Functional profiling of the Saccharomyces cerevisiae genome.
958 *nature* 418: 387

959 Gifford JL, Hunter HN, Vogel HJ (2005) Lactoferricin: a lactoferrin-derived peptide with
960 antimicrobial, antiviral, antitumor and immunological properties. *Cellular and molecular life*
961 *sciences : CMLS* 62: 2588-2598

962 Gracy J, Chiche L (2011) Structure and modeling of knottins, a promising molecular scaffold
963 for drug discovery. *Curr Pharm Des* 17: 4337-4350

964 Hale J, Hancock R (2008) Alternative mechanisms of action of cationic antimicrobial
965 peptides on bacteria. *Expert review of anti-infective therapy* 5: 951-959

966 Halliday CL, Slavin MA, Chen SCA (2017) Antifungal Susceptibility Testing of Candida and
967 Cryptococcus Species and Mechanisms of Resistance: Implications for Clinical Laboratories.
968 *Current Fungal Infection Reports* 11: 124-133

969 Hancock RE, Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective
970 therapeutic strategies. *Nat Biotechnol* 24: 1551-1557

971 Harries E, Carmona L, Muñoz A, Ibeas JI, Read ND, Gandía M, Marcos JF (2013) Genes
972 involved in protein glycosylation determine the activity and cell internalization of the
973 antifungal peptide PAF26 in Saccharomyces cerevisiae. *Fungal genetics and biology* 58:
974 105-115

975 Harris M, Mora-Montes HM, Gow NAR, Coote PJ (2009) Loss of mannosylphosphate from
976 Candida albicans cell wall proteins results in enhanced resistance to the inhibitory effect of a
977 cationic antimicrobial peptide via reduced peptide binding to the cell surface. *Microbiology*
978 *(Reading)* 155: 1058-1070

979 Hawkins NJ, Fraaije BA (2016) Predicting Resistance by Mutagenesis: Lessons from 45
980 Years of MBC Resistance. *Frontiers in microbiology* 7: 1814-1814

981 Hayes BM, Anderson MA, Traven A, van der Weerden NL, Bleackley MR (2014a)
982 Activation of stress signalling pathways enhances tolerance of fungi to chemical fungicides
983 and antifungal proteins. *Cellular and molecular life sciences* 71: 2651-2666

984 Hayes BM, Anderson MA, Traven A, van der Weerden NL, Bleackley MR (2014b)
985 Activation of stress signalling pathways enhances tolerance of fungi to chemical fungicides
986 and antifungal proteins. *Cellular and molecular life sciences : CMLS* 71: 2651-2666

987 Hayes BME, Bleackley MR, Anderson MA, van der Weerden NL (2018) The Plant Defensin
988 NaD1 Enters the Cytoplasm of Candida Albicans via Endocytosis. *Journal of fungi (Basel,*
989 *Switzerland)* 4

990 Hayes BME, Bleackley MR, Wiltshire JL, Anderson MA, Traven A, van der Weerden NL
991 (2013) Identification and Mechanism of Action of the Plant Defensin NaD1 as a New
992 Member of the Antifungal Drug Arsenal against Candida albicans. *Antimicrobial Agents and*
993 *Chemotherapy* 57: 3667-3675

994 Heilmann CJ, Schneider S, Barker KS, Rogers PD, Morschhäuser J (2010) An A643T
995 mutation in the transcription factor Upc2p causes constitutive ERG11 upregulation and
996 increased fluconazole resistance in Candida albicans. *Antimicrob Agents Chemother* 54: 353-
997 359

998 Helmerhorst EJ, Breeuwer P, van't Hof W, Walgreen-Weterings E, Oomen LC, Veerman
999 EC, Amerongen AVN, Abee T (1999) The cellular target of histatin 5 on Candida albicans is
1000 the energized mitochondrion. *Journal of Biological Chemistry* 274: 7286-7291

1001 Henzler-Wildman KA, Martinez GV, Brown MF, Ramamoorthy A (2004) Perturbation of the
1002 hydrophobic core of lipid bilayers by the human antimicrobial peptide LL-37. *Biochemistry*
1003 43: 8459-8469

1004 Ho CH, Magtanong L, Barker SL, Gresham D, Nishimura S, Natarajan P, Koh JL, Porter J,
1005 Gray CA, Andersen RJ (2009) A molecular barcoded yeast ORF library enables mode-of-
1006 action analysis of bioactive compounds. *Nature biotechnology* 27: 369

1007 Ho CH, Piotrowski J, Dixon SJ, Baryshnikova A, Costanzo M, Boone C (2011) Combining
1008 functional genomics and chemical biology to identify targets of bioactive compounds.
1009 *Current opinion in chemical biology* 15: 66-78

1010 Hokken MWJ, Zwaan BJ, Melchers WJG, Verweij PE (2019) Facilitators of adaptation and
1011 antifungal resistance mechanisms in clinically relevant fungi. *Fungal Genetics and Biology*
1012 132: 103254

1013 Hollmann A, Martinez M, Maturana P, Semorile LC, Maffía PC (2018) Antimicrobial
1014 Peptides: Interaction With Model and Biological Membranes and Synergism With Chemical
1015 Antibiotics. *Frontiers in Chemistry* 6

1016 Hsu C-H, Chen C, Jou M-L, Lee AY-L, Lin Y-C, Yu Y-P, Huang W-T, Wu S-H (2005)
1017 Structural and DNA-binding studies on the bovine antimicrobial peptide, indolicidin:
1018 evidence for multiple conformations involved in binding to membranes and DNA. *Nucleic*
1019 *acids research* 33: 4053-4064

1020 Hull CM, Parker JE, Bader O, Weig M, Gross U, Warrilow AG, Kelly DE, Kelly SL (2012)
1021 Facultative sterol uptake in an ergosterol-deficient clinical isolate of Candida glabrata
1022 harboring a missense mutation in ERG11 and exhibiting cross-resistance to azoles and
1023 amphotericin B. *Antimicrob Agents Chemother* 56: 4223-4232

1024 Jang WS, Bajwa JS, Sun JN, Edgerton M (2010) Salivary histatin 5 internalization by
1025 translocation, but not endocytosis, is required for fungicidal activity in Candida albicans.
1026 *Molecular microbiology* 77: 354-370

1027 Järvå M, Lay F, Phan TK, Humble C, Poon I, Bleackley M, Anderson M, Hulett M,
1028 Kvansakul M (2018a) X-ray structure of a carpet-like antimicrobial defensin–phospholipid
1029 membrane disruption complex. *Nature Communications* 9

1030 Järvå M, Phan TK, Lay F, Caria S, Kvansakul M, Hulett M (2018b) Human -defensin 2 kills
1031 Candida albicans through phosphatidylinositol 4,5-bisphosphate–mediated membrane
1032 permeabilization. *Science Advances* 4: eaat0979

1033 Joo H-S, Fu C-I, Otto M (2016) Bacterial strategies of resistance to antimicrobial peptides.
1034 *Philos Trans R Soc Lond B Biol Sci* 371: 20150292

1035 Kai-Larsen Y, Agerberth B (2008) The role of the multifunctional peptide LL-37 in host
1036 defense. *Front Biosci* 13: 3760-3767

1037 Kang X, Dong F, Shi C, Liu S, Sun J, Chen J, Li H, Xu H, Lao X, Zheng H (2019) DRAMP
1038 2.0, an updated data repository of antimicrobial peptides. *Sci Data* 6: 148

1039 Kemmer D, McHardy LM, Hoon S, Rebérioux D, Giaever G, Nislow C, Roskelley CD,
1040 Roberge M (2009) Combining chemical genomics screens in yeast to reveal spectrum of
1041 effects of chemical inhibition of sphingolipid biosynthesis. *BMC microbiology* 9: 9

1042 Komatsu T, Salih E, Helmerhorst EJ, Offner GD, Oppenheim FG (2011) Influence of
1043 Histatin 5 on Candida albicans Mitochondrial Protein Expression Assessed by Quantitative
1044 Mass Spectrometry. *Journal of Proteome Research* 10: 646-655

1045 Kosikowska P, Lesner A (2016) Antimicrobial peptides (AMPs) as drug candidates: a patent
1046 review (2003-2015). *Expert Opin Ther Pat* 26: 689-702

1047 Kovaleva V, Bukhteeva I, Kit OY, Nesmelova IV (2020) Plant Defensins from a Structural
1048 Perspective. *International Journal of Molecular Sciences* 21

1049 Krishnakumari V, Rangaraj N, Nagaraj R (2009) Antifungal activities of human beta-
1050 defensins HBD-1 to HBD-3 and their C-terminal analogs Phd1 to Phd3. *Antimicrob Agents*
1051 *Chemother* 53: 256-260

1052 Kumar P, Kizhakkedathu JN, Straus SK (2018) Antimicrobial Peptides: Diversity,
1053 Mechanism of Action and Strategies to Improve the Activity and Biocompatibility In Vivo.
1054 *Biomolecules* 8: 4

1055 Kumar R, Chadha S, Saraswat D, Bajwa JS, Li RA, Conti HR, Edgerton M (2011) Histatin 5
1056 uptake by Candida albicans utilizes polyamine transporters Dur3 and Dur31 proteins. *The*
1057 *Journal of biological chemistry* 286: 43748-43758

1058 Lamoth F, Alexander BD, Juvvadi PR, Steinbach WJ (2015) Antifungal activity of
1059 compounds targeting the Hsp90-calcineurin pathway against various mould species. *The*
1060 *Journal of antimicrobial chemotherapy* 70: 1408-1411

1061 Lamoth F, Juvvadi PR, Gehrke C, Steinbach WJ (2013) In vitro activity of calcineurin and
1062 heat shock protein 90 Inhibitors against Aspergillus fumigatus azole- and echinocandin-
1063 resistant strains. *Antimicrob Agents Chemother* 57: 1035-1039

1064 Lay FT, Brugliera F, Anderson MA (2003) Isolation and Properties of Floral Defensins from
1065 Ornamental Tobacco and Petunia. *Plant Physiology* 131: 1283-1293

1066 Lay FT, Mills GD, Poon IKH, Cowieson NP, Kirby N, Baxter AA, van der Weerden NL,
1067 Dogovski C, Perugini MA, Anderson MA *et al* (2012) Dimerization of Plant Defensin NaD1
1068 Enhances Its Antifungal Activity. *Journal of Biological Chemistry* 287: 19961-19972

1069 Lee DG, Kim DH, Park Y, Kim HK, Kim HN, Shin YK, Choi CH, Hahm KS (2001)
1070 Fungicidal effect of antimicrobial peptide, PMAP-23, isolated from porcine myeloid against
1071 Candida albicans. *Biochem Biophys Res Commun* 282: 570-574

1072 Lee DG, Kim PI, Park Y, Park SC, Woo ER, Hahm KS (2002) Antifungal mechanism of
1073 SMAP-29 (1-18) isolated from sheep myeloid mRNA against Trichosporon beigelii. *Biochem*
1074 *Biophys Res Commun* 295: 591-596

1075 Lee H, Lee DG (2018) Novel Approaches for Efficient Antifungal Drug Action. *J Microbiol*
1076 *Biotechnol* 28: 1771-1781

1077 Lee Y, Puumala E, Robbins N, Cowen LE (2020) Antifungal Drug Resistance: Molecular
1078 Mechanisms in Candida albicans and Beyond. *Chemical Reviews*

1079 Li M, Cha DJ, Lai Y, Villaruz AE, Sturdevant DE, Otto M (2007) The antimicrobial peptide-
1080 sensing system aps of Staphylococcus aureus. *Molecular microbiology* 66: 1136-1147

1081 Li R, Kumar R, Tati S, Puri S, Edgerton M (2013) Candida albicans Flu1-Mediated Efflux of
1082 Salivary Histatin 5 Reduces Its Cytosolic Concentration and Fungicidal Activity.
1083 *Antimicrobial Agents and Chemotherapy* 57: 1832

1084 Li XS, Sun JN, Okamoto-Shibayama K, Edgerton M (2006) Candida albicans cell wall ssa
1085 proteins bind and facilitate import of salivary histatin 5 required for toxicity. *The Journal of*
1086 *biological chemistry* 281: 22453-22463

1087 Lis M, Bhatt S, Schoenly NE, Lee AY, Nislow C, Bobek LA (2013) Chemical genomic
1088 screening of a Saccharomyces cerevisiae genomewide mutant collection reveals genes
1089 required for defense against four antimicrobial peptides derived from proteins found in
1090 human saliva. *Antimicrobial agents and chemotherapy* 57: 840-847

1091 Lis M, Bobek LA (2008) Proteomic and metabolic characterization of a Candida albicans
1092 mutant resistant to the antimicrobial peptide MUC7 12-mer. *FEMS Immunology & Medical*
1093 *Microbiology* 54: 80-91

1094 Lis M, Fuss JR, Bobek LA (2009) Exploring the mode of action of antimicrobial peptide
1095 MUC7 12-mer by fitness profiling of Saccharomyces cerevisiae genomewide mutant
1096 collection. *Antimicrobial agents and chemotherapy* 53: 3762-3769

1097 Lum PY, Armour CD, Stepaniants SB, Cavet G, Wolf MK, Butler JS, Hinshaw JC, Garnier
1098 P, Prestwich GD, Leonardson A (2004) Discovering modes of action for therapeutic
1099 compounds using a genome-wide screen of yeast heterozygotes. *Cell* 116: 121-137

1100 Magana M, Pushpanathan M, Santos AL, Leanse L, Fernandez M, Ioannidis A, Giulianotti
1101 MA, Apidianakis Y, Bradfute S, Ferguson AL *et al* (2020) The value of antimicrobial
1102 peptides in the age of resistance. *The Lancet Infectious Diseases* 20: e216-e230

1103 Mahlapuu M, Håkansson J, Ringstad L, Björn C (2016a) Antimicrobial Peptides: An
1104 Emerging Category of Therapeutic Agents. *Frontiers in Cellular and Infection Microbiology*
1105 6

1106 Mahlapuu M, Håkansson J, Ringstad L, Björn C (2016b) Antimicrobial Peptides: An
1107 Emerging Category of Therapeutic Agents. *Front Cell Infect Microbiol* 6: 194-194

1108 Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M,
1109 Ramaekers FCS, Odds FC, Vanden Bossche H (1999) Contribution of mutations in the
1110 cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in Candida
1111 albicans. *Microbiology (Reading, England)* 145 (Pt 10): 2701-2713

1112 Markovich S, Yekutiel A, Shalit I, Shadkchan Y, Osherov N (2004) Genomic approach to
1113 identification of mutations affecting caspofungin susceptibility in Saccharomyces cerevisiae.
1114 *Antimicrobial agents and chemotherapy* 48: 3871-3876

1115 Martin SW, Konopka JB (2004) Lipid raft polarization contributes to hyphal growth in
1116 Candida albicans. *Eukaryotic cell* 3: 675-684

1117 Mattila J-P, Sabatini K, Kinnunen PKJ (2008) Oxidized phospholipids as potential molecular
1118 targets for antimicrobial peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes*
1119 1778: 2041-2050

1120 McColl AI, Bleackley MR, Anderson MA, Lowe RGT (2018) Resistance to the Plant
1121 Defensin NaD1 Features Modifications to the Cell Wall and Osmo-Regulation Pathways of
1122 Yeast. *Frontiers in Microbiology* 9: 1648

1123 McColl AI, Lowe RGT, McKenna JA, Anderson MA, Bleackley MR (2020) Resistance to
1124 the antifungal activity of Aprotinin occurs through mutations in genes that function in cation
1125 homeostasis. *bioRxiv*: 2020.2006.2022.164863

1126 Mello EO, Ribeiro SF, Carvalho AO, Santos IS, Da Cunha M, Santa-Catarina C, Gomes VM
1127 (2011) Antifungal activity of PvD1 defensin involves plasma membrane permeabilization,
1128 inhibition of medium acidification, and induction of ROS in fungi cells. *Current*
1129 *microbiology* 62: 1209-1217

1130 Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ (2020) Antimicrobial host
1131 defence peptides: functions and clinical potential. *Nature Reviews Drug Discovery* 19: 311-
1132 332

1133 Muñoz A, Chu M, Marris PI, Sagaram US, Kaur J, Shah DM, Read ND (2014) Specific
1134 domains of plant defensins differentially disrupt colony initiation, cell fusion and calcium
1135 homeostasis in Neurospora crassa. *Molecular microbiology* 92: 1357-1374

1136 Munoz A, López-García B, Marcos JF (2006) Studies on the mode of action of the antifungal
1137 hexapeptide PAF26. *Antimicrobial agents and chemotherapy* 50: 3847-3855

1138 Nguyen LT, Haney EF, Vogel HJ (2011) The expanding scope of antimicrobial peptide
1139 structures and their modes of action. *Trends in biotechnology* 29: 464-472

1140 O'Meara TR, Robbins N, Cowen LE (2017) The Hsp90 Chaperone Network Modulates
1141 Candida Virulence Traits. *Trends in microbiology* 25: 809-819

1142 O'Shaughnessy EM, Lyman CA, Walsh TJ (2009) Amphotericin B: Polyene Resistance
1143 Mechanisms. In: *Antimicrobial Drug Resistance: Mechanisms of Drug Resistance*, Mayers
1144 D.L. (ed.) pp. 295-305. Humana Press: Totowa, NJ

1145 Ordonez SR, Amarullah IH, Wubbolts RW, Veldhuizen EJA, Haagsman HP (2014)
1146 Fungicidal Mechanisms of Cathelicidins LL-37 and CATH-2 Revealed by Live-Cell
1147 Imaging. *Antimicrobial Agents and Chemotherapy* 58: 2240

1148 Parisi K, Doyle SR, Lee E, Lowe RG, van der Weerden NL, Anderson MA, Bleackley MR
1149 (2019a) Screening the Saccharomyces cerevisiae Nonessential Gene Deletion Library
1150 Reveals Diverse Mechanisms of Action for Antifungal Plant Defensins. *Antimicrobial agents*
1151 *and chemotherapy* 63: e01097-01019

1152 Parisi K, Shafee TM, Quimbar P, van der Weerden NL, Bleackley MR, Anderson MA,
1153 2019b. The evolution, function and mechanisms of action for plant defensins, Seminars in
1154 cell & developmental biology. Elsevier, pp. 107-118.

1155 Parisi K, Shafee TMA, Quimbar P, van der Weerden NL, Bleackley MR, Anderson MA
1156 (2018) The evolution, function and mechanisms of action for plant defensins. *Seminars in*
1157 *cell & developmental biology*

1158 Park K, Oh D, Shin SY, Hahm KS, Kim Y (2002) Structural studies of porcine myeloid
1159 antibacterial peptide PMAP-23 and its analogues in DPC micelles by NMR spectroscopy.
1160 *Biochem Biophys Res Commun* 290: 204-212

1161 Parsons AB, Lopez A, Givoni IE, Williams DE, Gray CA, Porter J, Chua G, Sopko R, Brost
1162 RL, Ho C-H (2006) Exploring the mode-of-action of bioactive compounds by chemical-
1163 genetic profiling in yeast. *Cell* 126: 611-625

1164 Pasupuleti M, Schmidtchen A, Malmsten M (2012) Antimicrobial peptides: key components
1165 of the innate immune system. *Critical reviews in biotechnology* 32: 143-171

1166 Pathirana RU, Friedman J, Norris HL, Salvatori O, McCall AD, Kay J, Edgerton M (2018)
1167 Fluconazole-Resistant Candida auris Is Susceptible to Salivary Histatin 5 Killing and to
1168 Intrinsic Host Defenses. *Antimicrob Agents Chemother* 62

1169 Payne J, Bleackley M, Lee T-H, Shafee T, Poon I, Hulett M, Aguilar M-I, van der Weerden
1170 N, Anderson M (2016a) The plant defensin NaD1 introduces membrane disorder through a
1171 specific interaction with the lipid, phosphatidylinositol 4,5 bisphosphate. *Biochimica et*
1172 *Biophysica Acta (BBA) - Biomembranes* 1858

1173 Payne JA, Bleackley MR, Lee TH, Shafee TM, Poon IK, Hulett MD, Aguilar MI, van der
1174 Weerden NL, Anderson MA (2016b) The plant defensin NaD1 introduces membrane
1175 disorder through a specific interaction with the lipid, phosphatidylinositol 4,5 bisphosphate.
1176 *Biochimica et biophysica acta* 1858: 1099-1109

1177 Pea F, Lewis RE (2018) Overview of antifungal dosing in invasive candidiasis. *Journal of*
1178 *Antimicrobial Chemotherapy* 73: i33-i43

1179 Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A (2017) The global problem of
1180 antifungal resistance: prevalence, mechanisms, and management. *The Lancet Infectious*
1181 *diseases* 17: e383-e392

1182 Pianalto KM, Alspaugh JA (2016) New Horizons in Antifungal Therapy. *Journal of fungi*
1183 *(Basel, Switzerland)* 2

1184 Pierce SE, Davis RW, Nislow C, Giaever G (2007) Genome-wide analysis of barcoded
1185 Saccharomyces cerevisiae gene-deletion mutants in pooled cultures. *Nature protocols* 2: 2958

1186 Pinto E, Monteiro C, Maia M, Faria MA, Lopes V, Lameiras C, Pinheiro D (2018)
1187 Aspergillus Species and Antifungals Susceptibility in Clinical Setting in the North of
1188 Portugal: Cryptic Species and Emerging Azoles Resistance in A. fumigatus. *Front Microbiol*
1189 9: 1656

1190 Piotrowski JS, Simpkins SW, Li SC, Deshpande R, McIlwain SJ, Ong IM, Myers CL, Boone
1191 C, Andersen RJ (2015) Chemical genomic profiling via barcode sequencing to predict
1192 compound mode of action. In: *Chemical biology*, pp. 299-318. Springer:

1193 Poon IKH, Baxter AA, Lay FT, Mills GD, Adda CG, Payne JAE, Phan TK, Ryan GF, White
1194 JA, Veneer PK *et al* (2014) Phosphoinositide-mediated oligomerization of a defensin induces
1195 cell lysis. *eLife* 3: e01808

1196 Posteraro B, Sanguinetti M, Sanglard D, La Sorda M, Boccia S, Romano L, Morace G, Fadda
1197 G (2003) Identification and characterization of a Cryptococcus neoformans ATP binding
1198 cassette (ABC) transporter-encoding gene, CnAFR1, involved in the resistance to
1199 fluconazole. *Molecular microbiology* 47: 357-371

1200 Puri S, Edgerton M (2014) How does it kill?: understanding the candidacidal mechanism of
1201 salivary histatin 5. *Eukaryotic cell* 13: 958-964

1202 Raheem N, Straus SK (2019) Mechanisms of Action for Antimicrobial Peptides With
1203 Antibacterial and Antibiofilm Functions. *Frontiers in Microbiology* 10

1204 Ramamoorthy V, Cahoon EB, Li J, Thokala M, Minto RE, Shah DM (2007)
1205 Glucosylceramide synthase is essential for alfalfa defensin-mediated growth inhibition but
1206 not for pathogenicity of Fusarium graminearum. *Molecular microbiology* 66: 771-786

1207 Rautenbach M, Troskie AM, Vosloo JA (2016a) Antifungal peptides: To be or not to be
1208 membrane active. *Biochimie* 130: 132-145

1209 Rautenbach M, Troskie AM, Vosloo JA, Dathe ME (2016b) Antifungal membranolytic
1210 activity of the tyrocidines against filamentous plant fungi. *Biochimie* 130: 122-131

1211 Rees DC, Lipscomb WN (1982) Refined crystal structure of the potato inhibitor complex of
1212 carboxypeptidase A at 2.5 A resolution. *Journal of molecular biology* 160: 475-498

1213 Rego A, Trindade D, Chaves SR, Manon S, Costa V, Sousa MJ, Côrte-Real M (2014) The
1214 yeast model system as a tool towards the understanding of apoptosis regulation by
1215 sphingolipids. *FEMS yeast research* 14: 160-178

1216 Reinoso-Martín C, Schüller C, Schuetzer-Muehlbauer M, Kuchler K (2003) The yeast protein
1217 kinase C cell integrity pathway mediates tolerance to the antifungal drug caspofungin through
1218 activation of Slt2p mitogen-activated protein kinase signaling. *Eukaryotic cell* 2: 1200-1210

1219 Revie NM, Iyer KR, Robbins N, Cowen LE (2018) Antifungal drug resistance: evolution,
1220 mechanisms and impact. *Current opinion in microbiology* 45: 70-76

1221 Robbins N, Caplan T, Cowen LE (2017a) Molecular Evolution of Antifungal Drug
1222 Resistance. *Annual Review of Microbiology* 71: 753-775

1223 Robbins N, Caplan T, Cowen LE (2017b) Molecular Evolution of Antifungal Drug
1224 Resistance. *Annu Rev Microbiol* 71: 753-775

1225 Rosana Y, Yasmon A, Lestari DC (2015) Overexpression and mutation as a genetic
1226 mechanism of fluconazole resistance in Candida albicans isolated from human
1227 immunodeficiency virus patients in Indonesia. *J Med Microbiol* 64: 1046-1052

1228 Roy H (2009) Tuning the properties of the bacterial membrane with aminoacylated
1229 phosphatidylglycerol. *IUBMB Life* 61: 940-953

1230 Sagaram US, El-Mounadi K, Buchko GW, Berg HR, Kaur J, Pandurangi RS, Smith TJ, Shah
1231 DM (2013) Structural and Functional Studies of a Phosphatidic Acid-Binding Antifungal
1232 Plant Defensin MtDef4: Identification of an RGFRRR Motif Governing Fungal Cell Entry.
1233 *PLOS ONE* 8: e82485

1234 Sagaram US, Pandurangi R, Kaur J, Smith TJ, Shah DM (2011) Structure-Activity
1235 Determinants in Antifungal Plant Defensins MsDef1 and MtDef4 with Different Modes of
1236 Action against *Fusarium graminearum*. *PLoS ONE* 6: e18550

1237 Schuber F (1989) Influence of polyamines on membrane functions. *Biochemical Journal* 260:
1238 1

1239 Sengupta D, Leontiadou H, Mark AE, Marrink S-J (2008) Toroidal pores formed by
1240 antimicrobial peptides show significant disorder. *Biochimica et Biophysica Acta (BBA) -*
1241 *Biomembranes* 1778: 2308-2317

1242 Sevcsik E, Pabst G, Jilek A, Lohner K (2007) How lipids influence the mode of action of
1243 membrane-active peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1768:
1244 2586-2595

1245 Seyfi R, Kahaki FA, Ebrahimi T, Montazersaheb S, Eyvazi S, Babaeipour V, Tarhriz V
1246 (2020) Antimicrobial Peptides (AMPs): Roles, Functions and Mechanism of Action.
1247 *International Journal of Peptide Research and Therapeutics* 26: 1451-1463

1248 Shafee T, Anderson MA (2019) A quantitative map of protein sequence space for the cis-
1249 defensin superfamily. *Bioinformatics* 35: 743-752

1250 Shafee TMA, Lay FT, Hulett MD, Anderson MA (2016) The Defensins Consist of Two
1251 Independent, Convergent Protein Superfamilies. *Molecular Biology and Evolution* 33: 2345-
1252 2356

1253 Shafee TMA, Lay FT, Phan TK, Anderson MA, Hulett MD (2017) Convergent evolution of
1254 defensin sequence, structure and function. *Cellular and Molecular Life Sciences* 74: 663-682

1255 Shiver AL, Osadnik H, Kritikos G, Li B, Krogan N, Typas A, Gross CA (2016) A chemical-
1256 genomic screen of neglected antibiotics reveals illicit transport of kasugamycin and
1257 blasticidin S. *PLoS genetics* 12

1258 Shoemaker DD, Lashkari DA, Morris D, Mittmann M, Davis RW (1996) Quantitative
1259 phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar–coding
1260 strategy. *Nature genetics* 14: 450

1261 Sierotzki H, Parisi S, Steinfeld U, Tenzer I, Poirey S, Gisi U (2000) Mode of resistance to
1262 respiration inhibitors at the cytochrome bc1 enzyme complex of Mycosphaerella fijiensis
1263 field isolates. *Pest Management Science* 56: 833-841

1264 Singh A, Prasad R (2011) Comparative lipidomics of azole sensitive and resistant clinical
1265 isolates of Candida albicans reveals unexpected diversity in molecular lipid imprints. *PLoS*
1266 *One* 6: e19266

1267 Singh SD, Robbins N, Zaas AK, Schell WA, Perfect JR, Cowen LE (2009) Hsp90 governs
1268 echinocandin resistance in the pathogenic yeast Candida albicans via calcineurin. *PLoS*
1269 *Pathog* 5: e1000532

1270 Situ H, Wei G, Smith CJ, Mashhoon S, Bobek LA (2003) Human salivary MUC7 mucin
1271 peptides: effect of size, charge and cysteine residues on antifungal activity. *The Biochemical*
1272 *journal* 375: 175-182

1273 Skerlavaj B, Benincasa M, Risso A, Zanetti M, Gennaro R (1999) SMAP-29: a potent
1274 antibacterial and antifungal peptide from sheep leukocytes. *FEBS letters* 463: 58-62

1275 Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IS, Denning DW (2002)
1276 Increased expression of a novel Aspergillus fumigatus ABC transporter gene, atrF, in the
1277 presence of itraconazole in an itraconazole resistant clinical isolate. *Fungal genetics and*
1278 *biology : FG & B* 36: 199-206

1279 Spelbrink RG, Dilmac N, Allen A, Smith TJ, Shah DM, Hockerman GH (2004) Differential
1280 Antifungal and Calcium Channel-Blocking Activity among Structurally Related Plant
1281 Defensins. *Plant Physiology* 135: 2055

1282 Sun JN, Li W, Jang WS, Nayyar N, Sutton MD, Edgerton M (2008) Uptake of the antifungal
1283 cationic peptide Histatin 5 by Candida albicans Ssa2p requires binding to non-conventional
1284 sites within the ATPase domain. *Molecular microbiology* 70: 1246-1260

1285 Swidergall M, Ernst JF (2014) Interplay between Candida albicans and the antimicrobial
1286 peptide armory. *Eukaryot Cell* 13: 950-957

1287 Szafranski-Schneider E, Swidergall M, Cottier F, Tielker D, Román E, Pla J, Ernst JF (2012)
1288 Msb2 shedding protects Candida albicans against antimicrobial peptides. *PLoS pathogens* 8:
1289 e1002501-e1002501

1290 Taipale M, Jarosz DF, Lindquist S (2010) HSP90 at the hub of protein homeostasis:
1291 emerging mechanistic insights. *Nature reviews Molecular cell biology* 11: 515-528

1292 Takahashi D, Shukla SK, Prakash O, Zhang G (2010) Structural determinants of host defense
1293 peptides for antimicrobial activity and target cell selectivity. *Biochimie* 92: 1236-1241

1294 Takeshita N, Higashitsuji Y, Konzack S, Fischer R (2008) Apical sterol-rich membranes are
1295 essential for localizing cell end markers that determine growth directionality in the
1296 filamentous fungus Aspergillus nidulans. *Mol Biol Cell* 19: 339-351

1297 Thevissen K, Cammue BP, Lemaire K, Winderickx J, Dickson RC, Lester RL, Ferket KK,
1298 Van Even F, Parret AH, Broekaert WF (2000a) A gene encoding a sphingolipid biosynthesis
1299 enzyme determines the sensitivity of Saccharomyces cerevisiae to an antifungal plant
1300 defensin from dahlia (Dahlia merckii). *Proceedings of the National Academy of Sciences of*
1301 *the United States of America* 97: 9531-9536

1302 Thevissen K, de Mello Tavares P, Xu D, Blankenship J, Vandenbosch D, Idkowiak-Baldys J,
1303 Govaert G, Bink A, Rozental S, de Groot PW *et al* (2012) The plant defensin RsAFP2
1304 induces cell wall stress, septin mislocalization and accumulation of ceramides in Candida
1305 albicans. *Molecular microbiology* 84: 166-180

1306 Thevissen K, Francois IE, Takemoto JY, Ferket KK, Meert EM, Cammue BP (2003a)
1307 DmAMP1, an antifungal plant defensin from dahlia (Dahlia merckii), interacts with
1308 sphingolipids from Saccharomyces cerevisiae. *FEMS Microbiol Lett* 226: 169-173

1309 Thevissen K, François IE, Takemoto JY, Ferket KK, Meert EM, Cammue BP (2003b)
1310 DmAMP1, an antifungal plant defensin from dahlia (Dahlia merckii), interacts with
1311 sphingolipids from Saccharomyces cerevisiae. *FEMS Microbiol Lett* 226: 169-173

1312 Thevissen K, Osborn RW, Acland DP, Broekaert WF (1997) Specific, high affinity binding
1313 sites for an antifungal plant defensin on Neurospora crassa hyphae and microsomal
1314 membranes. *Journal of Biological Chemistry* 272: 32176-32181

1315 Thevissen K, Osborn RW, Acland DP, Broekaert WF (2000b) Specific Binding Sites for an
1316 Antifungal Plant Defensin from Dahlia (Dahlia merckii) on Fungal Cells Are Required for
1317 Antifungal Activity. *Molecular Plant-Microbe Interactions®* 13: 54-61

1318 Thevissen K, Warnecke DC, François IE, Leipelt M, Heinz E, Ott C, Zähringer U, Thomma
1319 BP, Ferket KK, Cammue BP (2004) Defensins from insects and plants interact with fungal
1320 glucosylceramides. *The Journal of biological chemistry* 279: 3900-3905

1321 Tobias NJ, Wolff H, Djahanschiri B, Grundmann F, Kronenwerth M, Shi Y-M, Simonyi S,
1322 Grün P, Shapiro-Ilan D, Pidot SJ *et al* (2017) Natural product diversity associated with the
1323 nematode symbionts Photorhabdus and Xenorhabdus. *Nature microbiology* 2: 1676-1685

1324 Troskie AM, Rautenbach M, Delattin N, Vosloo JA, Dathe M, Cammue BP, Thevissen K
1325 (2014) Synergistic activity of the tyrocidines, antimicrobial cyclodecapeptides from Bacillus

aneurinolyticus, with amphotericin B and caspofungin against Candida albicans biofilms. *Antimicrob Agents Chemother* 58: 3697-3707

Tsai P-W, Cheng Y-L, Hsieh W-P, Lan C-Y (2014a) Responses of Candida albicans to the human antimicrobial peptide LL-37. *Journal of Microbiology* 52: 581-589

Tsai P-W, Yang C-Y, Chang H-T, Lan C-Y (2011) Characterizing the role of cell-wall β-1,3-exoglucanase Xog1p in Candida albicans adhesion by the human antimicrobial peptide LL-37. *PloS one* 6: e21394-e21394

Tsai PW, Cheng YL, Hsieh WP, Lan CY (2014b) Responses of Candida albicans to the human antimicrobial peptide LL-37. *J Microbiol* 52: 581-589

Tsao S, Rahkhoodaee F, Raymond M (2009) Relative contributions of the Candida albicans ABC transporters Cdr1p and Cdr2p to clinical azole resistance. *Antimicrobial agents and chemotherapy* 53: 1344-1352

Tzeng YL, Stephens DS (2015) Antimicrobial peptide resistance in Neisseria meningitidis. *Biochimica et biophysica acta* 1848: 3026-3031

van der Weerden NL, Bleackley MR, Anderson MA (2013) Properties and mechanisms of action of naturally occurring antifungal peptides. *Cellular and Molecular Life Sciences* 70: 3545-3570

van der Weerden NL, Hancock REW, Anderson MA (2010) Permeabilization of Fungal Hyphae by the Plant Defensin NaD1 Occurs through a Cell Wall-dependent Process. *Journal of Biological Chemistry* 285: 37513-37520

van der Weerden NL, Lay FT, Anderson MA (2008) The Plant Defensin, NaD1, Enters the Cytoplasm of Fusarium Oxysporum Hyphae. *Journal of Biological Chemistry* 283: 14445-14452

Vandamme D, Landuyt B, Luyten W, Schoofs L (2012) A comprehensive summary of LL-37, the factotum human cathelicidin peptide. *Cell Immunol* 280: 22-35

Vincent BM, Lancaster AK, Scherz-Shouval R, Whitesell L, Lindquist S (2013) Fitness trade-offs restrict the evolution of resistance to amphotericin B. *PLoS biology* 11: e1001692

1353 Wang G (2014) Human antimicrobial peptides and proteins. *Pharmaceuticals (Basel)* 7: 545-
1354 594

1355 Wiederhold NP (2017) Antifungal resistance: current trends and future strategies to combat.
1356 *Infect Drug Resist* 10: 249-259

1357 Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, Bangham R,
1358 Benito R, Boeke JD, Bussey H (1999) Functional characterization of the S. cerevisiae
1359 genome by gene deletion and parallel analysis. *science* 285: 901-906

1360 Xu D, Jiang B, Ketela T, Lemieux S, Veillette K, Martel N, Davison J, Sillaots S, Trosok S,
1361 Bachewich C (2007) Genome-wide fitness test and mechanism-of-action studies of inhibitory
1362 compounds in Candida albicans. *PLoS pathogens* 3

1363 Yeaman MR, Büttner S, Thevissen K (2018) Regulated Cell Death as a Therapeutic Target
1364 for Novel Antifungal Peptides and Biologics. *Oxidative Medicine and Cellular Longevity*
1365 2018: 5473817

1366 Yeaman MR, Yount NY (2003) Mechanisms of antimicrobial peptide action and resistance.
1367 *Pharmacological reviews* 55: 27-55

1368 Zhang L-j, Gallo RL (2016) Antimicrobial peptides. *Current Biology* 26: R14-R19

1369 Zoran T, Sartori B, Sappl L, Aigner M, Sánchez-Reus F, Rezusta A, Chowdhary A, Taj-
1370 Aldeen SJ, Arendrup MC, Oliveri S *et al* (2018) Azole-Resistance in Aspergillus terreus and
1371 Related Species: An Emerging Problem or a Rare Phenomenon? *Frontiers in Microbiology* 9:
1372 516
1373