

Antifungal Activity of Synthetic Peptides Targeting Apoptosis in *Candida auris*

SIHAM SHABAN



Supervisors

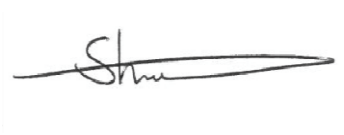
Associated Professor Aijaz Ahmad & Professor Mrudula Patel

**A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand
in fulfilment of the requirements for the degree of Doctor of Philosophy**

Johannesburg, 2024

DECLARATION

I, Siham Shaban, declare that this thesis is my own work. It has been submitted for the degree of Doctor of Philosophy in the Faculty of Health Sciences in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



.....

Signed this 12 day of August 2024

PUBLICATIONS

1. Shaban, S., Patel, M., & Ahmad, A. (2023). Fungicidal activity of human antimicrobial peptides and their synergistic interaction with common antifungals against multidrug-resistant *Candida auris*. *International microbiology: the official journal of the Spanish Society for Microbiology*, 2023 May;26(2):165-177. <https://doi.org/10.1007/s10123-022-00290-5>. Epub 2022 Nov 3. PMID: 36329309.
2. Shaban, S., Patel, M., & Ahmad, A. (2024). Antifungal activity of human antimicrobial peptides targeting apoptosis in *Candida auris*. *Journal of medical microbiology*, 73(5), 10.1099/jmm.0.001835. <https://doi.org/10.1099/jmm.0.001835>. PMID: 38743468.
3. Shaban, S., Patel, M., & Ahmad, A. Anti-virulence and anti-efflux pump activity of synthetic defensins and histatin in *Candida auris*. Manuscript submitted to *Journal of Microbial Pathogenesis*, July 2024.

RESEARCH PRESENTATIONS

1. Shaban, S., Patel, M. & Ahmad, A. Antifungal activity of synthetic peptides against *Candida auris*. Oral and poster presentations at the Faculty of Health Sciences Research Day and Postgraduate Expo 2020. University of the Witwatersrand, Johannesburg, South Africa, October 15, 2020.
2. Shaban, S., Patel, M. & Ahmad, A. The fungicidal activity of antimicrobial peptides and their synergistic effect in combination with common antifungal drugs against *Candida auris*. Poster presented at the Pathology Research and Development (PathReD) Congress 2021. August 19th- 22nd, 2021.
3. Shaban, S., Patel, M. & Ahmad, A. Effect of human antimicrobial peptides on membrane permeability and viability of *Candida auris*. Oral presentations at the Life Sciences Imaging Facility-Research Day. University of the Witwatersrand, Johannesburg, South Africa, June 1, 2022.
4. Shaban, S., Patel, M. & Ahmad, A. Fungicidal effect of human antimicrobial peptides by disrupting membrane integrity in *Candida auris*. Poster presentations at the 13th Cross-Faculty Graduate Symposium, University of the Witwatersrand, Johannesburg, South Africa, July 11 - 13, 2022.
5. Shaban, S., Patel, M. & Ahmad, A. Human defensins induced apoptosis in *Candida auris*. Poster presentations at the Faculty of Health Sciences Research Day and Postgraduate Expo 2022. University of the Witwatersrand, Johannesburg, South Africa, September 15, 2022

ABSTRACT

Background: Nosocomial infections caused by the multidrug-resistant (MDR) yeast *Candida auris* pose a significant healthcare threat. This pathogen presents a major clinical challenge due to its resistance profile, virulence factors, including biofilm formation, and the overexpression of efflux pumps. Current antifungal treatments are often limited in efficacy against MDR fungal pathogens, particularly *C. auris*. Therefore, novel strategies to address these challenges are urgently needed. Apoptosis, a form of programmed cell death, represents a potential target for novel antifungal approaches. Furthermore, strategies targeting virulence and efflux pump activity could be a valuable approach to combat *C. auris* infections. Human antimicrobial peptides (AMPs) hold promises due to their broad-spectrum activity, including activity against various fungi. Thus, this study investigated the potential of selected human AMPs against *C. auris* and the role of apoptosis induction in their antimicrobial activity. These peptides were also evaluated for their ability to inhibit virulence and efflux pump in *C. auris*.

Methods: Antifungal efficacy of seven distinct human AMPs, including HNP-1-3, hBD-1-3 and His 5, against ten *C. auris* clinical isolates, was determined adhering to the established CLSI guidelines for antifungal susceptibility testing. The antifungal activity of the AMPs was further assessed via time-kill curves and cell viability assays. The potential synergy with established antifungals (fluconazole, amphotericin B, and caspofungin) was explored using combination assay. To understand their antifungal mechanism, propidium iodide staining and confocal imaging were employed to evaluate their effects on *C. auris* membrane permeability. The three most active AMPs (HNP-1, hBD-3, and His 5) were further investigated for their ability to induce apoptosis in two *C. auris* isolates. Specific markers for apoptosis, including phosphatidylserine externalization (FITC Annexin-V/PI assay) and DNA fragmentation (TUNEL assay), were employed. Additionally, mitochondrial membrane potential (JC-10 staining), cytochrome c release (spectrophotometry), and reactive oxygen species (ROS) production (H₂DCFDA staining) were assessed to understand potential intracellular triggers of apoptosis. Furthermore, the study investigated the impact of the most active AMPs on *C. auris* virulence factors and efflux pumps. Adherence and proteinase activity were assessed using adherence assays and bovine serum albumin (BSA) plates, respectively. The effects on biofilm formation, maturation and structure were evaluated using the MTT (3-(4, 5-dimethylthiazolyl-

2)-2, 5-diphenyltetrazolium bromide) reduction test and confocal microscopy. The influence on efflux pump activity was determined by measuring rhodamine-6-G accumulation and efflux. Reverse transcription quantitative real-time PCR (RT-qPCR) was employed to investigate the effects on the expression of virulence and efflux pump-related genes. Finally, their safety was evaluated through a haemolytic assay.

Results: All examined AMPs exhibited fungicidal activity against *C. auris*, with hBD-3 demonstrating the most potent effect. Time-kill curves confirmed the fungicidal activity of all tested peptides and viability assays demonstrated a significant decrease in viable cells upon exposure to AMPs. Notably, AMPs displayed synergistic interactions with conventional antifungals, particularly caspofungin. Mechanistically, AMPs disrupted *C. auris* membrane permeability, correlating with growth inhibition. Furthermore, FITC Annexin-V/PI staining and TUNEL assays demonstrated that HNP-1 and hBD-3 induced both early and late apoptosis in *C. auris*, while His 5 primarily caused necrosis. HNP-1 and hBD-3 triggered significant mitochondrial membrane depolarization and cytochrome c release. In contrast, His 5 displayed minimal impact on mitochondrial function. All peptides significantly increased ROS production. In addition, subinhibitory concentrations of HNP-1, hBD-3, and His 5 significantly reduced *C. auris* adherence and proteinase activity. All three peptides effectively inhibited biofilm formation and reduced the density of established biofilms. They also impaired efflux pump activity and downregulated genes associated with virulence and efflux pumps. Furthermore, haemolytic assays confirmed the safety of these peptides towards mammalian red blood cells.

Conclusion: These findings highlight the potential of human AMPs as broad-spectrum antifungal agents against *C. auris*. Their ability to target multiple mechanisms, including direct killing, apoptosis induction, virulence inhibition and efflux pump reversal, suggests a multifaceted approach to combat *C. auris* infections. Additionally, the synergistic interaction with conventional antifungals suggests potential for combination therapy. Overall, this study findings provide a strong rationale for further investigation and development of human AMPs as novel antifungal agents against this MDR pathogen.

DEDICATION

With deepest appreciation to God, this thesis is dedicated to my parents, who instilled in me a lifelong love of learning and the drive to achieve academic success.

ACKNOWLEDGEMENTS

The completion of a doctoral thesis is a testament to the collective effort of many individuals. While the research journey can be solitary at times, it is ultimately shaped by the guidance and support of a network of mentors and colleagues. I am deeply grateful to all those who have contributed to this work in various ways.

First and foremost, my deepest gratitude goes to my God ALLAH for the countless blessings that have guided me throughout my life.

My deepest gratitude goes to my supervisors, Professor Aijaz Ahmad and Professor Mrudula Patel, for their mentorship, invaluable guidance, and support throughout this project. Their patience, enthusiasm, and immense knowledge were invaluable assets during both the research and thesis writing stages. Professor Ahmad's continuous encouragement and scientific expertise were instrumental in shaping my research. Professor Patel's insightful comments and meticulous attention to detail significantly improved the quality of my thesis.

My gratitude extends to Professor Nelesh Govender from the Mycology Reference Laboratory at the National Institute for Communicable Diseases for providing the essential clinical isolates used throughout this research project.

I would like to thank Dr. Aurelie Deroubaix from the Life Sciences Imaging Facility (LSIF), University of the Witwatersrand, for her expert guidance and assistance with confocal microscopy.

I would like to extend my gratitude to Professor Pascaline Fru from the Department of Surgery, University of the Witwatersrand, for her assistance with flow cytometry.

I am also grateful to Dr. Musa Marimani from the Anatomical Pathology Department, University of the Witwatersrand, for his assistance with molecular study.

I am also grateful to my broader laboratory colleagues within the Department of Clinical Microbiology and Infectious Diseases (CMID), University of the Witwatersrand, for their invaluable assistance on many occasions. Additionally, I would like to thank the staff of the

Infection Control Laboratory (National Health Laboratory Services and University of the Witwatersrand) for allowing me to utilize their equipment during my research.

A special thanks goes to all my senior and junior colleagues in the CMID Department, their assistance with my research was instrumental, especially during my initial adjustment to a new environment and culture. Their presence made those early days much more comfortable.

My deepest gratitude extends to my family, who have provided unwavering support throughout my life. My parents instilled in me the values and work ethic that made this achievement possible. Their constant encouragement and belief in me have been a source of immense strength.

I am especially grateful to my husband, Dr Hisham Issaadi, and my children, for their unwavering love and support throughout my doctoral journey. Their understanding and encouragement during long days of research were invaluable.

My heartfelt thanks also go to my brothers for their unwavering support and presence throughout my journey. Their belief in me has been a constant source of motivation.

In closing, I extend my sincere gratitude to all those who have contributed to the successful completion of this thesis. While it is not possible to acknowledge everyone individually, I am deeply grateful for the support of each person involved.

I acknowledge any shortcomings in this work and take full responsibility for any errors or omissions.

TABLE OF CONTENTS

DECLARATION.....	i
PUBLICATIONS	ii
RESEARCH PRESENTATIONS.....	iii
ABSTRACT	iv
DEDICATION.....	vi
ACKNOWLEDGEMENTS	vii
TABLE OF CONTENTS	ix
LIST OF FIGURES	xvi
LIST OF TABLES	xviii
LIST OF ABBREVIATIONS	xix
Chapter 1: Introduction & literature review.....	1
1.1. Introduction	1
1.2. literature review	3
1.2.1. Candidiasis	3
1.2.2. <i>Candida auris</i>	3
1.2.2.1. Discovery of <i>C. auris</i>	4
1.2.2.2. Risk factors for <i>C. auris</i> infections	4
1.2.2.3. Prevalence of <i>C. auris</i> in South Africa	4
1.2.2.4. Phenotypic characteristics of <i>C. auris</i>	5
	ix

1.2.2.5. Genomic characteristics of <i>C. auris</i>	5
1.2.3. Antifungal drugs: mode of action and mechanisms of resistance.....	6
1.2.3.1. Azoles.....	6
1.2.3.2. Polyenes.....	7
1.2.3.3. Echinocandins.....	8
1.2.3.4. Therapeutic options and resistance mechanisms in <i>C. auris</i>	9
1.2.3.5. Combination antifungal therapy	10
1.2.4. Novel perspective in antifungal drug development.....	11
1.2.4.1. Virulence factors.....	11
1.2.4.1.1. Adhesion	12
1.2.4.1.2. Morphogenesis.....	13
1.2.4.1.3. Hydrolytic enzymes	14
1.2.4.1.4. Biofilm formation.....	16
1.2.4.2. Types of cell death in yeast	17
1.2.4.2.1. Apoptosis in yeast	17
1.2.4.2.2. Necrosis in yeasts.....	21
1.2.5. Antimicrobial peptides.....	21
1.2.5.1. Human defensins	23
1.2.5.2. Human histatin.....	24
1.3. Justification of the study.....	25
1.4. References.....	26

Chapter 2: Aim and Objectives	46
2.1. Aim and Objectives.....	46
2.2. Layout of the Thesis	46
Chapter 3: Fungicidal activity of human antimicrobial peptides and their synergistic interaction with common antifungals against multidrug resistant <i>Candida auris</i>	50
3.1. Introduction	50
3.2. Materials and Methods.....	51
3.2.1. Strains and growth conditions.....	51
3.2.2. Peptides and antifungal drugs	52
3.2.3. Antifungal susceptibility assay	52
3.2.4. Time-kill curves assay	53
3.2.5. <i>Candida</i> cell counting and viability assay	53
3.2.6. Combination assay	53
3.2.7. Assessment of plasma membrane permeability	54
3.2.8. Statistical analysis	54
3.3. Results.....	54
3.3.1. Susceptibility profile of <i>C. auris</i> isolate to peptides and antifungals	54
3.3.2. Killing kinetics of peptides against <i>C. auris</i>	57
3.3.3. Peptides rapidly reduce the viability of <i>C. auris</i> cells.....	57
3.3.4. Combination activity of peptides with antifungal drugs	61
3.3.5. Peptides disrupt the <i>C. auris</i> membrane permeability	65
3.4. Discussion.....	68

3.5. Conclusion	71
3.6. References.....	72
Chapter 4: Antifungal activity of human antimicrobial peptides targeting apoptosis in <i>Candida auris</i>.....	80
4.1. Introduction	80
4.2. Materials and Methods.....	82
4.2.1. <i>Candida</i> strains and media.....	82
4.2.2. Antimicrobial peptides.....	82
4.2.3. Antifungal susceptibility testing.....	82
4.2.4. Preparation of protoplasts for cell death studies.....	83
4.2.5. FITC Annexin V/PI double labelling assay.	83
4.2.6. TUNEL (terminal deoxynucleotidyl transferase nick-end labeling) assay.....	84
4.2.7. Measurement of mitochondrial membrane potential/ membrane depolarization assay.....	84
4.2.8. Evaluation of cytochrome c release.	85
4.2.9. Evaluation of intracellular reactive oxygen species (ROS) accumulation.	85
4.2.10. Data analysis	85
4.3. Results.....	86
4.3.1. Susceptibility testing for peptides and antifungals.....	86
4.3.2. Effect of AMPs on PS externalization in <i>C. auris</i>	86
4.3.3. Effect of AMPs on DNA fragmentation in <i>C. auris</i>	89
4.3.4. Effect of AMPs on mitochondrial membrane potential.....	91

4.3.5. Effect of AMPs on cytochrome c release.....	92
4.3.6. Effect of AMPs on intracellular ROS overproduction.....	93
4.4. Discussion.....	94
4.5. Conclusion	98
4.6. References.....	100
Chapter 5: Anti-virulence and anti-efflux pump activity of synthetic defensins and histatin in <i>Candida auris</i>	106
5.1. Introduction	106
5.2. Materials and Methods.....	108
5.2.1 <i>Candida</i> strains, media and antimicrobial peptides.....	108
5.2.2. Effect of peptides on the virulence factors of <i>C. auris</i>	108
5.2.2.1. Viability assay	108
5.2.2.2. Adherence to epithelial cells assay	109
5.2.2.2. Proteinase assay	109
5.2.2.3. Biofilm formation assay.....	110
5.2.2.4. Mature biofilms assay.....	110
5.2.2.5. Confocal microscopy imaging of <i>C. auris</i> biofilms.....	111
5.2.3. Rhodamine-6G extracellular efflux assay.....	111
5.2.4. Rhodamine-6G intracellular accumulation assay.....	112
5.2.5. Quantitative Reverse Transcription PCR (RT-qPCR) assay.....	112
5.2.6. Cytotoxicity assay.....	113
5.2.7. Statistics.....	113

5.3. Results.....	113
5.3.1. Effect of peptides on <i>C. auris</i> cell viability	113
5.3.2. Inhibition of <i>C. auris</i> adherence to BECs.....	113
5.3.2. Inhibition of proteinase secretion in <i>C. auris</i>	115
5.3.3. Inhibition of the metabolic activity of <i>C. auris</i> biofilm cells.....	116
5.3.4. CLSM examination of biofilm.....	117
5.3.5. Inhibition of efflux pump activity in <i>C. auris</i>	118
5.3.6. Peptides downregulate the expression of genes linked to virulence and resistance of <i>C. auris</i>	120
5.3.7. Cytotoxicity assay.....	120
5.4. Discussion.....	123
5.5. Conclusion	126
5.6. References.....	127
Chapter 6: General discussion & clinical implications.....	133
6.1. General discussion.....	133
6.1.1. Effectiveness at low concentration for low toxicity and cost effectiveness.....	135
6.1.2. Multiple drug target to maximise benefit and for fast effectiveness.....	136
6.1.2.1. Targeting cell membrane.....	136
6.1.2.2. Targeting apoptosis.....	137
6.1.2.3. Targeting virulence factors.....	138
6.1.2.4. Targeting biofilm.....	138

6.1.3. Reversal of drug resistance mechanism	139
6.1.4. Enhanced efficacy with combination therapy.....	140
6.1.5. Minimum cytotoxicity.....	140
6.2. Recommendations and Clinical implications	141
6.3. References.....	143
Chapter 7: Conclusions, limitations & future research.....	149
7.1. Conclusion	149
7.2. Limitations & future research.....	150
 APPENDICES	
Appendix A: Ethical Clearance Certificate.....	151
Appendix B: Clinical isolates used in this study	152
Appendix C: Supplementary data for chapter 3.....	154
Appendix D: Supplementary data for chapter 4.....	157
Appendix E: Supplementary data for chapter 5.....	158

LIST OF FIGURES

Figure 2.1	Diagram illustrating the study outline.....	49
Figure 3.1	Time-kill curves for <i>Candida auris</i> following exposure to antimicrobial peptides.....	58
Figure 3.2	Effect of the antimicrobial peptides on cell count and viability of <i>Candida auris</i>	60
Figure 3.3	Confocal microscopy images of the effect of the tested peptides on <i>Candida auris</i> cells.	66
Figure 3.4	Confocal microscopy images of the effect of the tested peptides on <i>Candida auris</i> cells.....	67
Figure 4.1	Flow cytometric analysis of phosphatidylserine exposure using FITC Annexin V/PI double labelling assay	89
Figure 4.2	Confocal scanning fluorescence images represent DNA fragmentation caused by tested peptides in <i>Candida auris</i> cells as shown by TUNEL assay.....	90
Figure 4.3	The proportion of TUNEL positive cells represent DNA fragmentation caused by tested peptides in <i>Candida auris</i> cells.	91
Figure 4.4	The effect of the tested peptides on mitochondrial membrane potential of <i>Candida auris</i> as determined by JC-10 staining	92
Figure 4.5	The effect of the tested peptides on cytochrome c release from the mitochondria to the cytoplasm in <i>Candida auris</i>	93
Figure 4.6	The effect of the tested peptides on ROS production in <i>Candida auris</i> as determined by H2DCFDA staining.....	94

Figure 5.1	The effect of various concentrations of the tested peptides on cell count and viability of <i>Candida auris</i>	114
Figure 5.2	The effect of various concentrations of the tested peptides on <i>Candida auris</i> adherence to BECs.....	115
Figure 5.3	The effect of various concentration of the tested peptides on proteinase secretion of <i>Candida auris</i> using BSA plates.	116
Figure 5.4	The effect of various concentration of the tested peptides on <i>Candida auris</i> biofilm metabolic activity using MTT reduction assay.....	117
Figure 5.5	Confocal microscopy images of the effect of the tested peptides on forming and mature biofilm of <i>Candida auris</i>	119
Figure 5.6	The effect of various concentration of the tested peptides on efflux pump activity of <i>Candida auris</i> using Rhodamine-6G (R6G) efflux assay.....	120
Figure 5.7	The effect of various concentration of the tested peptides on efflux pump activity of <i>Candida auris</i> CAU-R strain using Fluorescence microscopy..	121
Figure 5.8	The effect of the tested peptides on gene expression of <i>Candida auris</i> CAU-R Strain, <i>CDR1</i> , <i>CDR2</i> , <i>MDR1</i> , <i>SAP3</i> , <i>SNQ2</i> , <i>PGA26</i> , <i>PGA7</i> and <i>PGA52</i> using RT-qPCR.	122
Figure 5.9	Cytotoxicity assay of the tested peptides by haemolytic activity.	122
Figure 6.1	Flow diagram of study on anti- <i>C. auris</i> activity of Synthetic AMPs (hBD-3, HNP-1 and His 5).....	142

LIST OF TABLES

Table 3.1	Antifungal activity of synthetic peptides against <i>Candida auris</i> isolates....	55
Table 3.2	Antifungal drug susceptibility profile of <i>Candida auris</i>	56
Table 3.3	Percentage reduction in viability of <i>Candida auris</i> cells following 4 h treatment with different peptides	59
Table 3.4	<i>In vitro</i> antifungal activity of peptides in combination with fluconazole (FLC) against <i>Candida auris</i>	62
Table 3.5	<i>In vitro</i> antifungal activity of peptides in combination with amphotericin B (AmB) against <i>Candida auris</i>	63
Table 3.6	<i>In vitro</i> antifungal activity of peptides in combination with caspofungin (CAS) against <i>Candida auris</i>	64
Table 4.1	Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of peptides and antifungals against <i>Candida auris</i> isolates ($n=3$).....	87
Table 4.2	The proportion of <i>Candida auris</i> cells showing early apoptosis, late apoptosis and necrosis after being exposed to 0.5 MIC, MIC and MFC of hBD-3, HNP- 1 and His 5, as determined by FITC Annexin V/PI double labelling assay ($n=3$).....	88

LIST OF ABBREVIATIONS AND ACRONYMNS

ABC:	ATP-binding cassette
AIF:	Apoptosis-inducing factor
ALS:	Agglutinin-like Sequence
AmB:	Amphotericin B
AMPs:	Antimicrobial peptides
ANOVA:	Analysis of Variances
AST:	Antifungal susceptibility testing
ATP:	Adenosine triphosphate
BEC:	Buccal Epithelial Cells
BMD:	Broth microdilution
BSA:	Bovine serum albumin
CAS:	Caspofungin
CDC:	The Centres for Diseases Control and Prevention
cDNA:	Complementary deoxyribonucleic acid
CFU/ml:	Colony Forming Units per millilitre
CLSI:	The Clinical and Laboratory Standards Institute
DMSO:	Dimethyl sulfoxide
FICI:	Fractional inhibitory concentration index
FLC:	Fluconazole
GPI:	Glycosylphosphatidylinisotol
H₂DCFDA:	2',7'-Dichlorofluorescein diacetate
H₂O₂:	Hydrogen peroxide

hBD:	Human beta-defensins
HNP:	Human neutrophil peptides
HPLC:	High performance liquid chromatography
HWP1:	Hyphal wall protein 1
MDR:	Multi-drug resistant
MFC:	Minimum Fungicidal Concentration
MFS:	Major facilitator superfamily
MIC:	Minimum inhibitory concentrations
MS:	Mass spectrometric
NAC:	Non-albicans <i>Candida</i>
NCCD:	Nomenclature Committee on Cell Death
PBS:	Phosphate buffer solution
PCD:	Programmed cell death
PI:	Propidium Iodide
PS:	Phosphatidyl-serine
ROS:	Reactive oxygen species
rpm:	Revolutions per minute
SAP:	Secreted Aspartyl Proteinase
SD:	Standard Deviation
SDA:	Sabouraud dextrose agar
SDB:	Sabouraud dextrose broth
TUNEL:	Terminal deoxynucleotidyl transferase nick-end labelling
Yca1p:	Yeast caspase-1

Chapter 1: Introduction and literature review

1.1. Introduction

Invasive fungal infections, particularly candidiasis, represent a serious health concern with a high risk of death (1). While *Candida albicans* endures as the most commonly isolated species, there actually has been an increasing worry about non-*albicans* *Candida* (NAC) species. These are becoming more frequent and pose a significant challenge because they are often resistant to existing antifungal drugs (2). Current options for treating *Candida* infections include azoles, echinocandins, and polyenes, however, the rise of drug-resistant NAC species, particularly *Candida auris*, poses a significant challenge (3). *C. auris* has garnered significant attention for causing severe hospital outbreaks with high morbidity and mortality rates worldwide (4). This multidrug-resistant (MDR) yeast exhibits intrinsic resistance to azoles, a commonly used antifungal class, and displays concerning trends of developing resistance to other available antifungal agents (5). This poses a substantial challenge in healthcare settings, highlighting the necessity for developing innovative antifungal strategies.

Given the limitations of conventional antifungals and the emergence of MDR fungi, antimicrobial peptides (AMPs) have evolved as an attractive alternative for developing innovative antifungal therapeutics (6). These naturally occurring biomolecules exhibit strong effectiveness against a variety of pathogens, which include viruses, bacteria and fungi (7). Among them, human defensins and histatins are particularly noteworthy for their potent antifungal properties against various human fungal pathogens, particularly different *Candida* species (8). These biomolecules employ various mechanisms to fight fungi, such as disrupting cell wall integrity, increasing membranes permeability, and interacting with intracellular targets, ultimately leading to fungal cell demise (9).

Alongside the discovery of new antifungal molecules, combination therapy has emerged as a promising approach in treating resistant fungal pathogens (10). This approach combines a well-established antifungal with another compound possessing antifungal action, aiming to prevent drug resistance emergence by utilizing lower dosages of both agents, thereby reducing potential side effects. It also enhances the therapeutic efficacy by targeting different fungal processes (11). Some AMPs have demonstrated encouraging synergistic effects in combination

with conventional antifungal agents towards various *Candida* species (12). This synergistic effect highlights their potential as valuable components in combination antifungal therapy.

Furthermore, induction of apoptosis, a regulated kind of cellular death, presents an attractive avenue for developing novel antifungal agents (13). Apoptotic cells exhibit distinct hallmarks that differentiate them from necrotic cells, including phosphatidylserine externalization, cytochrome c release from mitochondria and condensation of chromatin leading to DNA degradation (14). Conversely, necrotic cells exhibit ruptured membranes, releasing their contents and triggering inflammatory responses (14). Notably, distinct classes of AMPs have been shown to effectively induce apoptosis in *C. albicans*, highlighting its promise as a target for novel antifungal agents (6).

Another alternative strategy to combat fungal infections is targeting virulence factors, which are responsible for the pathogenicity and establishing the infection process (15). *C. auris* virulence is mediated by various factors, including adhesion, biofilm development and hydrolytic enzymes secretion (16). These factors could potentially be implemented as novel therapeutic targets for developing potent antifungal agents. AMPs have also demonstrated the ability to modulate fungal virulence by inhibiting the expression of key virulence characteristics (17). Furthermore, overexpression of efflux pumps has been suggested to establish a crucial role in drug resistance in *C. auris*. These transmembrane proteins actively expel antifungal drugs from the cell, reducing intracellular concentrations and efficacy (18). This highlights the importance of developing new strategies to combat these pumps.

This study assessed the antifungal efficacy of chosen individual AMPs and their combinations with commonly used antifungal drugs against *C. auris*. It further investigated their effects on membrane permeability of *C. auris*. Then, the three most active peptides were selected to study their potential to induce apoptosis in this pathogen. The impact of the active peptides on *C. auris* virulence factors (adhesion, proteinase secretion and biofilm formation), efflux pump activity and the expression of linked genes was also studied. The results provide promising evidence highlighting the potential of AMPs, both individually and in combination with existing drugs, as a novel strategy against fungal infections, including those attributed to MDR pathogens like *C. auris*.

1.2. Literature review

1.2.1. Candidiasis

Candidiasis, a fungal infection arising from an overgrowth of *Candida* species, represents a significant global health burden (19). These species, a diverse group of yeasts, are ubiquitous commensals colonizing various mucosal surfaces including the mouth, throat, gut, and vagina, as well as the skin. However, disruptions in host immunity or environmental factors can trigger their transformation into opportunistic pathogens (20). Candidiasis, a common hospital-acquired infection, exhibits a wide clinical spectrum, ranging from minimal mucosal involvement to severe, potentially fatal systemic dissemination (21). Invasive candidiasis is a serious fungal infection attributed to substantial fatalities of over 70%. This systemic infection can involve the bloodstream, heart, brain, eyes, bones, and other organs (1). While over 200 *Candida* species exist, only a select few, predominantly *C. albicans*, hold pathogenic potential for humans (22). Other NAC species are recognized as opportunistic pathogens in humans, such as *Candida glabrata*, *Candida parapsilosis* and *Candida krusei*. Compared to *C. albicans*, these species often exhibit resistance to antifungals commonly used for treating candidiasis (23). Recently, the detection of *C. auris* has prompted significant worries considering its propensity to cause serious infections with substantial mortality rates (24).

1.2.2. *Candida auris*

C. auris has been acknowledged as a noteworthy worldwide health menace, owing to its inherent and acquired resistance to different types of antifungals (18). It has been linked to severe disseminated diseases and hospital outbreaks in over 40 countries since its first recorded case, with high morbidities and mortalities (25). The rapid emergence of *C. auris* in many different countries, combined with its robust antifungal properties, has raised substantial concerns (26). Moreover, *C. auris* poses a diagnostic hurdle due to limitations in standard laboratory methods, often leading to misidentification and inappropriate patient management (18).

The growing concern surrounding *C. auris* has spurred the Centers for Disease Control and Prevention (CDC) to classify this yeast to be a global health hazard with pandemic potential in 2016. This designation highlights the urgency for immediate public health interventions to combat this pathogen (27). Furthermore, during the COVID-19 pandemic, several concerning *C. auris* outbreaks emerged in intensive care units globally (28-30). To address this, the World Health Organization (WHO) categorized it within the critical group of its first-ever global list

of concerning fungal pathogens in 2022 (31). All these challenges necessitate continuous monitoring of its virulence and highlight the critical urge for innovative and effective therapeutic strategies against *C. auris* infections.

1.2.2.1. Discovery of *C. auris*

The first ever infection with *C. auris* has been documented in 2009, after its detection in the outer ear canal secretions of a Japanese patient (32). In 2011, The first documented bloodstream infections (candidemia) with *C. auris* have been documented from South Korea, highlighting its potential for serious invasive infections (33). Since then, it has been established as an underlying cause of candidemia and other serious fungal infections in numerous countries worldwide (34). Subsequently, the CDC announced *C. auris* as a nationally notifiable disease in 2018 (35). Interestingly, four samples were retrospectively identified after being either misclassified as *Candida haemulonii*, or not recognized to the species level . One of these cases originated from a sample collected in 1996, further suggesting potential earlier presence of *C. auris* (24).

1.2.2.2. Risk factors for *C. auris* infections

This yeast exhibits a predilection for immunocompromised individuals and those with prolonged healthcare exposure. Notably, a high prevalence is observed in organ transplant recipients receiving immunosuppressive therapy. Additionally, patients with pre-existing comorbidities including diabetes mellitus, heart disease, chronic kidney diseases, malignancies and liver dysfunction are at increased risk (34). Further risk factors include recent broad-spectrum antibiotic usage, prolonged hospitalization, and a history of invasive medical procedures such as surgery and catheterization can also contribute to the acquisition of *C. auris* (36).

1.2.2.3. Prevalence of *C. auris* in South Africa

C. auris candidemia was first detected in South Africa in 2014. This prompted a retrospective analysis of archived isolates, leading to the identification of a previously unrecognized case from 2009 at a Johannesburg hospital (37). A nationwide surveillance study in South Africa revealed a concerning prevalence of *C. auris* infections. The yeast emerged as the third most frequent cause of candidemia, contributing to over 10% of all cases. Notably, the incidence was particularly elevated in private hospitals within the Gauteng area; it has been suggested that this situation is the result of excessive fluconazole prophylactic use (38). Furthermore, *C. auris* has exhibited a concerning trend of causing outbreaks within neonatal intensive care units, and

has become endemic in numerous South African hospitals, signifying its persistent presence within these healthcare settings (39, 40). To date, *C. auris* has already been identified among nearly 100 hospitals, with some facilities experiencing significant nosocomial outbreaks. This concerning trend has prompted the designation of *C. auris* as a priority pathogenic organism for ongoing surveillance in South Africa. This initiative aims to keep track of the development of antifungal resistance across various infection locations (41).

1.2.2.4. Phenotypic characteristics of *C. auris*

C. auris displays remarkable phenotypic versatility with regards to cellular morphological characteristics and nutrient utilization. Colonies appear differently on various culture media, with CHROMagar typically showing pale purple or pink colonies and Sabouraud Dextrose Agar showing smooth white to creamy in colour colonies. Sugar fermentation and assimilation patterns, along with nitrogen source utilization, further distinguish it from other *Candida* species (42). These traits were recently exploited to develop specific media for *C. auris* isolation. Interestingly, *C. auris* exhibits two distinct cellular morphologies (aggregating and non-aggregating). Aggregating cells resist separation by mechanical or chemical means, suggesting an inherent incapacity of daughter cells to detach following budding. Studies using *Galleria mellonella* models suggest that non-aggregating strains are more pathogenic compared to aggregating variants, possibly due to enhanced biofilm formation (43). Notably, *C. auris* exhibits thermotolerance, allowing growth at 42°C. Additionally, it possesses halotolerance, with documented growth observed at salt concentrations as high as 10%. These unique characteristics might contribute to its environmental persistence and potential spread. These variations can be attributed to strain differences and the geographical origin of the isolate, however, the capacity to thrive at high temperatures tends to be a consistent feature across *C. auris* strains globally (44).

1.2.2.5. Genomic characteristics of *C. auris*

Whole genome sequencing has revealed unique characteristics of *C. auris*. Its genome size and composition differ significantly from *C. albicans*. Interestingly, *C. auris* shares the closest genetic similarity with *Candida lusitanae*. *C. auris* clusters into five distinct genetic clades, each originating from a specific geographical region, including South Asia, East Asia, South America, South Africa, and Iran (45), and potentially a sixth clade in Singapore (46). This suggests these clades evolved independently under unique environmental pressures in their respective locations. The genetic variation between these clades is significant, while strains

within each clade are highly similar. Furthermore, the *C. auris* genome itself hints at the possibility that environmental factors may have influenced the development of antifungal resistance genes within this pathogen (47). Fundamental knowledge regarding the molecular processes underlying its phenotypic diversification and cell biology, however, is still lacking.

1.2.3. Antifungal drugs: mechanisms of action and resistance

Despite major advances in fighting infectious diseases with new drugs, developing effective antifungal agents has been slow. Ever since the first antifungal drug was discovered in 1950, only a handful of others have been developed over several decades. This is much slower progress compared to the frequent discovery of new antibiotics. One of the main reasons for this is that fungi share some similarities with humans on a cellular level. This makes it difficult to develop drugs that target the fungus without also harming human cells (48). This is a serious concern as fungal infections are becoming more common, and some fungal species, such as *C. auris*, are even developing resistance to multiple antifungals.

Additionally, there are only three primary groups of antifungals, the polyenes, azoles, and echinocandins, which limit treatment options. While some antifungal drugs, like azoles, are more tolerable in humans than others, the rise of antifungal resistance is making these treatments less effective (49). This lack of new drugs and growing resistance is a major concern as fungal infections become difficult to treat and can lead to serious complications and even death. Furthermore, these antifungal drugs often have limitations, like high toxicity or a narrow range of effectiveness, making them difficult to use in some cases (50). These current antifungals have been broadly categorized base upon their modes of action and targets.

1.2.3.1. Azoles

In clinical practice, azoles comprise the most popular type of antifungal drugs administered for both therapeutic and preventative purposes. They are generally less toxic than other antifungal drugs. There are two main categories of azoles: imidazoles and triazoles (51). Imidazoles have two nitrogens and include drugs like miconazole, clotrimazole, and ketoconazole. They are primarily used for mucosal infections but have limitations due to high toxicity and side effects. Triazoles have three nitrogens and encompass fluconazole, itraconazole, voriconazole, and posaconazole. Triazoles offer broader antifungal activity compared to imidazoles and are safer for use in both mucosal and systemic infections. They are generally preferred given their wide range of action and safety profile. However, a significant drawback of azoles is their fungistatic nature, meaning they inhibit fungal growth but may not necessarily kill the fungus. This

necessitates prolonged treatment, which can contribute to the development of drug resistance (52).

Azoles exert their antifungal effect by targeting a critical pathway for fungal cell membrane integrity, which is ergosterol biosynthesis. Ergosterol is a crucial sterol molecule that makes up the fungal cell membrane and serves as a regulator, preserving its permeability, elasticity, and overall structural integrity. Azoles target a specific enzyme called lanosterol 14 α -demethylase, encoded by the *ERG11* gene, which is essential for converting lanosterol to ergosterol. The altered balance of ergosterol and its precursors disrupts the proper functioning of the fungal cell membrane, ultimately hindering fungal cell growth and replication (53). Fluconazole is a particularly popular triazole, due to its low toxicity and effectiveness against various fungal infections. However, a growing concern is the increasing resistance among fungal pathogens to azole drugs (54). This resistance poses a significant challenge for healthcare providers in effectively treating fungal infections and selecting the most appropriate antifungal therapy.

Among the most prominent ways by which *C. albicans* develops resistance to azoles involves the upregulation of efflux pumps. These pumps actively efflux drugs out of the cell, reducing their intracellular levels and effectiveness. Two main families of efflux pumps are entailed in this resistance strategy. The ATP-binding cassette (ABC) transporters, encoded by the *CDR1* and *CDR2* genes, which are believed to have a broader substrate specificity, meaning they can expel a wider range of azole antifungals. And the Major facilitator superfamily (MFS) transporters, encoded by the *MDR1* and *MDR2* genes, which are particularly effective in expelling fluconazole, but not necessarily other azoles (55). The other mechanism by which *Candida* can develop resistance to azoles through *ERG11* gene mutations. These mutations affect the structure of the protein, making it harder for the azole drug to bind effectively. Overexpression of the *ERG11* gene can also lead to higher enzyme levels that require much higher drug concentrations for inhibition, again resulting in resistance (56, 57).

1.2.3.2. Polyenes

Polyenes represent a well-established antifungal drug class that has been used for over five decades to combat life-threatening invasive fungal infections. These drugs are derived from *Streptomyces* bacteria and belong to a category of molecules called amphipathic macrolides, consist of a long hydrophobic hydrocarbon chain and multiple hydrophilic hydroxyl groups (58). Polyenes exhibit fungicidal activity, meaning they directly kill fungi. They possess a wide

range of antifungal activity, effectively combating a diverse group of fungal pathogens. Despite their broad spectrum, only a few polyenes are currently used in clinical antifungal therapy. The most prominent member is amphotericin B, followed by nystatin (59).

Amphotericin B stands out as a broad-spectrum antifungal drug, effectively targeting a variety of invasive fungal infections like aspergillosis, candidiasis, and cryptococcosis. Its intravenous administration allows it to reach and combat fungal infections throughout the body. However, its potential severe side effects include, renal toxicity, hepatotoxicity, and neurotoxicity necessitate a careful evaluation of risks and benefits (60). Fortunately, advancements have resulted in developing lipid formulations of amphotericin B, which offer a crucial advantage of reduced toxicity compared to the traditional form. This makes them a more tolerable option for patients. However, their high cost can restrict their widespread use and availability (61).

For decades, the antifungal action of polyenes was believed that they directly adhere to ergosterol in the cell membrane, creating holes that leak cell contents. However, recent research has challenged this view, suggesting a "sterol sponge" model. In this model, polyenes form clumps outside the membrane, pulling ergosterol molecules out and disrupting the membrane's function, ultimately killing the fungus (62). Although very rare, certain species, like *C. albicans* and *C. glabrata*, can develop resistance to polyenes, which has been attributed to mutations in genes that regulate ergosterol production. These mutations can either decrease the ergosterol amount, making it a less effective target for the drug, or alter enzymes that interact with the drug, lowering its binding affinity (63). Interestingly, antifungal azoles can also indirectly contribute to polyene resistance by depleting ergosterol levels, making the fungus less susceptible to both drug classes (64).

1.2.3.3. Echinocandins

Echinocandins represent the latest group of antifungal agents which have been approved for medical usage. These drugs are amphiphilic lipopeptides, meaning they possess both hydrophobic and hydrophilic regions in their molecular structure. Echinocandins are natural products obtained through the fermentation of specific fungi (65). Currently, three agents have been approved to use in clinical applications, which are caspofungin, micafungin and anidulafungin. Echinocandins demonstrate strong antifungal activity against multiple *Candida* species, however, their effectiveness is limited towards specific species like *C. parapsilosis*. Despite their limited spectrum against some fungi, echinocandins are generally safe and well-

tolerated in humans. However, their low gastrointestinal absorption and short half-life necessitate intravenous administration (66).

Echinocandins target a critical fungal enzyme called 1,3- β -glucan synthase, which is required for synthesizing β -1,3-glucan, a key element of the fungal cell wall that provides structural strength. By inhibiting this enzyme, echinocandins effectively disrupt the cell wall formation process, ultimately leading to cell death (67). In addition, they may also indirectly affect the production of other cellular components like lanosterol and ergosterol while increasing the chitin content within the cell wall. This further disrupts fungal cell wall structure and function (53).

Candida species can develop resistance to this group by acquiring mutations in their *FKS* genes, the blueprint for the crucial enzyme 1,3- β -glucan synthase. The mutations typically occur in two specific regions of the genes: *FKS1* for most *Candida* species and *FKS2* in *C. glabrata*. These lead to reduced glucan synthase activity, thicker cell walls with abnormal structures, and ultimately treatment failure due to decreased drug susceptibility (68). Despite this resistance risk, echinocandins remain widely used in antifungal therapy due to their minimal interactions with other drugs and overall good safety profile.

1.2.3.4. Therapeutic options and resistance mechanisms in *C. auris*

C. auris is especially alarming given its strong resistance to antifungal drugs. Fluconazole resistance has been observed in up to 90% of *C. auris* isolates, between 20 and 50% are resistant to amphotericin B, and a smaller percentage (5-7%) are resistant to echinocandins (47). Therefore, echinocandins have been the preferred primary treatment for *C. auris* infections. However, the issue of echinocandin resistance developing during treatment has been a significant contributor to therapeutic failure, leaving no choices for treating these infections (69). Even more concerning is the emergence of strains resistant to multiple drugs, or even all antifungal therapeutics available. This shows much higher resistance than other *Candida* species and highlights the complex and concerning ability of *C. auris* to evolve and resist treatment.

Interestingly, resistance varies depending on the *C. auris* clade. Some clades are almost always resistant to fluconazole, while others are more susceptible. According to recent research, strains from the South Asian clade, known as Clade I, have the highest susceptibility to all antifungals, whereas isolates from the East Asian and South African clade, known as Clade II and III

respectively, exhibit strong resistance to most antifungals (40, 47). These variations are linked to alterations in the genes encoding efflux pumps or drug targets (70).

According to studies, *C. auris* has developed drug resistance mechanisms that are comparable to those of *C. albicans*. The overexpression of efflux pump has also been demonstrated to be a contributor in azole resistance in *C. auris*. Both ABC and MFS transporters have been identified in *C. auris* isolates (71), in which *CDR1* performs an integral part in resistance to triazole antifungals (72). This highlights the significance of these pumps in compromising the efficacy of azole drugs. Understanding these efflux pumps is crucial for overcoming azole resistance, improving antifungal treatment efficacy and developing more targeted antifungal therapies (73). In addition, mutations in the *ERG11* gene, particularly at Y132, K143 and F126 positions, have been attributed to fluconazole resistance in *C. auris*. Interestingly, the specific mutation observed seems to differ according to the geographical origin of the *C. auris* strain (74, 75).

For amphotericin B resistance, the exact mechanisms are still being studied, but it seems to involve changes in the cell membrane (76). Studies have shown that strains resistant to amphotericin B have a different genetic profile compared to non-resistant strains. Specifically, these resistant strains have higher activity of several genes (*ERG1*, *ERG2*, *ERG6*, and *ERG13*) involved in the production of ergosterol. This suggests that *C. auris* may be producing more ergosterol, potentially making the drug less effective (71). Regarding echinocandin resistance, studies on *C. auris* have revealed mutation in the *FKS1* gene as a potential mechanism, which is similar to the one known to cause echinocandin resistance in *C. albicans* (74). An animal model provided more evidence of the significance of this mutation (77).

1.2.3.5. Combination antifungal therapy

Combination approach has been used to combat severe fungal infections (78), which offers several advantages including reduction in the dosage needed for each individual drug, thereby increasing efficacy and lowering toxicity (79). Additionally, targeting multiple antifungal pathways simultaneously aligns with the understanding of diseases as complex processes with various contributing factors. This multipronged approach can also help slow down the emergence of resistance in fungal pathogen (80). The concept of combining antifungal drugs has emerged over 40 years ago. A case report indicated success with the flucytosine and amphotericin B combination for treating meningitis caused by *Cryptococcus neoformans* (81). This promising approach was supported by a landmark clinical trial that compared

amphotericin B monotherapy to a combination regimen with flucytosine for the same condition. The combination therapy resulted in a higher cure rate, fewer relapses, and surprisingly, lower kidney toxicity (82). For invasive candidiasis, the combination of echinocandin and azole antifungals has been used in clinics for a long time, however, clinical trials to verify its effectiveness as well as safety in patients are lacking (83). For *C. auris* infections, particularly those resistant to multiple drugs and posing treatment challenges, echinocandin antifungals are sometimes used in combination with amphotericin B. This combination approach aims to improve treatment efficacy against these stubborn fungal infections (84). Due to the challenges in treating *C. auris* infections, researchers are exploring combination therapies using existing antifungal drugs (85, 86), or combining antifungals with antibiotics (87, 88). These combinations have shown encouraging signs of synergistic activity. Our previous work has shown that combination of common antifungals with carvacrol, a natural compound found in oregano oil, has enhanced treatment efficacy, by substantially reducing the dosage of each individual drug (89). These combinations offer a promising treatment regime to overcome antifungal resistance and improvement in treatment efficacy.

1.2.4. Novel perspective in antifungal drug development

The rise of antifungal resistance and the restricted arsenal of antifungals pose a serious health threat. To address these challenges, researchers are actively exploring various strategies for developing new antifungal therapies. This includes developing new drugs that target established antifungal mechanisms or identifying novel targets within fungal cells (48). One promising approach involves targeting fungal virulence, anti-virulence agents aim to disarm fungi by hindering their ability to cause disease (90). Additionally, targeting the apoptotic cell death pathway within the fungus represents a novel and exciting approach. This strategy could bypass the mechanisms fungi use to resist traditional antifungals (91). Furthermore, investigating immunotherapy strategies like AMPs offers a promising new direction (92). By focusing on these novel targets and potentially combining these strategies, researchers can develop a fresh class of antifungal drugs to address the rising challenge of resistance.

1.2.4.1. Virulence factors

Pathogenicity refers to the inherent capacity of pathogens to cause disease or harm within a host. Commensal microbes and opportunistic pathogens lack this intrinsic ability. They can reside harmlessly within the host, and only cause illness under specific predisposing conditions (93). For instance, *C. albicans*, a common commensal yeast in humans, may convert into a

pathogenic organism in immunocompromised patients. Unlike most *Candida* species, *C. auris* is classified as an opportunistic pathogen that is not harmful to healthy individuals with a functioning immune system. However, *C. auris* can become virulent and cause infections in immunocompromised patients, particularly in healthcare settings (93).

The transformation of *C. albicans* from commensal to pathogenic yeast frequently involves activation of various virulence factors, which are biochemical and morphological changes that enhance the ability to cause disease. These virulence factors can encompass a range of mechanisms, allowing the microbe to evade the host, acquire nutrients, and ultimately cause damage. The key virulence characteristics of *C. albicans* include host recognition, adhesion, morphological switching, hydrolytic enzyme production, and biofilm development. These are crucial for colonization, immune system evasion, and tissue damage (94). Although extensive knowledge is available on *C. albicans* pathogenicity mechanisms, fewer studies have addressed the virulence capacity of NAC species, particularly *C. auris*. Few studies, including our previous study, have provided some insights on *C. auris* virulence factors (89, 95). To understand virulence factors in the emerging pathogen *C. auris*, researchers have used *C. albicans* as a reference. While detailed descriptions of virulence factors are lacking for *C. auris* and similar species, *C. haemulonii*, comparative genomic analysis between these and *C. albicans* allows for predictions.

Intriguingly, these factors hold promise as emerging drug targets for novel antifungal therapies (48). Targeting virulence factors puts less pressure on the fungus to evolve resistance mechanisms compared to traditional drugs that directly target growth. Drugs targeting virulence can utilize various points of action, which makes it harder for the fungus to acquire resistance. Moreover, by targeting virulence rather than fungal growth, these drugs are less likely to disrupt the natural microbiota (15). Therefore, focusing on virulence factors presents a promising strategy for developing effective antifungal treatments against *C. auris* infections.

1.2.4.1.1. Adhesion

Adherence to host tissues and surfaces is the crucial first step for fungal pathogens to establish colonization and infections (96). This initial attachment is well documented for *C. albicans* and other NAC species. These fungi can adhere to various host cells, including endothelium and epithelial cells, or even medical devices within the body (97). Importantly, adherence is also a crucial early step in biofilm formation, which is linked to increased virulence and antifungal resistance (98). *C. albicans* achieves adherence using specific cell wall proteins called

adhesins. Among these adhesins, the agglutinin-like sequence (ALS) family, which includes (Als1-7 and Als9), has been particularly well-studied. Each of the eight members of this class of glycosylphosphatidylinositol (GPI)-anchored cell surface proteins aids in adhesion. However, Als3 (hypha-associated adhesin) plays a particularly important role, with its expression being upregulated in oral and vaginal infections (99).

Another key adhesin is the GPI-linked hyphal wall protein (Hwp1). This protein interacts with mammalian transglutaminases, potentially forming a covalent bond between *C. albicans* and host cells (94). Notably, both Als3 and Hwp1 function as complimentary adhesins to promote the production of biofilms (100). Upon adherence to host, *C. albicans* can penetrate its cells through two main ways: induced endocytosis or active penetration. Induced endocytosis involves specific cell wall proteins including Als3 (which also functions in adherence) and Ssa1 (a heat shock protein). Active penetration, on the other hand, is thought to be facilitated by secreted hydrolases, particularly secreted aspartic proteases (Saps) (101).

While these virulence factors are well-characterized in *C. albicans*, limited research exists for *C. auris*. Several studies, including our previous work, have shown that *C. auris* exhibits decreased adherence ability to epithelial cells and catheter materials in comparison to *C. albicans*, suggesting potential differences in adhesion abilities (89, 102). Studies suggest that *C. auris* utilizes distinct strategies compared to other *Candida* species. The exact mechanisms, however, are still being explored (103). *C. auris* likely shares some adhesins with *C. albicans* based on genomic analysis, although their functions have not been fully characterized yet. A study indicates that Als3 is crucial for *C. auris* adherence, and antibodies targeting Als3 have been shown to prevent biofilm formation, highlighting its importance in adherence (104). Interestingly, *C. auris* lacks a counterpart for the Hwp1 adhesin found in *C. albicans*, which might be related to its inability to form true hyphae. However, the *IFF4* gene shows increased expression during initial stage of biofilm formation in *C. auris*, suggesting a potential role in adherence, which has a similar function in *C. albicans* (105). This highlights the need for further investigation to fully explore the specific mechanisms of adhesion in *C. auris* and develop effective antifungal strategies.

1.2.4.1.2. Morphogenesis

C. albicans undergoes a well-studied and tightly controlled transformation called morphogenesis, where it reversibly switches between its yeast form and hyphal forms, both true and pseudohyphae. This switch is governed by multiple transcriptional factors and relies

on various biochemical pathways (106). The capacity to change between these two forms is regarded as a critical virulence characteristic in *C. albicans*, making it a key target for research into novel antifungal drugs (90). Notably, both forms are essential for *C. albicans* pathogenicity, each playing distinct roles in establishing infections. While the yeast form is primarily responsible for dissemination, allowing the fungus to spread within the host, hyphal growth is crucial for breaching host barriers, invading tissues, and causing damage (107). Interestingly, it has been demonstrated that yeast cell adhesion is essential for subsequent hyphal formation (108). Furthermore, hyphal formation triggers the expression of various virulence genes, include those encoding Als3, Hwp1 and Sap4-6 (109).

C. albicans and *Candida dubliniensis* are distinct among *Candida* species in their capacity to form both true hyphae and pseudohyphae (110). In contrast, other pathogenic species like *C. parapsilosis*, *C. tropicalis*, and *C. haemulonii* are restricted to pseudohyphal growth (110). *C. auris* generally lacks the ability to form pseudohyphae, germ tubes, chlamydoconidia, or chlamydospores (44). However, limited studies suggest rudimentary pseudohyphae formation might occur in some strains, suggesting potential strain-specificity or environmental influences warranting further investigation (111). Unlike *C. albicans*, which transitions to hyphae with serum exposure, *C. auris* isolates from China exhibited morphological changes in response to high salt concentrations (10% NaCl) or passage through an animal model. Notably, these studies describe "filamentous growth" or "pseudohyphal-like forms" rather than true hyphae due to morphological differences between the two (112). These findings suggest that *C. auris* morphogenesis might be more complex than previously thought, warranting further investigation. Nevertheless, its higher virulence despite the lack of these structures remains unclear.

1.2.4.1.3. Hydrolytic enzymes

C. albicans produces several kinds of hydrolytic enzymes that are crucial for its virulence. These enzymes play a multifaceted part in various aspects of its pathogenic behaviour (113). They contribute to adherence by aiding the attachment to host cells, facilitate invasion and penetration by breaking down host cell components, and might even interfere with the host immune system. Notably, these enzymes are essential for the absorption of nutrients as well, as their degradative abilities convert complex materials in host tissue into usable nutrients for *Candida* growth and survival. The most well-studied classes of enzymes include secreted

aspartyl proteinases and phospholipases (114). Other enzymes like haemolysin, coagulase, and lipase also play a role in *C. albicans* pathogenicity (115).

Secreted aspartyl proteinases (SAPs) are a particularly important class of hydrolytic enzymes secreted by *C. albicans* and other related species. These enzymes are key contributors to *C. albicans* virulence in various ways. During infection, proteinases play a role in adhesion by disrupting host epithelial and mucosal membrane components, facilitating *Candida* attachment to host cells (116). They also inflict damage on host tissues by hydrolyzing various proteins like immunoglobulins, albumin and haemoglobin, compromising the host innate immune defences. Additionally, proteinases might be involved in morphogenesis and hyphal formation in *C. albicans* (117). This multifaceted role highlights the importance of SAPs in *Candida* pathogenesis.

C. albicans possesses a family of ten genes encoding SAPs, named *SAP1-10*. They play diverse roles throughout the course of the infection (118). Interestingly, their expression varies depending on the fungal form. The yeast form exhibits significant expression of *SAP1-3*, while *SAP4-6* are more prominent during the hyphal form. *SAP9* and *SAP10*, however, are expressed in both forms (119). The exact contribution of individual *SAPs* to pathogenesis remains under investigation due to variations in their expression during infection. Among them, *SAP4-6* appear to be particularly important for virulence (120). Overall, the *SAPs* family exhibits a complex and dynamic expression pattern, with each member likely playing a particular position in different stages and sites of *C. albicans* infection.

While SAPs are well-characterized virulence factors in *C. albicans*, their presence extends to other *Candida* species. *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis* and even *C. auris*, have also been shown to exhibit these enzymes (116). The production of proteinases in *C. auris* remains under investigation. While some studies report strong enzyme activity (121), others, including our previous study, found that proteinase activity might vary among *C. auris* strains (89, 102), potentially influencing their virulence. Interestingly, *C. auris* demonstrates significantly higher SAPs activity at 42°C in contrast to *C. albicans*, suggesting a potential advantage for maintaining virulence at elevated temperatures (122). *C. auris* also possesses four *SAPs* orthologs, *SAP3* was the most prominent, making proteinases the largest enzyme group in its genome (123, 124). This highlights the need for further research on proteinase secretion in *C. auris* and NAC species to understand their role in pathogenesis and to identify potential targets for antifungal therapies.

Phospholipases are another key virulence factor secreted by *C. albicans* and other fungal pathogens. These enzymes damage host cell membranes by hydrolyzing the phospholipid molecules, facilitating adherence and invasion into host tissues (125). Phospholipases classified into four groups (A, B, C and D) based on their phospholipid cleavage sites. However, only class B phospholipases (PLB) are considered significant virulence factors. These PLBs, specifically PLB1-5, have been suggested to have a critical role in pathogenesis by disrupting host cell membranes (126).

Furthermore, *C. auris* has also been shown to produce phospholipases. These enzymes, however, seems to be less prevalent. While *C. albicans* exhibits the greatest phospholipase production among pathogenic *Candida* species (127), *C. auris* appears to have a reduced and strain-dependent ability to produce these enzymes (102). Our previous study also demonstrated that none of our *C. auris* isolates secreted phospholipase (89). This suggests that proteinase production might be more consistent across *C. auris* strains, while phospholipase activity might be more variable. Despite this variation, the production of a diverse array of enzymes likely contributes to the pathogenesis of *C. auris* infections. However, further investigations are required to evaluate the degree to which phospholipases contribute to *C. auris* virulence and pathogenicity.

1.2.4.1.4. Biofilm formation

Candida species possesses another key virulence characteristic which is the capacity to establish biofilms on both living and non-living surfaces. These biofilms are organized populations of fungal cells that adhere to a surface and encased in an extracellular matrix (128). Crucially, microbial cells within a biofilm exhibit distinct properties compared to the free-floating (planktonic) cells. These biofilm-associated cells often exhibit increased resistance to antifungals and host immune defences. This heightened resistance is a major concern, particularly as biofilm formation is associated with increased mortality rates in hospitalized patients using medical devices (129).

C. albicans reigns supreme in biofilm formation in comparison to other *Candida* species, including *C. auris*. Studies have consistently shown its superior ability to create these structured communities (127). Conversely, *C. auris* forms biofilms to a considerably lower degree compared to *C. albicans*, but biofilm formation in *C. auris* is still more substantial than in *C. glabrata* (130). Interestingly, these observations are reflected in the physical structure of the biofilms. *C. albicans* biofilms are tightly clustered with yeast and hyphal cells enclosed in

a substantial extracellular matrix. In contrast, *C. auris* biofilms exhibit a more modest amount of extracellular matrix, primarily containing budding yeasts with occasional pseudohyphae. And *C. glabrata* forms the least impressive biofilm composed of a thin film of yeast cells devoid of any extracellular matrix (130).

Furthermore, a study using transcriptome analysis of developing *C. auris* biofilms revealed that genes involved in adhesion, specifically GPI-anchored cell wall genes (*HYR3*, *CSA1*, *PGA7*, *IFF4*, *PGA26*, and *PGA52*), have elevated expression throughout all phases of biofilm formation. In addition, as the biofilm matures, the transcription of genes that encode efflux pumps belonging to the ABC family (*CDR1*, *SNQ2*, and *YHD3*) and the MFS transporters (*MDR1* and *RDC3*) increases as well (105). This suggests that adherence and efflux pump activity play key roles in both the early stages of biofilm development and its subsequent maturation. Alarming, *C. auris* biofilms exhibit lower susceptibility to antifungals like caspofungin and amphotericin B in comparison to *C. albicans* biofilms (130). Therefore, developing novel drugs that can either hinder the formation of biofilms or penetrate existing biofilms to reach the fungal cells is an urgent priority.

1.2.4.2. Types of cell death in yeast

Cell death is defined as the irreversible cessation of life functions due to a breakdown in internal organization. For a cell to be considered truly dead, it must pass a "point of no return" where critical changes have occurred. This typically involves degradation of the plasma membrane, complete disintegration including the nucleus, or engulfment by neighbouring cells. The Nomenclature Committee on Cell Death (NCCD) suggested a categorization system based on both morphological features and measurable biochemical/molecular characteristics. They recommend using at least two detection methods to definitively classify a specific cell death event, which can be broadly classified as controlled forms like apoptosis and autophagy, and uncontrolled forms like necrosis (131).

1.2.4.2.1. Apoptosis in yeast

Apoptosis, a controlled form of cell death crucial for maintaining balance in multicellular organisms, can also occur in yeast cells. The discovery of yeast apoptosis in 1997 using *Saccharomyces cerevisiae* involved studying mutants of the *CDC48* gene. These mutants displayed key markers of apoptosis typically observed in mammalian cells (132). Further research revealed that these mutations might trigger a form of apoptosis involving the mitochondria (133). Since then, researchers have identified numerous yeast apoptosis

regulators and pathways with similarities to mammalian systems. This makes yeast an ideal model system to explore the process of programmed cell death (PCD) and developing antifungal drugs that target fungal apoptotic regulators, potentially minimizing side effects on human cells.

During PCD in yeast, cells exhibit several hallmarks reminiscent of mammalian apoptosis (134). These markers include:

- Phosphatidylserine (PS) externalization: PS normally located on the interior surface of the cell membrane, however, it gets externalized on the outside surface during PCD.
- Mitochondrial depolarization: The electrical potential across the mitochondrial membrane decreases, disrupting energy production and potentially triggering further cell death events.
- Cytochrome c: Its release from the mitochondria is an important checkpoint in the intrinsic apoptotic pathway. It acts as a signal for caspase activation, initiating the cell death cascade.
- Reactive oxygen species (ROS) accumulation: PCD can be accompanied by increased levels of ROS, which can damage cellular components.
- Chromatin margination: The genetic material (chromatin) condenses and aggregates at the nuclear periphery.
- Nuclear fragmentation: The nucleus breaks down into smaller fragments, a characteristic late-stage event in PCD.
- DNA degradation: The genetic material itself undergoes breakdown, a hallmark of cell death.

Yeasts share some key apoptotic regulators with mammalian cells. Bax, a pro-apoptotic member of the Bcl-2 group in humans, is also present in yeast and can trigger apoptosis by altering mitochondrial membrane permeability, leading to cytochrome c release (135). Interestingly, mutant forms of the anti-apoptotic protein Bcl-xL can block cell death induction in yeast (136). Yeast caspase-1 (Yca1p), the sole essential caspase in yeast apoptosis, functions similarly to mammalian caspases. During oxidative stress, cells lacking Yca1 exhibit diminished formation of apoptotic markers (137). Further evidence for conservation of apoptotic machinery comes from the presence of an AIF (apoptosis-inducing factor) homology

in yeast (AIF1). Similar to its mammalian equivalent, AIF1 migrates from the mitochondria to the nuclei during apoptosis due to factors like hydrogen peroxide, acetate, and aging (138). Notably, AIF in both yeast and mammals plays a dual role, being essential for both apoptosis and optimal oxidative phosphorylation.

Apoptosis involves intricate mechanisms operating through two main pathways: extrinsic and intrinsic. While distinct, these pathways can influence each other. Yeast cells can undergo apoptosis in response to multiple stimuli, which can be categorized as either extrinsic (exogenous) or intrinsic (endogenous). These stimuli trigger distinct signalling cascades that ultimately result in characteristic features of programmed cell death (91). Understanding these pathways and their triggers is crucial for further research into yeast programmed cell death.

The extrinsic apoptotic pathway in yeast cells can be activated by a diverse range of external stressors and chemicals. These include hydrogen peroxide, acetic acid, hypochlorous acid, ethanol, ultraviolet radiation, salt stress, oxidative stress, and heat stress (139). Interestingly, Yca1 seems to be crucial to this process. Strains lacking Yca1 exhibit increased tolerance to external stressors, while overexpression of Yca1 enhances apoptosis induced by these agents (140). Similarly, hydrogen peroxide and acetic acid can trigger apoptosis-like cell death with characteristic nuclear markers, but this can be prevented by either producing the anti-apoptotic protein Bcl-xl or deleting Yca1 (141). Other stressors like hypochlorous acid induce apoptosis by generating harmful oxygen radicals within the cell (142). Notably, hyperosmotic stress has also been shown to induce a caspase-dependent, partially cytochrome c-mediated apoptotic-like pathway (143). This highlights the diverse range of external stimuli that can trigger the extrinsic apoptotic pathway in yeast and the complex interplay between various cellular components during this process.

Furthermore, yeast cells can undergo apoptosis when exposed to low doses of their mating pheromone, when mating is unsuccessful or a suitable partner is missing (144). The pheromone binds to its receptor, activating a signalling pathway that increases intracellular calcium and mitochondrial activity. This is followed by the release of cytochrome c, a key enzyme in the electron transport chain located on the outer mitochondrial membrane, which is frequently linked to yeast apoptosis, with its release serving as a marker for this process (145). Moreover, amphotericin B has been demonstrated to directly trigger apoptosis in *C. albicans* (146). Its antifungal activity seems to be linked to its ability to increase caspase activity. Studies further

support this connection by showing that caspase inhibitors can protect *Candida* cells from its effects (147).

The intrinsic apoptotic pathway in yeast is triggered by various internal signals independent of cell surface receptors. These include disruption in protein modification, chromosome stability, mRNA stability, and issues with replication (148-150). Unlike the extrinsic pathway, this route relies heavily on mitochondrial function. The significance of mitochondria in yeast apoptosis is well-established (151). During intrinsic apoptosis, oxidative stress triggers the creation of the mitochondrial permeability transition pore, facilitating the release of cytochrome c and other pro-apoptotic factors like Bax, AIF1 and Yca1p. These factors then activate a cascade of processes resulting in cell death (91, 138).

In yeast, mitochondria are central to both generating and responding to ROS, which are known triggers of apoptosis and necrosis (152). Studies suggest that ROS acts as a key initiator, rather than simply a byproduct of yeast apoptosis. ROS accumulation damages proteins, nucleic acids, and lipids, pushing the cell towards either apoptosis or necrosis depending on the concentration (153). AIF1, Yca1p, and other key apoptosis-inducing factors are also involved in ROS-mediated cell death. Interestingly, ROS can directly activate the metacaspase-dependent apoptotic pathway, highlighting its central role in this process (154).

The ability to induce apoptosis in fungi, particularly yeasts, holds significant promise for antifungal drug development. Several existing antifungal drugs, including amphotericin B and ciclopirox olamine, are known to trigger apoptosis in yeast. However, these drugs often exhibit high human toxicity (155). To address this challenge, researchers are actively searching for novel antifungal molecules that can induce apoptosis in yeast with minimal to no side effects in humans. This focus on apoptosis-inducing antifungals could lead to the discovery of targeted and safer agents to combat fungal infections.

Numerous studies have explored the potential of natural products as antimicrobial peptides that induce apoptosis in yeast. These include compounds like lycopene, psacotoxin, plant defensin RsAFP2, human lactoferrin and melittin (156-160). While initial studies exploring natural products as antifungal agents are promising, further in-depth studies are needed to develop new, targeted antifungal drugs that specifically exploit the apoptotic pathway in yeast and potentially other fungi (161).

1.2.4.2.2. Necrosis in yeasts

Necrosis is a type of cell death that differs from apoptosis and has unique morphological features (162). Necrotic cells exhibit swelling of organelles and the entire cell and ultimately burst, releasing their contents. They also rapidly lose energy, suffer random DNA damage, and trigger inflammation in surrounding tissues by attracting immune cells like macrophages and neutrophils. This inflammatory response also involves the release of signalling molecules called cytokines (139). Because of these characteristics, necrosis was previously known as accidental uncontrolled cell death. However, recent research suggests a more controlled form of necrosis called necroptosis. This distinction is important because it suggests necrosis can be a programmed process, not just random cell damage. Necroptosis plays a role in various diseases and is triggered by factors similar to those causing apoptosis, but at much higher concentrations. These factors include hydrogen peroxide, acetic acid and some pheromones (163). While the various factors contributing to necrosis are being identified, the exact mechanisms and pathways of necrosis are still under debate. Various cellular components and processes are potentially involved, including mitochondrial dysfunction, nuclear alterations and ROS production. Identifying necrosis is still challenging because of the lack of precise biochemical hallmarks. However, researchers often identify it by the absence of markers specific to apoptosis like PS exposure (131).

Distinguishing between necrosis and apoptosis is crucial because these cell death pathways can occur independently, sequentially, or even simultaneously. In many cases, the type of cell death triggered (necrosis or apoptosis) depends on the specific stressor and its intensity. Factors like heat, radiation, lack of oxygen and certain medications can all influence which cell death pathway gets activated (164).

1.2.5. Antimicrobial peptides

Antimicrobial peptides (AMPs) are natural molecules produced by various living things and humans as an element of their innate immunity, acting as a primary phase of defence against invading pathogens (165). They were discovered in 1939, and there are now over 3,000 known AMPs, according to the Antimicrobial Peptide Database (APD), have been identified in various organisms (166, 167). These AMPs exhibit a wide spectrum activity targeting bacteria, fungi, viruses, and also cancer cells (168). Notably, they often have minimal toxicity to human cells, making them attractive candidates for future antimicrobial therapy. Beyond directly killing microbes, AMPs can also indirectly boost the immune system in higher organisms. They can

influence wound healing and modulate the adaptive immune response, which helps the body create targeted defences (169). AMP production is typically concentrated in areas with high exposure to the environment, like skin, airways, and digestive tract in animals and the outer cell layer in plants. Interestingly, some AMPs are constantly produced for baseline protection, while others are produced only when needed in response to infection or inflammation (170). This adaptability allows organisms to deploy AMPs strategically for optimal defence.

AMPs are surprisingly diverse in their molecular structure despite their functional similarity. Most AMPs are short, ranging between 12 to 54 amino acids long, and have a positive charge at neutral pH. A common feature is their amphipathic nature, meaning they have both hydrophobic and hydrophilic regions, which allows them to interact with target membranes (171). There are four main classes of AMPs based on their structure: α -helical, β -sheet, cyclic, and residue-rich peptides (172). Alpha-helical AMPs are the most common and often fold into amphiphilic helices upon encountering membranes, including cathelicidins, cecropins, magainins, and melittin (173). Beta-sheet AMPs with disulfide bridges, most commonly defensins, have a well-defined structure stabilized by these bonds (174). Cyclic AMPs, like gramicidin S and cyclotides, have their peptide backbone cyclized for increased stability (175). Finally, residue-rich AMPs are defined by an abundance of a specific amino acid, such as tryptophan in indolicidin, histidine in histatin, and proline in arasin 1 (172). This structural diversity highlights the remarkable adaptability of AMPs in their fight against microbes.

AMPs are not only found in various organisms but also play a crucial role in human defence against microbial infections. These peptides are produced in multiple tissues and surfaces exposed to the environment, including skin, eyes, airways, and the digestive tract. The human AMP family is diverse, with over 100 identified members in the APD. These peptides exhibit a wide range in length (10-149 amino acids) and net charge (-3 to +20). On average, human peptides are typically longer as well as higher in positive charge compared to peptides from organisms. This difference can be attributed to the presence of larger, highly charged human proteins. This diversity in sequence translates to a variety of structures and functions for human AMPs, highlighting their multifaceted role in human health (176).

The concept of antimicrobial substances likely has a long history, but the scientific exploration began much later. While human lysozyme, discovered in saliva in 1922, is considered the first identified antimicrobial protein (177), a surge in AMP research came in the 1980s, with the isolation and characterization of numerous AMPs (178). This progress was driven by two key

methods: chromatographic techniques for isolating and characterizing new peptides, and later, bioinformatics approaches that use sequence patterns to identify AMPs within genomes (176).

The initial understanding of AMP activity focused solely on their ability to directly disrupt microbial membranes, leading to cell death. However, research has revealed intricate modes of action for several antifungal peptides. These peptides may interact with the microbial cell surface and target various intracellular processes, including the generation of harmful ROS, disruption of mitochondria and essential organelles, triggering apoptosis, and hindering the cell cycle, ultimately leading to microbial cell demise (165). This study focuses on two major families of human AMPs, including human defensins and human histatins.

1.2.5.1. Human defensins

Defensins are typically created as inactive forms that get processed by proteases into their mature active forms. In 1985, a family of developed alpha-defensins has been discovered in human blood, naming them human neutrophil peptides 1-3, HNPs 1-3, according to their length, characteristics, and origin (179). These three defensins show extremely comparable amino acid arrangements, with HNP-1 and HNP-3, differing from HNP-2 by a single extra amino acid. A fourth member, HNP-4, was discovered in 1989 using chromatographic purification methods (180). Notably, all HNPs have demonstrated *in vitro* activity against *C. albicans* and certain bacteria like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E. coli* and *Streptococcus faecalis*. Studies further revealed that alpha-defensins are primarily found in bone marrow but might also be found in leukocytes, spleen, and thymus (180).

Human beta-defensins (hBDs) emerged as a distinct AMP family in the 1990s (180). Unlike alpha-defensins, beta-defensins have a unique pattern of six bridges and a relatively longer pattern for an extra spiral area. The first member, hBD-1, was isolated from human plasma in 1995 and primarily produced in the kidneys (181). Following that, hBD-2 was isolated from psoriatic lesion and showed effectiveness against *C. albicans* and various bacteria (182). The discovery of hBD-3 and hBD-4 followed in 2001, with hBD-3 notably retaining activity against *C. albicans* and other bacteria such as *S. aureus* and *Enterococcus faecium* (183). Bioinformatic tools based on disulfide bond patterns aided in the identification of hBD-3 and revealed a vast potential for novel AMPs. This resulted in the discovery of an additional 28 human beta-defensin genes. Indeed, several of these predicted human beta-defensins have been confirmed to exhibit antimicrobial activity, highlighting the power of this approach for AMP discovery in any species with a sequenced genome (184).

The antifungal activity of several AMPs involves interaction with specific fungal targets. It has been demonstrated that hBD-2 and hBD-3 interact with a particular phospholipid called phosphatidylinositol 4,5-bisphosphate of the fungal cell membrane, resulting in increased permeability (185, 186). In addition, studies have shown that both beta-defensin and HNP-1 target heat shock proteins (Ssa1p and Ssa2p) located in the cell membrane of *C. albicans* as part of their antifungal mechanism (187). Moreover, it has been demonstrated that a number of AMPs, including hBD-2, cause apoptosis in eukaryotic cells, as this process is characterized by the appearance of PS on the cell surface (188). ATP efflux is another mechanism employed by some AMPs to kill fungal cells. hBD-2, and hBD-3, for instance, have been shown to induce ATP release from yeast cells with no significant membrane disruption (189). Furthermore, alpha and beta-defensins have also been demonstrated to exhibit inhibitory activity against *C. albicans* and *C. neoformans* biofilms (17).

1.2.5.2. Human histatins

Histatins, a family of AMPs abundant in the amino acid histidine, were first discovered in human saliva in 1988 (190). Histatins 1, 3, and 5 emerged as key players in the human immune system due to their antifungal activity against *C. albicans* and other NAC species (191), and some antibacterial properties (192). Beyond their direct antimicrobial activity, all three histatins contribute to the creation of a barrier on the tooth enamel, which aids in preventing colonization by microbes and promotes healthy mineral interactions (193). Among them, histatin 5 is well-studied for its strong antifungal efficacy against *C. albicans* and other medically relevant fungal pathogens like *C. neoformans* and *Aspergillus fumigatus* (193). Histatin 5 can inhibit *C. albicans* growth and prevent its transformation into its more virulent hyphal form (194).

Regarding their mechanism of action, recent research suggests that salivary histatin 5 binds to both β -glucan and heat shock proteins (Ssa1p and Ssa2p) in *C. albicans* (195). However, our understanding of antifungal mechanisms of AMPs has evolved beyond simple pore formation in the fungal membrane. Many AMPs appear to have more intricate modes of action, histatin 5, for instance, can be internalized by the fungus without causing immediate membrane disruption. This internalization can occur through three distinct pathways including endocytosis, which is energy-dependent transport via specific proteins, or even through membrane lysis at higher concentrations (196). Furthermore, some AMPs, including histatin 5, trigger the production of ROS within the fungus. An excessive buildup of ROS inside the cell can lead to oxidative damage to various cellular components like proteins, DNA, lipids,

membranes, and organelles (197). In *C. albicans*, histatin 5 triggers a significant drop in ATP levels inside the cells by causing it to efflux from the cell (198). Moreover, this peptide has been shown to reduce the biofilm metabolic activity of *C. albicans* and *C. glabrata* (199).

The human AMPs offer a promising starting point for developing new therapeutics. Given the long evolutionary history of AMPs and their effectiveness against resistant microbes, they serve as attractive templates for novel antifungal.

1.3. Justification of the study

The rise of MDR *C. auris* infections necessitates the exploration of novel antifungal therapies due to the ineffectiveness and limitations of existing drugs. These new agents should ideally target *C. auris* through multiple mechanisms, potentially including apoptosis induction and virulence factor inhibition. With a focus on broad-spectrum activity, minimal toxicity, and the possibility of combination therapy, the development of such innovative antifungal agents holds immense promise for combating the growing threat of MDR *C. auris* infections. This study addresses this critical need by investigating human AMPs as potential antifungal agents against *C. auris*. AMPs possess many of these desired characteristics and warrant further investigation for their potential as future antifungal agents.

1.4. References

1. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. Nat Rev Dis Primers. 2018;4(1):18026.
2. Sadeghi G, Ebrahimi-Rad M, Mousavi SF, Shams-Ghahfarokhi M, Razzaghi-Abyaneh M. Emergence of non-*Candida albicans* species: epidemiology, phylogeny and fluconazole susceptibility profile. J Mycol Med. 2018;28(1):51-58.
3. Frías-De-León MG, Hernández-Castro R, Vite-Garín T, Arenas R, Bonifaz A, Castañón-Olivares L, et al. Antifungal resistance in *Candida auris*: molecular determinants. Antibiotics (Basel). 2020;9(9):568.
4. Egger NB, Kainz K, Schulze A, Bauer MA, Madeo F, Carmona-Gutierrez D. The rise of *Candida auris*: from unique traits to co-infection potential. Microb Cell. 2022;9(8):141-144.
5. Al-Rashdi A, Al-Maani A, Al-Wahaibi A, Alqayoudhi A, Al-Jardani A, Al-Abri S. Characteristics, risk factors, and survival analysis of *Candida auris* cases: results of one-year national surveillance data from Oman. J Fungi (Basel). 2021;7(1):31.
6. Buda De Cesare G, Cristy SA, Garsin DA, Lorenz MC. Antimicrobial peptides: a new frontier in antifungal therapy. mBio. 2020;11(6):e02123-20.
7. Galzitskaya OV. Creation of new antimicrobial peptides. Int J Mol Sci. 2023;24(11):9451.
8. Moretta A, Scieuzo C, Petrone AM, Salvia R, Manniello MD, Franco A, et al. Antimicrobial peptides: a new hope in biomedical and pharmaceutical fields. Front Cell Infect Microbiol. 2021;11:668632.
9. Li X, Zuo S, Wang B, Zhang K, Wang Y. Antimicrobial mechanisms and clinical application prospects of antimicrobial peptides. Molecules. 2022;27(9):2675.
10. Shapiro RS, Gerstein AC. Powering up antifungal treatment: using small molecules to unlock the potential of existing therapies. mBio. 2023;14(4):e0107323.
11. Belanger ES, Yang E, Forrest GN. Combination antifungal therapy: when, where, and why. Curr Clin Microbiol Rep. 2015;2(2):67-75.

12. Bondaryk M, Staniszewska M, Zielińska P, Urbańczyk-Lipkowska Z. Natural Antimicrobial peptides as inspiration for design of a new generation antifungal compounds. *J Fungi (Basel)*. 2017;3(3):46.
13. Yeaman MR, Büttner S, Thevissen K. Regulated cell death as a therapeutic target for novel antifungal peptides and biologics. *Oxid Med Cell Longev*. 2018;2018:5473817.
14. Gonçalves AP, Heller J, Daskalov A, Videira A, Glass NL. Regulated forms of cell death in fungi. *Front Microbiol*. 2017;8:1837.
15. Ahmad Khan MS, Alshehrei F, Al-Ghamdi SB, Bamaga MA, Al-Thubiani AS, Alam MZ. Virulence and biofilms as promising targets in developing antipathogenic drugs against candidiasis. *Future Sci OA*. 2020;6(2):Fso440 -0027.
16. Ahmad S, Alfouzan W. *Candida auris*: epidemiology, diagnosis, pathogenesis, antifungal susceptibility, and infection control measures to combat the spread of infections in healthcare facilities. *Microorganisms*. 2021;9(4):807.
17. Oshiro KGN, Rodrigues G, Monges BED, Cardoso MH, Franco OL. Bioactive peptides against fungal biofilms. *Front Microbiol*. 2019;10:2169.
18. Sanyaolu A, Okorie C, Marinkovic A, Abbasi AF, Prakash S, Mangat J, et al. *Candida auris*: an overview of the emerging drug-resistant fungal infection. *Infect Chemother*. 2022;54(2):236-246 8.
19. Jorge Alberto C, Ivohne Fernanda C. Invasive candidiasis: epidemiology and risk factors. fungal infection. *IntechOpen*; 2018. p. Ch. 3.
20. Parslow BY, Thornton CR. Continuing shifts in epidemiology and antifungal susceptibility highlight the need for improved disease management of invasive Candidiasis. *Microorganisms*. 2022;10(6):1208.
21. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev*. 2007;20(1):133-163.
22. d'Enfert C, Kaune AK, Alaban LR, Chakraborty S, Cole N, Delavy M, et al. The impact of the fungus-host-microbiota interplay upon *Candida albicans* infections: current knowledge and new perspectives. *FEMS Microbiol Rev*. 2021;45(3):060.

23. Hirayama T, Miyazaki T, Ito Y, Wakayama M, Shibuya K, Yamashita K, *et al.* Virulence assessment of six major pathogenic *Candida* species in the mouse model of invasive candidiasis caused by fungal translocation. *Sci Rep.* 2020;10(1):3814.
24. Sabino R, Veríssimo C, Pereira Á A, Antunes F. *Candida auris*, an agent of hospital-associated outbreaks: which challenging issues do we need to have in mind? *Microorganisms.* 2020;8(2):181.
25. Ragusa P, Prinzivalli A, Pizzini S, Libero G, Lo Moro G, Brescia V, *et al.* *Candida auris*: A bibliometric analysis of an emerging global health threat. *J Infect Public Health.* 2023;16(10):1696-1702.
26. Geremia N, Brugnaro P, Solinas M, Scarparo C, Panese S. *Candida auris* as an emergent public health problem: a current update on european outbreaks and cases. *Healthcare.* 2023;11(3):425.
27. CDC. Centres for Disease Control and Prevention. 24 June 2016, posting date. Global emergence of invasive infections caused by the multidrug resistant yeast *Candida auris*. Centres for Disease Control and Prevention, Atlanta, GA. 2016.
28. Lee EH, Choi MH, Lee KH, Kim D, Jeong SH, Song YG, *et al.* Intrahospital transmission and infection control of *Candida auris* originating from a severely infected COVID-19 patient transferred abroad. *J Hosp Infect.* 2024;143:140-149.
29. Rajni E, Singh A, Tarai B, Jain K, Shankar R, Pawar K, *et al.* A High frequency of *Candida auris* blood stream infections in Coronavirus Disease 2019 patients admitted to intensive care units, northwestern india: a case control study. *Open Forum Infect Dis.* 2021;8(12): 452.
30. Koulenti D, Karvouniaris M, Paramythiotou E, Koliakos N, Markou N, Paranos P, *et al.* Severe *Candida* infections in critically ill patients with COVID-19. *J Intensive Med.* 2023;3(4):291-297.
31. World Health Organization. WHO fungal priority pathogens list to guide research, development and public health action. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO. 2022.

32. Satoh K, Makimura K, Hasumi Y, Nishiyama Y, Uchida K, Yamaguchi H. *Candida auris* sp. nov., a novel ascomycetous yeast isolated from the external ear canal of an inpatient in a Japanese hospital. *Microbiol Immunol*. 2009;53(1):41-44.
33. Lee WG, Shin JH, Uh Y, Kang MG, Kim SH, Park KH, et al. First three reported cases of nosocomial fungemia caused by *Candida auris*. *J Clin Microbiol*. 2011;49(9):3139-3142.
34. Lone SA, Ahmad A. *Candida auris*-the growing menace to global health. *Mycoses*. 2019;62(8):620-637.
35. Ragusa P, Prinzivalli A, Pizzini S, Libero G, Lo Moro G, Brescia V, et al. *Candida auris*: a bibliometric analysis of an emerging global health threat. *J Infect Public Health*. 2023;16(10):1696-1702.
36. Lockhart SR, Etienne KA, Vallabhaneni S, Farooqi J, Chowdhary A, Govender NP, et al. Simultaneous emergence of multidrug-resistant *Candida auris* on 3 continents confirmed by whole-genome sequencing and epidemiological analyses. *Clin Infect Dis*. 2017;64(2):134-140.
37. Magobo RE, Corcoran C, Seetharam S, Govender NP. *Candida auris*-associated candidemia, South Africa. *Emerg Infect Dis*. 2014;20(7):1250-1251.
38. van Schalkwyk E, Mpembe RS, Thomas J, Shuping L, Ismail H, Lowman W, et al. Epidemiologic shift in candidemia driven by *Candida auris*, South Africa, 2016-2017(1). *Emerg Infect Dis*. 2019;25(9):1698-1707.
39. Mashau RC, Meiring ST, Dramowski A, Magobo RE, Quan VC, Perovic O, et al. Culture-confirmed neonatal bloodstream infections and meningitis in South Africa, 2014-19: a cross-sectional study. *Lancet Glob Health*. 2022;10(8):e1170-e8.
40. Kekana D, Naicker SD, Shuping L, Velaphi S, Nakwa FL, Wadula J, et al. *Candida auris* clinical isolates associated with outbreak in neonatal unit of tertiary academic hospital, South Africa. *Emerg Infect Dis*. 2023;29(10):2044-2053.

41. Govender NP, Avenant T, Brink A, Chibabhai V, Cleghorn J, du Toit B, *et al.* Federation of infectious diseases societies of southern africa guideline: recommendations for the detection, management and prevention of healthcare-associated *Candida auris* colonisation and disease in South Africa. S Afr J Infect Dis. 2019;34(1):163.
42. Welsh RM, Bentz ML, Shams A, Houston H, Lyons A, Rose LJ, *et al.* Survival, persistence, and isolation of the emerging multidrug-resistant pathogenic yeast *Candida auris* on a plastic health care surface. J Clin Microbiol. 2017;55(10):2996-3005.
43. Sherry L, Ramage G, Kean R, Borman A, Johnson EM, Richardson MD, *et al.* Biofilm-forming capability of highly virulent, multidrug-resistant *Candida auris*. Emerg Infect Dis. 2017;23(2):328-331.
44. Osei Sekyere J. *Candida auris*: A systematic review and meta-analysis of current updates on an emerging multidrug-resistant pathogen. Microbiologyopen. 2018;7(4):e00578.
45. Spruijtenburg B, Badali H, Abastabar M, Mirhendi H, Khodavaisy S, Sharifisooraki J, *et al.* Confirmation of fifth *Candida auris* clade by whole genome sequencing. Emerg Microbes Infect. 2022;11(1):2405-2411.
46. Suphavilai C, Ko K, Lim K, Tan M, Boonsimma P, Chu J, *et al.* Discovery of the sixth *Candida auris* clade in Singapore2023.
47. Chow NA, Muñoz JF, Gade L, Berkow EL, Li X, Welsh RM, *et al.* Tracing the Evolutionary history and global expansion of *Candida auris* using population genomic analyses. mBio. 2020;11(2):19.
48. Ahmad A, Molepo J, Patel M. Challenges in the development of antifungal agents against *candida*: scope of phytochemical research. Curr Pharm Des. 2016;22(27):4135-4150.
49. Vandeputte P, Ferrari S, Coste AT. Antifungal resistance and new strategies to control fungal infections. Int J Microbiol. 2012;2012:713687.
50. Campoy S, Adrio JL. Antifungals. Biochem Pharmacol. 2017;133:86-96.
51. Hossain CM, Ryan LK, Gera M, Choudhuri S, Lyle N, Ali KA, *et al.* Antifungals and drug resistance. Encyclopedia. 2022;2(4):1722-1737.

52. Sheehan DJ, Hitchcock CA, Sibley CM. Current and emerging azole antifungal agents. Clin Microbiol Rev. 1999;12(1):40-79.
53. Ghannoum MA, Rice LB. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin Microbiol Rev. 1999;12(4):501-517.
54. Kanafani ZA, Perfect JR. Resistance to antifungal agents: mechanisms and clinical impact. Clin Infect Dis. 2008;46(1):120-128.
55. Lee Y, Puumala E, Robbins N, Cowen LE. Antifungal drug resistance: molecular mechanisms in *Candida albicans* and beyond. Chem Rev. 2021;121(6):3390-3411.
56. Lamb DC, Kelly DE, Schunck WH, Shyadehi AZ, Akhtar M, Lowe DJ, et al. The mutation T315A in *Candida albicans* sterol 14 α -demethylase causes reduced enzyme activity and fluconazole resistance through reduced affinity. J Biol Chem. 1997;272(9):5682-5688.
57. Robbins N, Caplan T, Cowen LE. Molecular evolution of antifungal drug resistance. Annu Rev Microbiol. 2017;71:753-775.
58. Ostrosky-Zeichner L, Casadevall A, Galgiani JN, Odds FC, Rex JH. An insight into the antifungal pipeline: selected new molecules and beyond. Nat Rev Drug Discov. 2010;9(9):719-727.
59. Carolus H, Pierson S, Lagrou K, Van Dijck P. Amphotericin B and other polyenes—discovery, clinical use, mode of action and drug resistance. J Fungi. 2020;6(4):321.
60. Robbins N, Wright GD, Cowen LE. Antifungal drugs: the current armamentarium and development of new agents. Microbiol Spectr. 2016;4(5).
61. Perfect JR. The antifungal pipeline: a reality check. Nat Rev Drug Discov. 2017;16(9):603-616.
62. Anderson TM, Clay MC, Cioffi AG, Diaz KA, Hisao GS, Tuttle MD, et al. Amphotericin forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol. 2014;10(5):400-406.

63. Ellis D. Amphotericin B: spectrum and resistance. *J Antimicrob Chemother.* 2002;49 Suppl 1:7-10.
64. Vazquez JA, Arganoza MT, Boikov D, Yoon S, Sobel JD, Akins RA. Stable phenotypic resistance of *Candida* species to amphotericin B conferred by preexposure to subinhibitory levels of azoles. *J Clin Microbiol.* 1998;36(9):2690-2695.
65. Bondaryk M, Kurzątkowski W, Staniszevska M. Antifungal agents commonly used in the superficial and mucosal candidiasis treatment: mode of action and resistance development. *Postepy Dermatol Alergol.* 2013;30(5):293-301.
66. Pfaller MA, Messer SA, Woosley LN, Jones RN, Castanheira M. Echinocandin and triazole antifungal susceptibility profiles for clinical opportunistic yeast and mold isolates collected from 2010 to 2011: application of new CLSI clinical breakpoints and epidemiological cutoff values for characterization of geographic and temporal trends of antifungal resistance. *J Clin Microbiol.* 2013;51(8):2571-2581.
67. Hasim S, Coleman JJ. Targeting the fungal cell wall: current therapies and implications for development of alternative antifungal agents. *Future Med Chem.* 2019;11(8):869-883.
68. Alexander BD, Johnson MD, Pfeiffer CD, Jiménez-Ortigosa C, Catania J, Booker R, *et al.* Increasing echinocandin resistance in *Candida glabrata*: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. *Clin Infect Dis.* 2013;56(12):1724-1732.
69. Cuomo CA, David P. The molecular basis of antifungal resistance in *Candida auris*. *Curr Opin Microbiol.* 2021;71:102695.
70. Rybak JM, Cuomo CA, Rogers PD. The molecular and genetic basis of antifungal resistance in the emerging fungal pathogen *Candida auris*. *Curr Opin Microbiol.* 2022;70:102208.
71. Muñoz JF, Gade L, Chow NA, Loparev VN, Juieng P, Berkow EL, *et al.* Genomic insights into multidrug-resistance, mating and virulence in *Candida auris* and related emerging species. *Nat Commun.* 2018;9(1):5346.

72. Rybak JM, Doorley LA, Nishimoto AT, Barker KS, Palmer GE, Rogers PD. Abrogation of triazole resistance upon deletion of CDR1 in a clinical isolate of *Candida auris*. *Antimicrob Agents Chemother*. 2019;63(4):10.1128/aac.00057-19.
73. Holmes AR, Cardno TS, Strouse JJ, Ivnitski-Steele I, Keniya MV, Lackovic K, *et al*. Targeting efflux pumps to overcome antifungal drug resistance. *Future Med Chem*. 2016;8(12):1485-1501.
74. Chowdhary A, Prakash A, Sharma C, Kordalewska M, Kumar A, Sarma S, *et al*. A multicentre study of antifungal susceptibility patterns among 350 *Candida auris* isolates (2009-17) in India: role of the *ERG11* and *FKS1* genes in azole and echinocandin resistance. *J Antimicrob Chemother*. 2018;73(4):891-899.
75. Healey KR, Kordalewska M, Jiménez Ortigosa C, Singh A, Berrío I, Chowdhary A, *et al*. Limited ERG11 mutations identified in isolates of *Candida auris* directly contribute to reduced azole susceptibility. *Antimicrob Agents Chemother*. 2018;62(10).
76. Carolus H, Sofras D, Boccarella G, Sephton-Clark P, Romero CL, Vergauwen R, *et al*. Acquired amphotericin B resistance and fitness trade-off compensation in *Candida auris*. 2023.
77. Kordalewska M, Lee A, Park S, Berrio I, Chowdhary A, Zhao Y, *et al*. Understanding echinocandin resistance in the emerging pathogen *Candida auris*. *Antimicrob Agents Chemother*. 2018;62(6)-18.
78. Johnson MD, Perfect JR. Use of antifungal combination therapy: agents, order, and timing. *Curr Fungal Infect Rep*. 2010;4(2):87-95.
79. Baddley JW, Poppas PG. Antifungal combination therapy. *Drugs*. 2005;65(11):1461-1480.
80. Spitzer M, Robbins N, Wright GD. Combinatorial strategies for combating invasive fungal infections. *Virulence*. 2017;8(2):169-185.
81. Tobias JS, Wrigley PF, Shaw E. Combination antifungal therapy for cryptococcal meningitis. *Postgrad Med J*. 1976;52(607):305-308.

82. Bennett JE, Dismukes WE, Duma RJ, Medoff G, Sande MA, Gallis H, *et al.* A comparison of amphotericin B alone and combined with flucytosine in the treatment of cryptococcal meningitis. *N Engl J Med.* 1979;301(3):126-131.
83. Cui J, Ren B, Tong Y, Dai H, Zhang L. Synergistic combinations of antifungals and anti-virulence agents to fight against *Candida albicans*. *Virulence.* 2015;6(4):362-371.
84. Chandramati J, Sadanandan L, Kumar A, Ponthenkandath S. Neonatal *Candida auris* infection: Management and prevention strategies - A single centre experience. *J Paediatr Child Health.* 2020;56(10):1565-1569.
85. Nagy F, Tóth Z, Nyikos F, Forgács L, Jakab Á, Borman AM, *et al.* *In vitro* and *in vivo* interaction of caspofungin with isavuconazole against *Candida auris* planktonic cells and biofilms. *Med Mycol.* 2021;59(10):1015-1023.
86. Fakhim H, Chowdhary A, Prakash A, Vaezi A, Dannaoui E, Meis JF, *et al.* *In vitro* interactions of echinocandins with triazoles against multidrug-resistant *Candida auris*. *Antimicrob Agents Chemother.* 2017;61(11):10.1128/aac.01056-17.
87. Bidaud A, Djenontin E, Botterel F, Chowdhary A, Dannaoui E. Colistin interacts synergistically with echinocandins against *Candida auris*. *Int J Antimicrob Agents.* 2020;55(3):105901.
88. Brennan-Krohn T, Friar L, Ditelberg S, Kirby JE. Evaluation of the synergistic activity of antibacterial and antifungal drugs against *Candida auris* using an inkjet printer-assisted method. *Antimicrob Agents Chemother.* 2021;65(10):10.1128/aac.00268-21.
89. Shaban S, Patel M, Ahmad A. Improved efficacy of antifungal drugs in combination with monoterpene phenols against *Candida auris*. *Sci Rep.* 2020;10(1):1162.
90. Ahmad Khan MS, Alshehrei F, Al-Ghamdi SB, Bamaga MA, Al-Thubiani AS, Alam MZ. Virulence and biofilms as promising targets in developing antipathogenic drugs against candidiasis. *Future Sci OA.* 2020;6(2):FSO440.
91. Carmona-Gutierrez D, Eisenberg T, Büttner S, Meisinger C, Kroemer G, Madeo F. Apoptosis in yeast: triggers, pathways, subroutines. *Cell Death Differ.* 2010;17(5):763-773.

92. Nicola AM, Albuquerque P, Paes HC, Fernandes L, Costa FF, Kioshima ES, *et al.* Antifungal drugs: New insights in research & development. *Pharmacol Ther.* 2019;195:21-38.
93. Tharp B, Zheng R, Bryak G, Litvintseva AP, Hayden MK, Chowdhary A, *et al.* Role of microbiota in the skin colonization of *Candida auris*. *mSphere.* 2023;8(1):e0062322.
94. Mayer FL, Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. *Virulence.* 2013;4(2):119-128.
95. Kean R, Brown J, Gulmez D, Ware A, Ramage G. *Candida auris*: A decade of understanding of an enigmatic pathogenic yeast. *J Fungi (Basel).* 2020;6(1):30.
96. de Groot PW, Bader O, de Boer AD, Weig M, Chauhan N. Adhesins in human fungal pathogens: glue with plenty of stick. *Eukaryot Cell.* 2013;12(4):470-481.
97. Moyes DL, Richardson JP, Naglik JR. *Candida albicans*-epithelial interactions and pathogenicity mechanisms: scratching the surface. *Virulence.* 2015;6(4):338-346.
98. Silva-Dias A, Miranda IM, Branco J, Monteiro-Soares M, Pina-Vaz C, Rodrigues AG. Adhesion, biofilm formation, cell surface hydrophobicity, and antifungal planktonic susceptibility: relationship among *Candida* spp. *Front Microbiol.* 2015;6:205.
99. Hoyer LL, Cota E. *Candida albicans* Agglutinin-Like Sequence (Als) family vignettes: a review of ALs protein structure and function. *Front Microbiol.* 2016;7:280.
100. Nobile CJ, Schneider HA, Nett JE, Sheppard DC, Filler SG, Andes DR, *et al.* Complementary adhesin function in *C. albicans* biofilm formation. *Curr Biol.* 2008;18(14):1017-1024.
101. Wächtler B, Citiulo F, Jablonowski N, Förster S, Dalle F, Schaller M, *et al.* *Candida albicans*-epithelial interactions: dissecting the roles of active penetration, induced endocytosis and host factors on the infection process. *PLoS One.* 2012;7(5):e36952.
102. Larkin E, Hager C, Chandra J, Mukherjee PK, Retuerto M, Salem I, *et al.* The emerging pathogen *Candida auris*: Growth Phenotype, virulence factors, activity of antifungals, and effect of SCY-078, a novel glucan synthesis inhibitor, on growth morphology and biofilm formation. *Antimicrob Agents Chemother.* 2017;61(5):e02396-16.

103. Gómez-Gaviria M, Martínez-Álvarez JA, Chávez-Santiago JO, Mora-Montes HM. *Candida haemulonii* complex and *Candida auris*: Biology, Virulence factors, immune response, and multidrug resistance. *Infect Drug Resist.* 2023;16:1455-1470.
104. Singh S, Uppuluri P, Mamouei Z, Alqarihi A, Elhassan H, French S, *et al.* The NDV-3A vaccine protects mice from multidrug resistant *Candida auris* infection. *PLoS Pathog.* 2019;15(8):e1007460.
105. Kean R, Delaney C, Sherry L, Borman A, Johnson EM, Richardson MD, *et al.* Transcriptome assembly and profiling of *Candida auris* reveals novel insights into biofilm-mediated resistance. *mSphere.* 2018;3(4):00334-18.
106. Chow EWL, Pang LM, Wang Y. From jekyll to hyde: The yeast-hyphal transition of *Candida albicans*. *Pathogens.* 2021;10(7):859.
107. Lopes JP, Lionakis MS. Pathogenesis and virulence of *Candida albicans*. *Virulence.* 2022;13(1):89-121.
108. McCall AD, Pathirana RU, Prabhakar A, Cullen PJ, Edgerton M. *Candida albicans* biofilm development is governed by cooperative attachment and adhesion maintenance proteins. *NPJ Biofilms Microbiomes.* 2019;5(1):21-0094-5.
109. Mroczyńska M, Brillowska-Dąbrowska A. Virulence of clinical *Candida* isolates. *Pathogens.* 2021;10(4):466.
110. Bliss JM, Sullivan MA, Malone J, Haidaris CG. Differentiation of *Candida albicans* and *Candida dubliniensis* by using recombinant human antibody single-chain variable fragments specific for hyphae. *J Clin Microbiol.* 2003;41(3):1152-1160.
111. Cortegiani A, Misseri G, Fasciana T, Giammanco A, Giarratano A, Chowdhary A. Epidemiology, clinical characteristics, resistance, and treatment of infections by *Candida auris*. *J Intensive Care.* 2018;6:69.
112. Wang X, Bing J, Zheng Q, Zhang F, Liu J, Yue H, *et al.* The first isolate of *Candida auris* in China: clinical and biological aspects. *Emerg Microbes Infect.* 2018;7(1):1-9.
113. Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev.* 2003;67(3):400-428.

114. Tsai P-W, Chen Y-T, Hsu P-C, Lan C-Y. Study of *Candida albicans* and its interactions with the host: A mini review. *BioMedicine*. 2013;3:51–64.
115. Park M, Do E, Jung WH. Lipolytic enzymes involved in the virulence of human pathogenic fungi. *Mycobiology*. 2013;41(2):67-72.
116. Rapala-Kozik M, Bochenska O, Zajac D, Karkowska-Kuleta J, Gogol M, Zawrotniak M, *et al*. Extracellular proteinases of *Candida* species pathogenic yeasts. *Mol Oral Microbiol* 2018;33(2):113-124.
117. Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev*. 2003;67(3):400-428.
118. Chin VK, Lee TY, Rusliza B, Chong PP. Dissecting *Candida albicans* infection from the perspective of c. albicans virulence and omics approaches on host-pathogen interaction: a review. *Int J Mol Sci*. 2016;17(10):43.
119. Felk A, Kretschmar M, Albrecht A, Schaller M, Beinhauer S, Nichterlein T, *et al*. *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. *Infect Immun*. 2002;70(7):3689-3700.
120. Lee SA, Jones J, Hardison S, Kot J, Khalique Z, Bernardo SM, *et al*. *Candida albicans* VPS4 is required for secretion of aspartyl proteases and *in vivo* virulence. *Mycopathologia*. 2009;167:55-63.
121. Kumar D, Banerjee T, Pratap CB, Tilak R. Itraconazole-resistant *Candida auris* with phospholipase, proteinase and hemolysin activity from a case of vulvovaginitis. *J Infect Dev Ctries*. 2015;9(04):435-437.
122. Wang X, Bing J, Zheng Q, Zhang F, Liu J, Yue H, *et al*. The first isolate of *Candida auris* in China: clinical and biological aspects. *Emerg Microbes Infect*. 2018;7(1):93.
123. Chatterjee S, Alampalli SV, Nageshan RK, Chettiar ST, Joshi S, Tatu US. Draft genome of a commonly misdiagnosed multidrug resistant pathogen *Candida auris*. *BMC genomics*. 2015;16:1-16.

124. Kim JS, Lee KT, Bahn YS. Secreted aspartyl protease 3 regulated by the Ras/cAMP/PKA pathway promotes the virulence of *Candida auris*. *Front Cell Infect Microbiol*. 2023;13:1257897.
125. Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev*. 2000;13(1):122-43.
126. Djordjevic JT. Role of phospholipases in fungal fitness, pathogenicity, and drug development - lessons from *Cryptococcus neoformans*. *Front Microbiol*. 2010;1:125.
127. Dabiri S, Shams-Ghahfarokhi M, Razzaghi-Abyaneh M. Comparative analysis of proteinase, phospholipase, hydrophobicity and biofilm forming ability in *Candida* species isolated from clinical specimens. *J Mycol Med*. 2018;28(3):437-442.
128. Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL. *Candida* biofilms: an update. *Eukaryotic cell*. 2005;4(4):633-638.
129. Rajendran R, Sherry L, Nile CJ, Sherriff A, Johnson EM, Hanson M, *et al*. Biofilm formation is a risk factor for mortality in patients with *Candida albicans* bloodstream infection—Scotland, 2012–2013. *Clin Microbiol Infect*. 2016;22(1):87-93.
130. Sherry L, Ramage G, Kean R, Borman A, Johnson EM, Richardson MD, *et al*. Biofilm-forming capability of highly virulent, multidrug-resistant *Candida auris*. *Emerg Infect Dis*. 2017;23(2):328.
131. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, *et al*. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ*. 2009;16(1):3-11.
132. Madeo F, Fröhlich E, Fröhlich KU. A yeast mutant showing diagnostic markers of early and late apoptosis. *J Cell Biol*. 1997;139(3):729-734.
133. Braun RJ. Mitochondrion-mediated cell death: dissecting yeast apoptosis for a better understanding of neurodegeneration. *Front Oncol*. 2012;2:182.
134. Ziegler U, Groscurth P. Morphological features of cell death. *News Physiol Sci*. 2004;19:124-128.

135. Rouchidane Eyitayo A, Gonin M, Arokium H, Manon S. Contribution of yeast studies to the understanding of BCL-2 family intracellular trafficking. *Int J Mol Sci.* 2021;22(8):086.
136. Jürgensmeier JM, Krajewski S, Armstrong RC, Wilson GM, Oltersdorf T, Fritz LC, *et al.* Bax- and Bak-induced cell death in the fission yeast *Schizosaccharomyces pombe*. *Mol Biol Cell.* 1997;8(2):325-339.
137. Grosfeld EV, Bidiuk VA, Mitkevich OV, Ghazy E, Kushnirov VV, Alexandrov AI. A systematic survey of characteristic features of yeast cell death triggered by external factors. *J Fungi (Basel).* 2021;7(11):86.
138. Wissing S, Ludovico P, Herker E, Büttner S, Engelhardt SM, Decker T, *et al.* An AIF orthologue regulates apoptosis in yeast. *J Cell Biol.* 2004;166(7):969-974.
139. Chaves SR, Rego A, Martins VM, Santos-Pereira C, Sousa MJ, Côrte-Real M. Regulation of cell death induced by acetic acid in yeasts. *Front Cell Dev Biol.* 2021;9:75.
140. Madeo F, Herker E, Maldener C, Wissing S, Lächelt S, Herlan M, *et al.* A caspase-related protease regulates apoptosis in yeast. *Mol Cell.* 2002;9(4):911-917.
141. Eisenberg T, Büttner S, Kroemer G, Madeo F. The mitochondrial pathway in yeast apoptosis. *Apoptosis.* 2007;12(5):1011-1023.
142. Kaluç N, Thomas P. Hypochlorous acid induces caspase dependent apoptosis in *Saccharomyces cerevisiae*. *J. Biosci. Med.* 2021;9:42-53.
143. Silva RD, Sotoca R, Johansson B, Ludovico P, Sansonetty F, Silva MT, *et al.* Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis in *Saccharomyces cerevisiae*. *Mol Microbio.* 2005;58(3):824-834.
144. Severin FF, Hyman AA. Pheromone induces programmed cell death in *S. cerevisiae*. *Curr Biol.* 2002;12(7):R233-235.
145. Pozniakovsky AI, Knorre DA, Markova OV, Hyman AA, Skulachev VP, Severin FF. Role of mitochondria in the pheromone- and amiodarone-induced programmed death of yeast. *J Cell Biol.* 2005;168(2):257-269.

146. Phillips AJ, Sudbery I, Ramsdale M. Apoptosis induced by environmental stresses and amphotericin B in *Candida albicans*. *Proc Natl Acad Sci*. 2003;100(24):14327-14332.
147. Al-Dhaheri RS, Douglas LJ. Apoptosis in *Candida* biofilms exposed to amphotericin B. *J Med Microbiol*. 2010;59(2):149-57.
148. Hauptmann P, Riel C, Kunz-Schughart LA, Fröhlich KU, Madeo F, Lehle L. Defects in N-glycosylation induce apoptosis in yeast. *Mol Microbiol*. 2006;59(3):765-778.
149. Mazzoni C, Herker E, Palermo V, Jungwirth H, Eisenberg T, Madeo F, *et al*. Yeast caspase 1 links messenger RNA stability to apoptosis in yeast. *EMBO Rep*. 2005;6(11):1076-1081.
150. Weinberger M, Ramachandran L, Feng L, Sharma K, Sun X, Marchetti M, *et al*. Apoptosis in budding yeast caused by defects in initiation of DNA replication. *J Cell Sci*. 2005;118(Pt 15):3543-3553.
151. Pereira C, Silva RD, Saraiva L, Johansson B, Sousa MJ, Côrte-Real M. Mitochondria-dependent apoptosis in yeast. *Biochim Biophys Acta*. 2008;1783(7):1286-1302.
152. Falcone C, Mazzoni C. External and internal triggers of cell death in yeast. *Cell Mol Life Sci*. 2016;73(11-12):2237-2250.
153. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim Biophys Acta*. 2016;1863(12):2977-2992.
154. Villalpando-Rodriguez GE, Gibson SB. Reactive oxygen species (ROS) regulates different types of cell death by acting as a rheostat. *Oxid Med Cell Longev*. 2021;2021:9912436.
155. Almeida B, Silva A, Mesquita A, Sampaio-Marques B, Rodrigues F, Ludovico P. Drug-induced apoptosis in yeast. *Biochim Biophys Acta*. 2008;1783(7):1436-1448.
156. Choi H, Lee DG. Lycopene induces apoptosis in *Candida albicans* through reactive oxygen species production and mitochondrial dysfunction. *Biochimie*. 2015;115:108-115.

157. Hwang B, Hwang JS, Lee J, Lee DG. The antimicrobial peptide, psacothasin induces reactive oxygen species and triggers apoptosis in *Candida albicans*. *Biochem Biophys Res Commun*. 2011;405(2):267-271.
158. Aerts AM, François IE, Meert EM, Li QT, Cammue BP, Thevissen K. The antifungal activity of RsAFP2, a plant defensin from *Raphanus sativus*, involves the induction of reactive oxygen species in *Candida albicans*. *J Mol Microbiol Biotechnol*. 2007;13(4):243-247.
159. Andrés MT, Viejo-Díaz M, Fierro JF. Human lactoferrin induces apoptosis-like cell death in *Candida albicans*: critical role of K⁺-channel-mediated K⁺ efflux. *Antimicrob Agents Chemother*. 2008;52(11):4081-4018.
160. Park C, Lee DG. Melittin induces apoptotic features in *Candida albicans*. *Biochem Biophys Res Commun*. 2010;394(1):170-172.
161. Bondaryk M, Staniszewska M, Zielińska P, Urbańczyk-Lipkowska Z. Natural antimicrobial peptides as inspiration for design of a new generation antifungal compounds. *J Fungi*. 2017;3(3):46.
162. Walker NI, Harmon BV, Gobé GC, Kerr JF. Patterns of cell death. *Methods Achiev Exp Pathol*. 1988;13:18-54.
163. Eisenberg T, Carmona-Gutierrez D, Büttner S, Tavernarakis N, Madeo F. Necrosis in yeast. *Apoptosis*. 2010;15(3):257-268.
164. Denton D, Nicolson S, Kumar S. Cell death by autophagy: facts and apparent artefacts. *Cell Death Differ*. 2012;19(1):87-95.
165. Struyfs C, Cammue BPA, Thevissen K. Membrane-interacting antifungal peptides. *Front Cell Dev Biol*. 2021;9:649875.
166. Dubos RJ. Studies on a bactericidal agent extracted from a soil *Bacillus* : protective effect of the bactericidal agent experimental *Pneumococcus* infections in mice. *J Exp Med*. 1939;70(1):11-17.
167. Wang G, Li X, Wang Z. APD3: the antimicrobial peptide database as a tool for research and education. *Nucleic Acids Res*. 2016;44(D1):D1087-93.

168. Kumar P, Kizhakkedathu JN, Straus SK. Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility *in vivo*. *Biomolecules*. 2018;8(1):04.
169. Boniotto M, Jordan WJ, Eskdale J, Tossi A, Antcheva N, Crovella S, et al. Human beta-defensin 2 induces a vigorous cytokine response in peripheral blood mononuclear cells. *Antimicrob Agents Chemother*. 2006;50(4):1433-1441.
170. Sørensen OE, Cowland JB, Theilgaard-Mönch K, Liu L, Ganz T, Borregaard N. Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *J Immunol*. 2003;170(11):5583-5589.
171. Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ. Antimicrobial host defence peptides: functions and clinical potential. *Nat Rev Drug Discov*. 2020;19(5):311-332.
172. Almeida PF, Pokorny A. 5.10 Interactions of antimicrobial peptides with lipid bilayers. *C Biophysics*. 2012;189-222.
173. Tossi A, Sandri L, Giangaspero A. Amphipathic, alpha-helical antimicrobial peptides. *Biopolymers*. 2000;55(1):4-30.
174. van der Weerden NL, Bleackley MR, Anderson MA. Properties and mechanisms of action of naturally occurring antifungal peptides. *Cell Mol Life Sci*. 2013;70(19):3545-3570.
175. Tam JP, Lu YA, Yang JL, Chiu KW. An unusual structural motif of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides. *Proc Natl Acad Sci U S A*. 1999;96(16):8913-8918.
176. Wang G. Human antimicrobial peptides and proteins. *Pharmaceuticals (Basel)*. 2014;7(5):545-594.
177. Fleming A. On a remarkable bacteriolytic element found in tissues and secretions. *Proceedings of the Royal Society of London, Series B*, 1922;93,306-317.
178. Wang G. Database-guided discovery of potent peptides to combat HIV-1 or superbugs. *Pharmaceuticals (Basel)*. 2013;6(6):728-758.

179. Selsted ME, Harwig SS, Ganz T, Schilling JW, Lehrer RI. Primary structures of three human neutrophil defensins. *J Clin Invest.* 1985;76(4):1436-1439.
180. Zhao C, Wang I, Lehrer RI. Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. *FEBS Lett.* 1996;396(2-3):319-322.
181. Bensch KW, Raida M, Mägert HJ, Schulz-Knappe P, Forssmann WG. hBD-1: a novel beta-defensin from human plasma. *FEBS Lett.* 1995;368(2):331-335.
182. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science.* 1998;282(5386):121-125.
183. Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem.* 2001;276(8):5707-5713.
184. Huang L, Leong SS, Jiang R. Soluble fusion expression and characterization of bioactive human beta-defensin 26 and 27. *Appl Microbiol Biotechnol.* 2009;84(2):301-308.
185. Järvå M, Phan TK, Lay FT, Caria S, Kvensakul M, Hulett MD. Human β -defensin 2 kills *Candida albicans* through phosphatidylinositol 4,5-bisphosphate-mediated membrane permeabilization. *Sci Adv.* 2018;4(7):eaat0979.
186. Phan TK, Lay FT, Poon IK, Hinds MG, Kvensakul M, Hulett MD. Human β -defensin 3 contains an oncolytic motif that binds PI(4,5)P₂ to mediate tumour cell permeabilisation. *Oncotarget.* 2016;7(2):2054-2069.
187. Vylkova S, Li XS, Berner JC, Edgerton M. Distinct antifungal mechanisms: beta-defensins require *Candida albicans* Ssa1 protein, while Trk1p mediates activity of cysteine-free cationic peptides. *Antimicrob Agents Chemother.* 2006;50(1):324-331.
188. Yount NY, Kupferwasser D, Spisni A, Dutz SM, Ramjan ZH, Sharma S, et al. Selective reciprocity in antimicrobial activity versus cytotoxicity of hBD-2 and crotamine. *Proc Natl Acad Sci U S A.* 2009;106(35):14972-7.

189. Vylkova S, Nayyar N, Li W, Edgerton M. Human beta-defensins kill *Candida albicans* in an energy-dependent and salt-sensitive manner without causing membrane disruption. *Antimicrob Agents Chemother*. 2007;51(1):154-161.
190. Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, *et al*. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on *Candida albicans*. *J Biol Chem*. 1988;263(16):7472-7477.
191. Nikawa H, Jin C, Fukushima H, Makihiro S, Hamada T. Antifungal activity of histatin-5 against non-albicans *Candida* species. *Oral Microbiol Immunol*. 2001;16(4):250-252.
192. Puri S, Edgerton M. How does it kill?: understanding the candidacidal mechanism of salivary histatin 5. *Eukaryot Cell*. 2014;13(8):958-964.
193. Siqueira WL, Margolis HC, Helmerhorst EJ, Mendes FM, Oppenheim FG. Evidence of intact histatins in the in vivo acquired enamel pellicle. *J Dent Res*. 2010;89(6):626-630.
194. Xu T, Levitz SM, Diamond RD, Oppenheim FG. Anticandidal activity of major human salivary histatins. *Infect Immun*. 1991;59(8):2549-2554.
195. Jang WS, Bajwa JS, Sun JN, Edgerton M. Salivary histatin 5 internalization by translocation, but not endocytosis, is required for fungicidal activity in *Candida albicans*. *Mol Microbiol*. 2010;77(2):354-370.
196. Kumar R, Chadha S, Saraswat D, Bajwa JS, Li RA, Conti HR, *et al*. Histatin 5 uptake by *Candida albicans* utilizes polyamine transporters Dur3 and Dur31 proteins. *J Biol Chem*. 2011;286(51):43748-43758.
197. Helmerhorst EJ, Troxler RF, Oppenheim FG. The human salivary peptide histatin 5 exerts its antifungal activity through the formation of reactive oxygen species. *Proc Natl Acad Sci*. 2001;98(25):14637-14642.
198. Koshlukova SE, Lloyd TL, Araujo MW, Edgerton M. Salivary histatin 5 induces non-lytic release of ATP from *Candida albicans* leading to cell death. *J Biol Chem*. 1999;274(27):18872-18879.

199. Konopka K, Dorocka-Bobkowska B, Gebremedhin S, Düzgüneş N. Susceptibility of *Candida* biofilms to histatin 5 and fluconazole. *Antonie Van Leeuwenhoek*. 2010;97:413-417.

Chapter 2: Aim and Objectives

2.1. Aim and Objectives

The aim of this study was to determine the antifungal, anti-virulence and apoptosis enhancing activity of selected AMP in *C. auris*. In addition, the cytotoxicity of selected AMPs was also studied. To achieve this aim in a practical manner, the following specific objectives were addressed through experimentation:

1. To evaluate the minimum inhibitory concentrations and minimum fungicidal concentrations of seven AMPs against *C. auris* isolates.
2. To evaluate the antifungal activity of seven AMPs in combination with the most commonly used antifungal drugs against *C. auris* isolates.
3. To study the concentration- and time- dependent antifungal effect of seven AMPs (time-kill curves) against *C. auris* isolates.
4. To determine the physiology and mode of cell death in response to the most active peptides by analyzing apoptotic markers (Annexin V, cytochrome c, TUNEL assay and mitochondrial membrane potential).
5. To determine the effect of the most active peptides on pathogenicity markers (adherence, proteinase production and biofilm formation) of *C. auris* isolates.
6. To evaluate the effect of the most active peptides on the expression profile of genes involved in pathogenicity in *C. auris* using Real Time PCR.
7. To determine the cytotoxicity of the active peptides by using *in vitro* haemolytic assay.

2.2. Layout of the Thesis

For a thorough comprehension of the research, the thesis is structured into interconnected chapters. These chapters systematically introduce the various methods used in the study, facilitating a coherent understanding of the overarching framework.

The study evaluated the *in vitro* antifungal activity of a panel of AMPs against *C. auris*. Seven peptides, comprising three alpha-defensins (HNP-1-3), three beta-defensins (hBD-1-3) and one histatin (His 5), were evaluated against 10 clinical *C. auris* isolates (**Objective 1**), this involved determining their minimum inhibitory concentration (MIC) and minimum fungicidal

concentrations (MFC). The susceptibility testing revealed the fungicidal efficacy of all tested peptides against *C. auris* isolates. Additionally, the study investigated the combined effects of these AMPs with three conventional antifungal agents: fluconazole, amphotericin B and caspofungin (**Objective 2**). Significant synergy was observed in nearly all antifungal-peptide combinations. Detailed methodology, results, and discussion are provided in **Chapter 3**.

Based on the antifungal susceptibility of *C. auris* isolates, two strains were selected for further investigation regarding the fungicidal impacts of AMPs and their underlying mechanisms of action. These strains are identified as CAU-02 (named CAU-S) exhibiting susceptibility to fluconazole, amphotericin B, and caspofungin and CAU-09 (named CAU-R) demonstrating resistance to all three antifungals (MDR).

Time-kill curve analyses and viability studies using the muse count & viability kits revealed that all peptide induced concentration and time- dependent cell death (**Objective 3**). Subsequently, to gain insights into the mechanism of action, the effect of the peptides on cell membrane was evaluated using a propidium iodide assay. All peptides induced membrane permeability in *C. auris*, as visualized using confocal microscopy. The complete methodology, results, and remarks are provided in **Chapter 3 (Publication 1)**.

Based on the initial screening, the three most potent AMPs (hBD-3, HNP-1 and His 5) were selected for further studies to focus on elucidating their specific mechanisms of action against *C. auris*. The type of cell death induced by the peptides was assessed by studying different apoptotic markers using various assays (**objective 4**). Our results indicate that hBD-3 and HNP-1 are potent inducers of apoptosis in *C. auris*, potentially targeting the mitochondria and activating a specific cell death pathway. In contrast, His-5 induces necrosis with minimal impact on *C. auris* mitochondrial function. Detailed methodology, findings and remarks are outlined in **Chapter 4 (Publication 2)**.

This study also investigated the effect of peptides on the virulence factors of *C. auris*, including adherence, proteinase secretion, and biofilm formation and mature biofilm (**Objective 5**). The results revealed that all three tested peptides, even at subinhibitory concentrations, were effective in hindering the virulence factors of *C. auris*. In addition, the study investigated their ability to counteract a major fungal resistance mechanism, efflux pumps. Results demonstrated that all the tested peptides effectively inhibited efflux pumps in *C. auris*. Detailed methodology and findings are discussed in **Chapter 5**.

Furthermore, the influence of the most active peptides on genes related to virulence (*SAP3*, *PGA26*, *PGA7*, *PGA52*) and efflux-pump (*CDR1*, *CDR2*, *MDR1*, and *SNQ2*) of *C. auris* was assessed in this study (**Objective 6**). The peptides significantly reduced the expression of the genes linked to these aspects. Finally, the potential toxicity of the peptides on mammalian cells was investigated using a haemolytic assay (**Objective 7**). Encouragingly, the results demonstrated minimal to no cytotoxic effects, as evidenced by low levels of haemolysis across all tested concentrations of the peptides. **Chapter 5** outlines the thorough methodology, findings, and discussion (**Manuscript ready to submit**).

Chapter 6 provides the discussion of the overall findings of this research, followed by the clinical application prospects of the AMPs. And **Chapter 7** includes the conclusions of the overall study, limitation of the current work and future research.

To encapsulate the study, the following outline comprehensively covers all sections of this research work (**Fig. 2.1**).

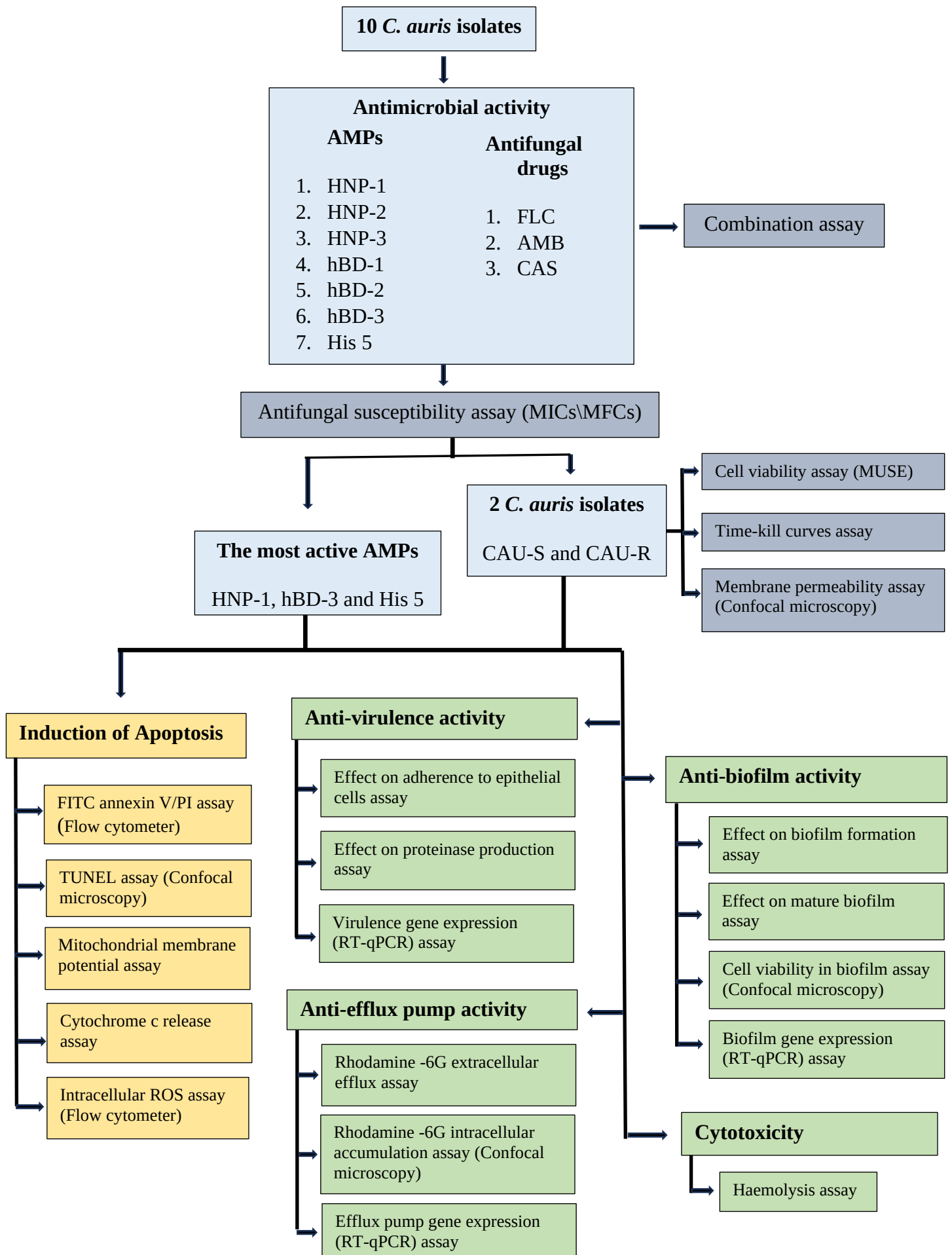


Figure 2.1: Diagram illustrating the study outline.

Chapter 3: Fungicidal activity of human antimicrobial peptides and their synergistic interaction with common antifungals against multidrug resistant *Candida auris*

Research in this chapter has been published and the publication format has been kept including referencing style.

3.1. Introduction

Since *Candida auris* emergence in 2009 (Sato et al. 2009), it has been identified as a causative agent of nosocomial infections in compromised individuals globally (Dahiya et al. 2020). This pathogen has been involved in colonization of multiple surfaces and equipment in healthcare facilities (Lockhart et al. 2017a) and is therefore responsible for several hospital outbreaks worldwide, with high mortality rates between 30 and 72% (Zhu et al. 2020).

Previous studies have reported that *C. auris* isolates are commonly resistant to azoles and polyenes (Osei Sekyere 2018). Echinocandins (e.g. caspofungin) exhibit a greater activity against *C. auris* isolates and currently used as a standard recommended treatment for these infections (Lyman et al. 2021). However, secondary resistance development among previously susceptible strains has been observed (Ostrowsky et al. 2020). These challenges have encouraged the urge to develop alternative antifungal compounds with a low potential for inducing fungal resistance. Therefore, the discovery of novel antifungals from natural sources, such as peptides produced by host defense system themselves, has been the focus of many studies (Mercer and O'Neil 2020).

Antimicrobial peptides (AMPs) are important constituents of the innate immune response produced in humans and most forms of life (Mahlpuu et al. 2016). Many AMPs typically exhibit a wide range of activity protecting the host from different pathogenic fungi, bacteria, enveloped viruses, and protozoa (Hancock and Chapple 1999). Furthermore, they have immunomodulatory activity by attracting the phagocytic cells to the sites of infection (Choi et al. 2012). Therefore, natural and synthetic AMPs have been considered as potential alternatives to the traditional antimicrobial drugs (Kang et al. 2017).

In general, AMPs are a group of relatively small cationic polypeptides (Malmsten 2014), commonly classified into three large families, including, i) α -helical, ii) β -sheet and iii) extended peptides (Nguyen et al. 2011). The human defensins, belonging to the β -sheet category, and human salivary histatin 5 peptide, belonging to the extended peptide, are of the major peptides present in humans (Buda De Cesare et al. 2020). The human defensins are further divided into two subfamilies; human α - and β -defensins (Schneider et al. 2005). Previously, different AMPs have been illustrated in several studies to have significant antifungal activities against different medically important fungal pathogens including *Candida*, *Cryptococcus* and *Aspergillus* species (Fernández de Ullivarri et al. 2020).

In the field of antifungal drug development, combination approach together with screening for novel antifungal molecules has been the focus of many researches in the hope of discovering better therapeutic option (Scorzoni et al. 2017). Combination antifungal therapy has become a common approach used to treat resistant fungal infections (Candoni et al. 2014). Combining an antifungal agent with another compound that has antifungal activity can overcome the development of drug resistance in pathogenic fungi by using a lower dosage of both agents, with a concurrent reduction in the associated side effects (Baddley and Poppas 2005). Several AMPs have shown promising synergetic activity when combined with commercial antifungals against *Candida albicans* (Krishnakumari et al. 2008) and other pathogenic non-*albicans* *Candida* (Lupetti et al. 2003; Singh et al. 2017; Wei and Bobek 2004).

Based upon the present knowledge of the antifungal properties of the major human AMPs, the present study evaluated the antifungal activity of seven peptides individually and combined with three common antifungal agents against clinical *C. auris* isolates. The results presented here have provided evidence of their promising role in the field of antifungal drug development.

3.2. Materials and Methods

3.2.1. Strains and growth conditions

Clinical *C. auris* isolates ($n = 10$) used in this study were previously identified and stored as glycerol stocks at $-70\text{ }^{\circ}\text{C}$ in the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand. Each strain was initially grown on Sabouraud Dextrose Agar (SDA) plates and incubated at $37\text{ }^{\circ}\text{C}$ for 24 h, and then pure colonies from these plates were suspended in 15 ml of Sabouraud Dextrose Broth (SDB) with continuous shaking at 200 rpm

for 18 h. After that, cells were centrifuged, washed three times with phosphate buffer solution (PBS), adjusted to 1×10^6 CFU/ml and used for the subsequent assays.

3.2.2. Peptides and antifungal drugs

Seven peptides from the different classes of human AMPs, which are human neutrophil peptides 1-3 (HNP-1, HNP-2 and HNP-3), histatin 5 (His 5), and human β -defensin 1-3 (hBD-1, hBD-2 and hBD-3) were used in this study. All peptides were purchased from Thermo Fisher Scientific, IL, USA. Mass spectrometric (MS) analyses of the peptides were performed using Applied Biosynthesis Voyager DE system, and purity of $\geq 95\%$ was measured by high performance liquid chromatography (HPLC) system (Biosynthesis, Inc) as per the certificate of analysis provided by the supplier. All peptides were dissolved in distilled water to make stock solution of 5 mg/ml and kept in a dark environment at -20°C until use. The following antifungal drugs (fluconazole, FLC; amphotericin B, AmB and caspofungin, CAS) procured from Sigma-Aldrich (St. Louis, MO, USA) were also used in this study.

3.2.3. Antifungal susceptibility assay

Antifungal activity of the selected peptides and three antifungal drugs was determined using broth microdilution method as per Clinical and Laboratory Standard Institute (CLSI) guidelines reference document M27-A3 (CLSI 2008) with modification. AMPs (800 $\mu\text{g/ml}$) and antifungal drugs (2000 $\mu\text{g/ml}$ of FLC; 32 $\mu\text{g/ml}$ of AmB; 16 $\mu\text{g/ml}$ of CAS) at volume of 100 μl were added to the first well of the 96 well plates, which were then serially diluted in SDB. One hundred microliters of standardized *Candida* suspensions, which further diluted the concentrations by one-fold, were added to each well and plates were incubated at 35°C for 24 h. The final concentration ranged from 200 to 1.56 $\mu\text{g/ml}$ for peptides, 500 to 4 $\mu\text{g/ml}$ for FLC, 8 to 0.063 $\mu\text{g/ml}$ for AmB and 4 to 0.031 $\mu\text{g/ml}$ for CAS. A culture control containing SDB and culture, a media control containing only SDB and negative control containing sterile water were also included. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the tested peptides\antifungals at which no visible growth was observed. Following MIC determination, the minimum fungicidal concentration (MFC) was determined as the lowest concentration which causes no visible growth on agar plates after sub-culturing for 24 h from wells.

3.2.4. Time-kill curves assay

To study killing curves of each peptide, the procedure described by Klepser and coauthors (1998) was followed. Two strains (sensitive and MDR) were chosen based on the MICs for the antifungal drugs. Standardized *Candida* suspensions were treated with the tested peptides at concentrations equal to their MIC/MFC levels for the strains being tested. Cultures with and without the test compounds (controls) were incubated at 37 °C with agitation at 200 rpm for 48 h. 100 µl samples were taken out at 0, 2, 4, 6, 8, 12, 24, 48 h, and then immediately washed and diluted in PBS, and 20 µl was plated onto SDA plates. Plates were kept for 24 h at 37 °C for colony count determination. The colony forming unit per ml (CFU/ml) was calculated and the average of three measurements was log₁₀ transformed and plotted against time for each peptide.

3.2.5. *Candida* cell counting and viability assay

To further quantify the killing effect of each peptide, total cell counting, and viability was assayed by the Muse™ cell analyzer (EMD Millipore, Germany), following manufacturer's instructions. *C. auris* cells were treated with the tested peptides at their MIC/MFC concentrations and incubated at 37 °C for 4 h. Both negative (untreated cells) and positive (cells treated with 10 mM H₂O₂) controls were also included. The treated cells were then washed and resuspended in PBS, and 20 µl of the cell suspension was added to 380 µl of count & viability reagent (EMD Millipore, USA), after 5 min incubation at room temperature the sample was analyzed for total cell counting and viability.

3.2.6. Combination assay

The peptide-antifungal interaction was evaluated using the microdilution method (Ahmad et al. 2015). The assay was carried out in 96-well microplates by combining 50 µl of the tested peptides and 50 µl of the antifungals (FLC, AmB and CAS) in a volume ratio of 1:1 against all the *C. auris* strains followed by serial dilution by transferring 100 µl as described previously. A total of 100 µl of *Candida* suspension was added to each well, and the plates were incubated at 35 °C for 24 h. After incubation, the MIC for each antifungal agent in combination with all the peptides was defined. The combination interaction was assessed by calculating the fractional inhibitory concentration index (FICI) using the following formula:

$$FICI = FIC_{Drug} + FIC_{Peptide} = \frac{(MIC_{Drug} \text{ in combination})}{(MIC_{Drug} \text{ alone})} + \frac{(MIC_{peptide} \text{ in combination})}{(MIC_{peptide} \text{ alone})}$$

The results interpretation was as follows: synergy (FICI ≤ 0.5); additive (FICI between 0.5 and 1.0); indifference (FICI between 1.0 and 4.0) and antagonistic (FICI > 4.0).

3.2.7. Assessment of plasma membrane permeability

The effect of the tested peptides on *C. auris* membrane integrity was assessed using propidium iodide (PI; Sigma-Aldrich) assay as described (Suchodolski et al. 2017), with modification. Standardized cell suspension was treated with the different peptides at MIC/MFC concentrations for 4 h, washed cells were then stained with 30 μ M of PI. After 30 min incubation in the dark at room temperature, cells were washed and resuspended in 200 μ l of BPS. A laser scanning confocal microscope (Zeiss LSM 780) with Airyscan detector was used for sample visualization and image acquisition. Both positive and negative controls were prepared as described above and included in this assay.

3.2.8. Statistical analysis

Graph Pad Prism 5 was used for statistical analysis. The data were presented as the mean of three independent replicates (mean $\pm$ SD) and analyzed using one-way analysis of variance (ANOVA) with Dunnett's comparison test (p value < 0.05).

3.3. Results

3.3.1. Susceptibility profile of *C. auris* isolate to peptides and antifungals

The susceptibility of 10 *C. auris* isolates to seven peptides was evaluated and the MIC/MFC values are shown in Table 3.1. For comparison three conventional antifungal agents were also included in this study and the antifungal susceptibility profiles are shown in Table 3.2. All peptides exhibited good antifungal potential against all *C. auris* isolates with variation in activity levels. Based on the MIC results, the order of antifungal efficacy was hBD-3 $>$ hBD-1 $>$ hBD-2 $>$ His 5 $>$ HNP-1 $>$ HNP-2 = HNP-3, with MIC values ranges of (3.125 – 12.5 μ g/ml), (6.25 – 25 μ g/ml), (12.5 – 50 μ g/ml), (25 – 50 μ g/ml), (25 – 100 μ g/ml) and (50 – 100 μ g/ml), respectively. In terms of MFC results, all peptides gave values 2-fold higher than their corresponding MICs for all strains evaluated, suggesting that all peptides have fungicidal activity against *C. auris*. As for the standard antifungal agents, CAS was the most active antifungal (MIC range; 0.25- 2 μ g/ml). For AmB and FLC, the MIC values were found between 0.125 to 4 μ g/ml and 16 to 500 μ g/ml, respectively. Based on the Centers for Disease Control and Prevention (CDC) tentative MIC Breakpoints (CDC 2019), out of 10 tested *C. auris*

isolates 8, 5 and 1 were classified resistant to FLC (MIC $\geq$ 32 μ g/ml), AmB (MIC $\geq$ 2 μ g/ml) and CAS (MIC $\geq$ 2 μ g/ml), respectively. Based on this classification, two isolates (CAU-02 and CAU-09) were chosen to further study the fungicidal activity of the selected peptides. The CAU-02 strain showed sensitivity (the lowest MICs) to all antifungals, and CAU-09 showed resistance (the highest MICs) to all antifungal drugs tested.

Table 3.1: Antifungal activity of synthetic antimicrobial peptides against *Candida auris* isolates ($n = 30$)

Peptide	MICs/MFCs (μ g/ml)	
	Median MIC (range)	Median MFC (range)
HNP-1	50 (25-100)	100 (50-200)
HNP-2	100 (50-100)	200 (100- 200)
HNP-3	100 (50-100)	200 (100- 200)
His 5	25 (25-50)	50 (50-100)
hBD-1	12.5 (6.25-25)	25 (12.5-50)
hBD-2	25 (12.5-50)	50 (25-100)
hBD-3	6.25 (3.125-12.5)	12.5 (6.25-25)

- Each value represents the median of three independent experiments.
- HNP-1, Human Neutrophil Peptide 1; HNP-2, Human Neutrophil Peptide 2; HNP-3, Human Neutrophil Peptide 3; His 5, Histatin 5; hBD-1, human β -Defensin-1; hBD-2, human β -Defensin-2; hBD-3, human β -Defensin-3

Table 3.2: Antifungal drug susceptibility profile of *Candida auris* (n=3)

<i>C. auris</i>	Median MIC/MFC (µg/ml)					
	Amphotericin B		Caspofungin		Fluconazole	
	MIC	MFC	MIC	MFC	MIC	MFC
CAU-01	0.5 (S)	1	0.25 (S)	0.5	16 (S)	NS
CAU-02	0.125 (S)	0.25	0.25 (S)	0.5	16 (S)	NS
CAU-03	2 (R)	4	0.25 (S)	0.5	250 (R)	NS
CAU-04	2 (R)	2	0.5 (S)	1	250 (R)	NS
CAU-05	2 (R)	2	0.25 (S)	0.5	500 (R)	NS
CAU-06	2 (R)	4	0.25 (S)	0.5	500 (R)	NS
CAU-07	0.5 (S)	1	0.25 (S)	1	125 (R)	NS
CAU-08	1 (S)	2	0.25 (S)	0.5	125 (R)	NS
CAU-09	4 (R)	8	2 (R)	4	500 (R)	NS
CAU-10	0.25 (S)	1	0.25 (S)	0.5	32 (R)	NS

- Each value represents the median of three independent experiments.
- S, sensitive; R, resistance. Classification based on CDC guidelines (Tentative MIC Breakpoints); FLC (S<32 µg/ml; R ≥32 µg/ml); AMP (S<2 µg/ml; R ≥2 µg/ml); CAS (S<2 µg/ml; R ≥2 µg/ml)

3.3.2. Killing kinetics of peptides against *C. auris*

The antifungal activity of AMPs was further assessed to determine their killing kinetics at MIC and MFC concentrations (Fig. 3.1). The fungicidal effect of the examined peptides was defined as $\geq 3 \log_{10}$ reduction in CFU/ml (≥ 99.99 killing) from the starting inoculum. All peptides had reached the maximum fungicidal endpoint (100% killing) at their highest concentration tested (MFC) at 12 and 24 h of the test period for both strains. However, the onset of the fungicidal effect differed between the two strains. Among the tested peptides, hBD-1, -2 and -3 exhibited the most rapid activity by killing *C. auris* within 6 h of treatment at their MFC concentrations and after 12 h at their MIC concentrations for both strains. Histatin 5 also exhibited rapid fungicidal effect at 6 and 24 h of incubation for MFC and MIC concentration respectively in the MDR strain. The sensitive strain was killed within 8 h at MFC concentration, while at the MIC level experienced no killing effect. The time kill curves for HNP1, -2 and -3 looked similar with almost no killing effect at MIC level in both strains, whereas at MFC values killing effect at 8 and 12 h in the MDR and sensitive strains was observed. This correlates well with the susceptibility results and revealed the time- and dose-dependent killing activity of the selected peptides towards *C. auris*.

3.3.3. Peptides rapidly reduce the viability of *C. auris* cells

Peptides' effect on cell viability of susceptible and MDR *C. auris* isolates was determined by using Muse™ Count & Viability kit. This method provides the absolute cell count and viable cells percentage in the sample, whereas the reagent can differentiate between viable and dead cells by simultaneous staining both populations based on their selective permeability to the two DNA-binding dyes. The results showed that treatment with the test peptides resulted in a decrease in the percentage of cell viability after 4 h exposure when compared to untreated control cells (Fig. 3.2, Table 3.3). The untreated negative control showed 95.8% and 96.0% live cells, while the positive control showed 0.5% and 0.9% live cells in CAU-02 and CAU-09 respectively. For the susceptible strain (CAU-02), hBD-1, -2 and -3 demonstrated the best killing effects and reduced the cell viability by 71.7 to 99.4% after treatment with MIC and MFC concentrations respectively. While HNP-1, -2 and -3 displayed the poorest killing effect and reduced the viability by 24.4 to 67.6% after treatment with the same concentrations. Likewise, hBD-1, -2 and -3 also demonstrated the best killing effects against the MDR strain (CAU-09) where the reduction in cell viability percentage was between 58.9 and 98.8% after treatment with MIC and MFC values respectively. However, HNP-1, -2 and -3 displayed better

killing effect (50.5–78.0%) than in the susceptible strain after treatment with the same concentrations. These are in accordance with the previous results indicating that the hBD-3 is the most active peptide. Finally, all peptides significantly inhibit the survival of *C. auris* cells ($p < 0.01$) compared to untreated negative controls in both strains tested (Fig. 3.2). The viability profile of *C. auris* strains after treatment with different concentrations of peptides are represented in Appendix C, Fig. C.1.

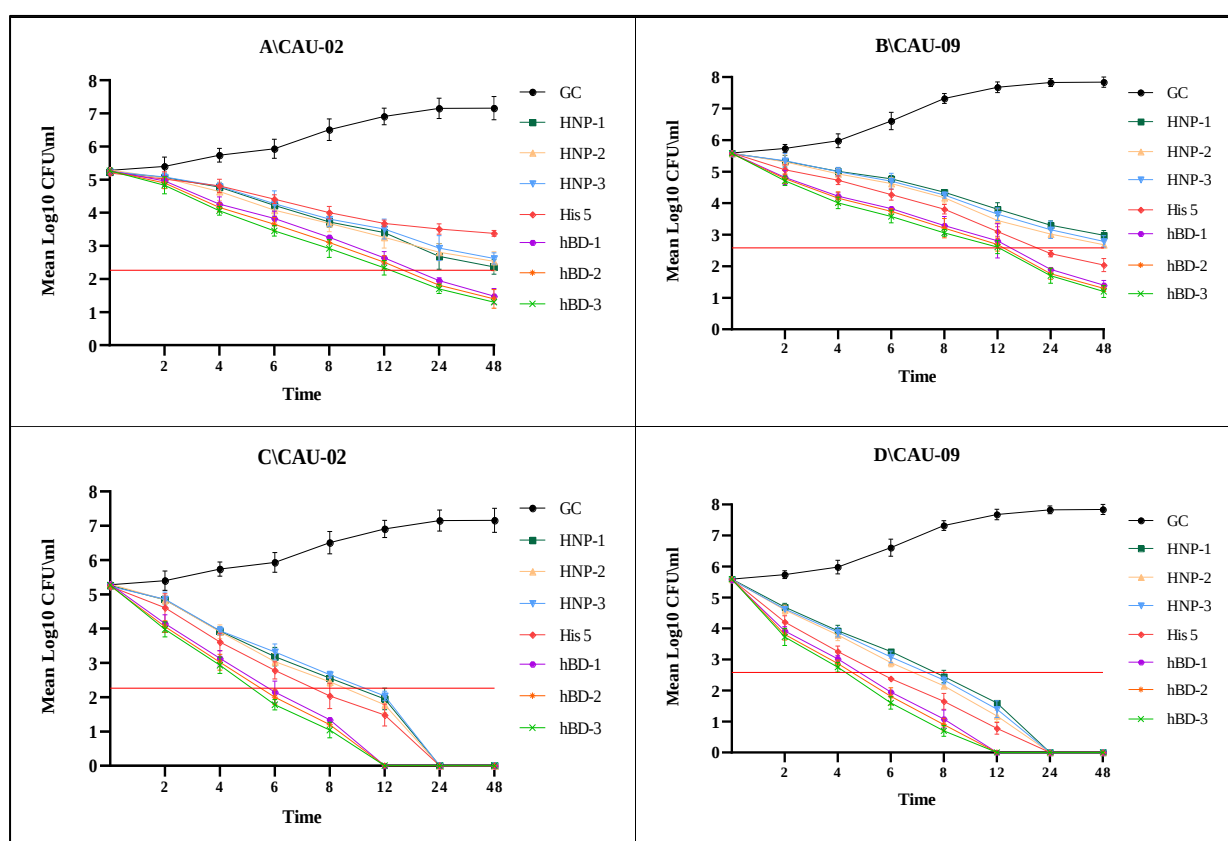


Figure 3.1: Time-kill curves for *Candida auris* following exposure to antimicrobial peptides. HNP-1, HNP-2, HNP-3, His 5, hBD-1, hBD-2 and hBD-3 at MIC concentrations against CAU-02 (A) and CAU-09 (B), and at MFC concentrations against CAU-02 (C) and CAU-09 (D) *C. auris* strains. The red solid line represents the 3 log₁₀ growth reduction compared with that of the initial inoculums (fungicidal effect). Each time point represents as the mean log₁₀ CFU/ml of three independent experiments.

Table 3.3: Percentage reduction in viability of *Candida auris* cells following 4 h treatment with different antimicrobial peptides.

Peptides	Percentage reduction in viability of <i>C. auris</i> after 4 h treatment (%)			
	CAU-02 (Sensitive)		CAU-09 (MDR)	
	MIC	MFC	MIC	MFC
HNP-1	39.3	66.1	58.9	78.0
HNP-2	24.4	61.6	54.9	77.6
HNP-3	31.2	67.6	50.5	70.4
His 5	43.5	72.5	43.4	84.2
hBD-1	71.7	94.5	58.9	94.6
hBD-2	74.2	98.1	61.0	97.5
hBD-3	77.6	99.4	68.4	98.8

- HNP-1, Human Neutrophil Peptide 1; HNP-2, Human Neutrophil Peptide 2; HNP-3, Human Neutrophil Peptide 3; His 5, Histatin 5; hBD-1, human β -Defensin-1; hBD-2, human β -Defensin-2; hBD-3, human β -Defensin-3

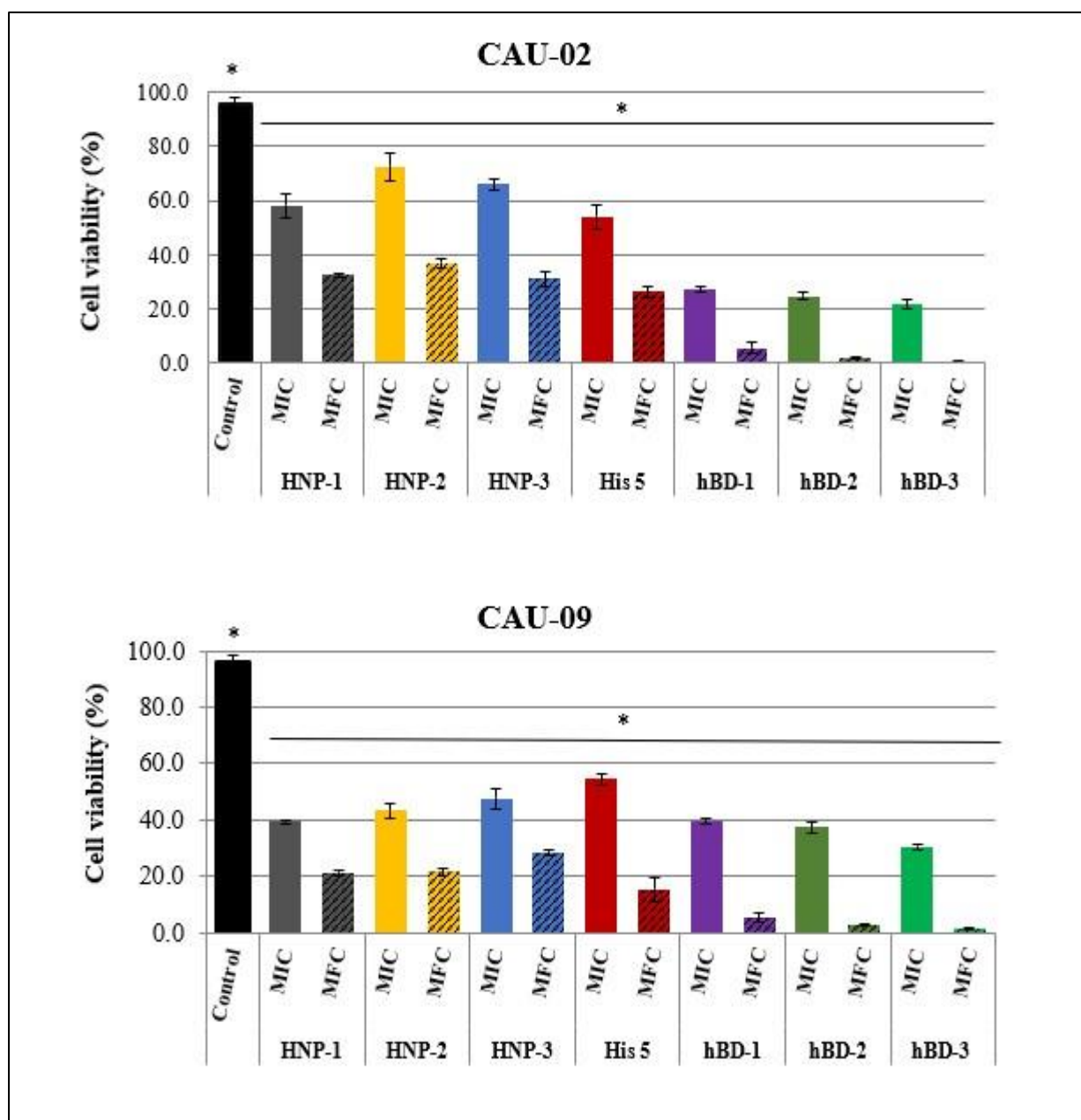


Figure 3.2: Effect of the antimicrobial peptides on cell count and viability of *Candida auris*. The cell viability percentage of *C. auris* cells in the absence and presence of the different peptides. *C. auris* strains CAU-02 and CAU-09 treated with HNP-1, HNP-2, HNP-3, His 5, hBD-1, hBD-2 and hBD-3 at different concentrations for 4 h and control represents untreated *C. auris* cells. Bar graphs represent the average percentage of three independent experiments (mean $\pm$ SD) and statistical significance was determined using one-way ANOVA and Dunnett's test. (* $p < 0.01$).

3.3.4. Combination activity of peptides with antifungal drugs

The microbroth dilution assay allowed to assess the antifungal effect of the peptides–antifungals combinations against *C. auris* strains. From the FIC indices, it has been observed that the combinations of FLC and AmB with the peptides displayed varied interaction among the isolates tested (Table 3.4 and 3.5). Synergistic effects were observed in 90% of the isolates when AmB was combined with HNP-1, HNP-3 and His 5, while only 10% of the isolates showed additive interactions. The combinations of AmB with hBD-1, -2 and -3, and HNP-2 produced FIC indices of synergism in 100% of the isolates. The FLC-peptides combinations were synergistic, additive and indifferent in 70 to 90 %, 20 to 10 % and up to 10% of the isolates, respectively. The calculated FIC indices of the combination of CAS with all the peptides were well 0.5 or less, pointing toward synergistic activity in all 10 strains tested (Table 3.6). No antagonistic activity was detected for any combination. The MICs of antifungal drugs and peptides in combination decreased by up to 4- and 8- fold when compared to their MICs alone. This indicates that the antifungal drugs in combination with the tested peptides inhibited the *Candida* growth at lower concentrations compared to the drugs alone.

Table 3.4: *In vitro* antifungal activity of antimicrobial peptides in combination with fluconazole (FLC) against *Candida auris*

Combinations	MIC in µg/ml				FICI	INT No. of isolates (%)		
	MIC A	PEP A	MIC B	PEP B	Mean ±SD (range)	SYN	ADD	IND
FLC- HNP-1	125	50	16	6.25	0.50 ±0.09 (0.25-1.25)	7 (70)	2 (20)	1 (10)
FLC- HNP-2	125	100	16	12.5	0.47 ±0.07 (0.25-1.25)	7 (70)	2 (20)	1 (10)
FLC- HNP-3	125	100	16	12.5	0.48 ±0.07 (0.25-1.25)	7 (70)	2 (20)	1 (10)
FLC- His 5	125	25	32	6.25	0.37 ±0.08 (0.25-0.63)	8 (80)	2 (20)	0 (0)
FLC- hBD-1	125	12.5	16	1.56	0.40 ±0.10 (0.19-1.12)	8 (80)	1 (10)	1 (10)
FLC- hBD-2	125	25	32	6.25	0.38 ±0.09 (0.25-0.56)	8 (80)	2 (20)	0 (0)
FLC- hBD-3	125	6.25	16	0.78	0.32 ±0.09 (0.19-0.62)	9 (90)	1 (10)	0 (0)

- MIC is the median MIC of three independent experiments. MIC A and MIC B are the median MIC of the drug alone and in combination respectively. PEP A and PEP B are the median MIC of the peptide alone and in combination respectively.
- FICI, fractional inhibitory concentration index; presented as mean ±standard deviation.
- INT, interpretation; SYN, synergy; IND, Indifference; ADD, additive.
- HNP-1, Human Neutrophil Peptide 1; HNP-2, Human Neutrophil Peptide 2; HNP-3, Human Neutrophil Peptide 3; His 5, Histatin 5; hBD-1, human β-Defensin-1; hBD-2, human β-Defensin-2; hBD-3, human β-Defensin-3.

Table 3.5: *In vitro* antifungal activity of antimicrobial peptides in combination with amphotericin B (AmB) against *Candida auris*

Combination	MIC in µg/ml				FICI	INT No. of isolates (%)		
	MIC A	PEP A	MIC B	PEP B		SYN	ADD	IND
AmB- HNP-1	1	50	0.25	12.5	0.44 ±0.05 (0.31-0.63)	9 (90)	1 (10)	0 (0)
AmB- HNP-2	1	100	0.125	12.5	0.35 ±0.02 (0.25-0.50)	10 (100)	0 (0)	0 (0)
AmB- HNP-3	1	100	0.125	12.5	0.39 ±0.01 (0.25-0.62)	9 (90)	1 (10)	0 (0)
AmB- His 5	1	25	0.125	3.125	0.36 ±0.10 (0.19-0.56)	9 (90)	1 (10)	0 (0)
AmB- hBD-1	1	12.5	0.125	1.56	0.34 ±0.06 (0.25-0.50)	10 (100)	0 (0)	0 (0)
AmB- hBD-2	1	25	0.125	3.125	0.34 ±0.06 (0.12-0.50)	10 (100)	0 (0)	0 (0)
AmB- hBD-3	1	6.25	0.125	0.78	0.30 ±0.05 (0.16-0.37)	10 (100)	0 (0)	0(0)

- MIC is the median MIC of three independent experiments. MIC A and MIC B are the median MIC of the drug alone and in combination respectively. PEP A and PEP B are the median MIC of the peptide alone and in combination respectively.
- FICI, fractional inhibitory concentration index; presented as mean ±standard deviation.
- INT, interpretation; SYN, synergy; IND, Indifference; ADD, additive.
- HNP-1, Human Neutrophil Peptide 1; HNP-2, Human Neutrophil Peptide 2; HNP-3, Human Neutrophil Peptide 3; His 5, Histatin 5; hBD-1, human β -Defensin-1; hBD-2, human β -Defensin-2; hBD-3, human β -Defensin-3.

Table 3.6: *In vitro* antifungal activity of antimicrobial peptides in combination with caspofungin (CAS) against *Candida auris*

Combinations	MIC in µg/ml				FICI	INT No. of isolates (%)		
	MIC A	PEP A	MIC B	PEP B	Mean ±SD (range)	SYN	ADD	IND
CAS- HNP-1	0.25	50	0.031	6.25	0.38 ±0.08 (0.19-0.50)	10 (100)	0 (0)	0 (0)
CAS- HNP-2	0.25	100	0.031	12.5	0.38 ±0.09 (0.25-0.50)	10 (100)	0 (0)	0 (0)
CAS- HNP-3	0.25	100	0.031	12.5	0.35±0.07 (0.25-0.50)	10 (100)	0 (0)	0 (0)
CAS- His 5	0.25	25	0.062	6.25	0.36 ±0.01 (0.25-0.50)	10 (100)	0 (0)	0 (0)
CAS- hBD-1	0.25	12.5	0.062	3.125	0.34 ±0.02 (0.25-0.50)	10 (100)	0 (0)	0 (0)
CAS- hBD-2	0.25	25	0.031	3.125	0.31 ±0.05 (0.19-0.50)	10 (100)	0 (0)	0 (0)
CAS- hBD-3	0.25	6.25	0.031	0.78	0.28 ±0.02 (0.19-0.50)	10 (100)	0 (0)	0(0)

- MIC is the median MIC of three independent experiments. MIC A and MIC B are the median MIC of the drug alone and in combination respectively. PEP A and PEP B are the median MIC of the peptide alone and in combination respectively.
- FICI, fractional inhibitory concentration index; presented as mean ±standard deviation.
- INT, interpretation; SYN, synergy; IND, Indifference; ADD, additive.
- HNP-1, Human Neutrophil Peptide 1; HNP-2, Human Neutrophil Peptide 2; HNP-3, Human Neutrophil Peptide 3; His 5, Histatin 5; hBD-1, human β-Defensin-1; hBD-2, human β-Defensin-2; hBD-3, human β-Defensin-3.

3.3.5. Peptides disrupt *C. auris* membrane permeability

The permeabilization effect of peptides on *C. auris* membrane was assessed using PI staining assay. PI is a DNA- staining dye which cannot cross the intact membrane; however, it is able to penetrate the cells with compromised membrane and stain the nucleic acid. As seen in Figs. 3.3 and 3.4, the negative control (untreated healthy cells) showed no fluorescence signal. However, the yeast cells showed a clear red fluorescence after 4 h of incubation with the different peptides as well as the positive control (cells exposed to H₂O₂) in both strains. Figs. 3.3 and 3.4 represent the impact of the most active peptides HNP-1, His 5, and hBD-3 on membrane permeabilization of the susceptible *C. auris* CAU-02 and MDR CAU-09 strains respectively. The impact of the other tested peptides HNP-2, HNP-3, hBD-1, and hBD-2 on the cell membranes of the susceptible *C. auris* CAU-02 and MDR CAU-09 strains are presented in Appendix C, Figs C.2 and C.3 respectively. The results suggest that the tested peptides were able to increase the permeability of *C. auris* membrane as shown by increasing PI fluorescence. Variation in the fluorescence intensity and the number of non-viable cells (PI-positive) was observed in a dose-dependent pattern in both sensitive and MDR strains, which correlates well with the other results in this study.

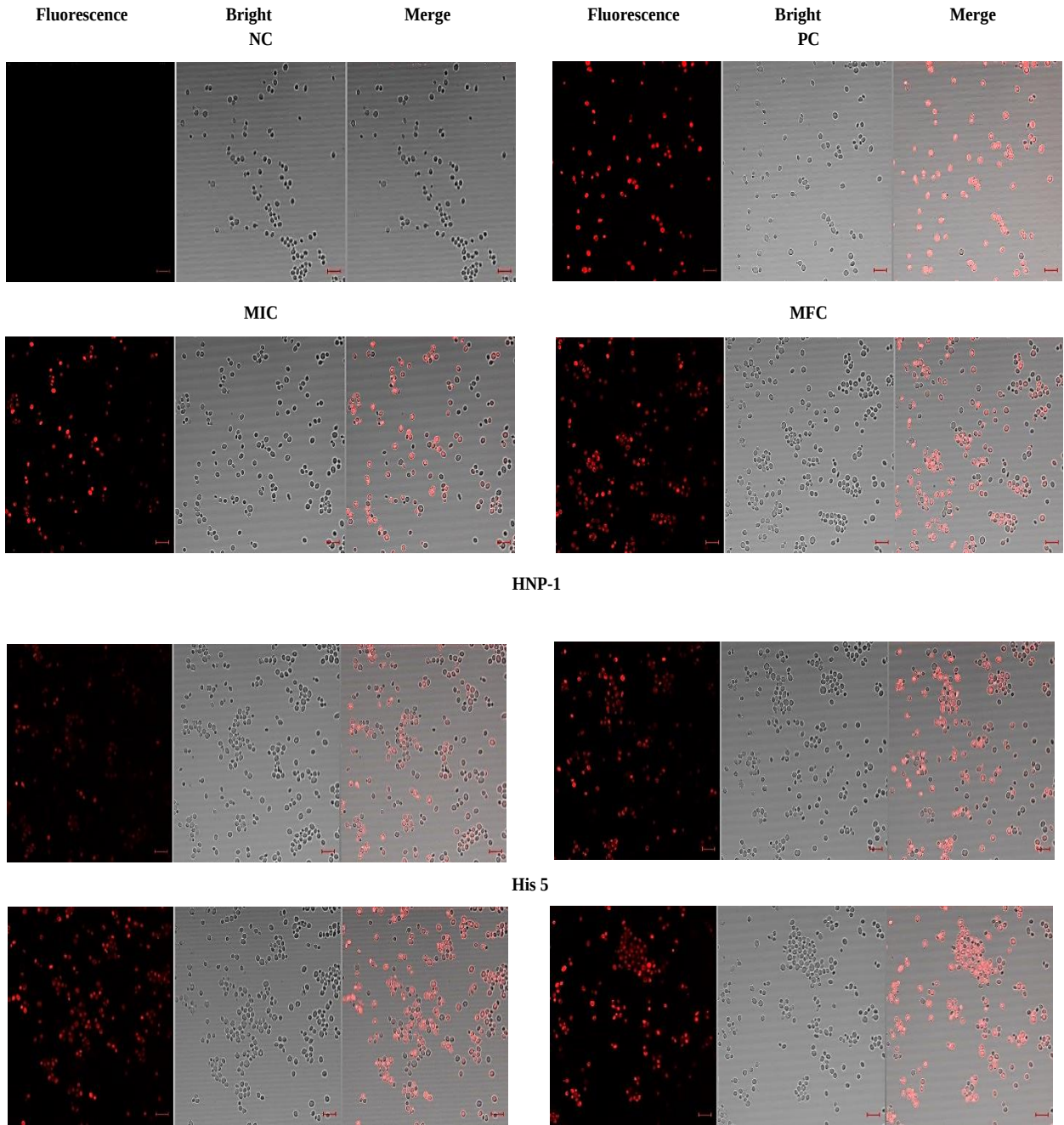


Figure 3.3: Confocal microscopy images of the effect of the tested peptides on *Candida auris* cells. The figures shown are representative data of three independent experiments. Permeabilization of the membrane of *C. auris* strains CAU-02 following exposure to the tested peptides (HNP-1, His 5 and hBD-3) at MIC/MFC concentrations for 4 hours. cells with membrane damage were stained with PI red; negative and positive and control represent untreated, and cells treated with 10 mM H₂O₂; Scale-bar: 10 μ m. Images of the effect of the tested peptides (HNP-2, HNP-3, hBD-1 and hBD-2) on *C. auris* strains CAU-02 are available in C.2.

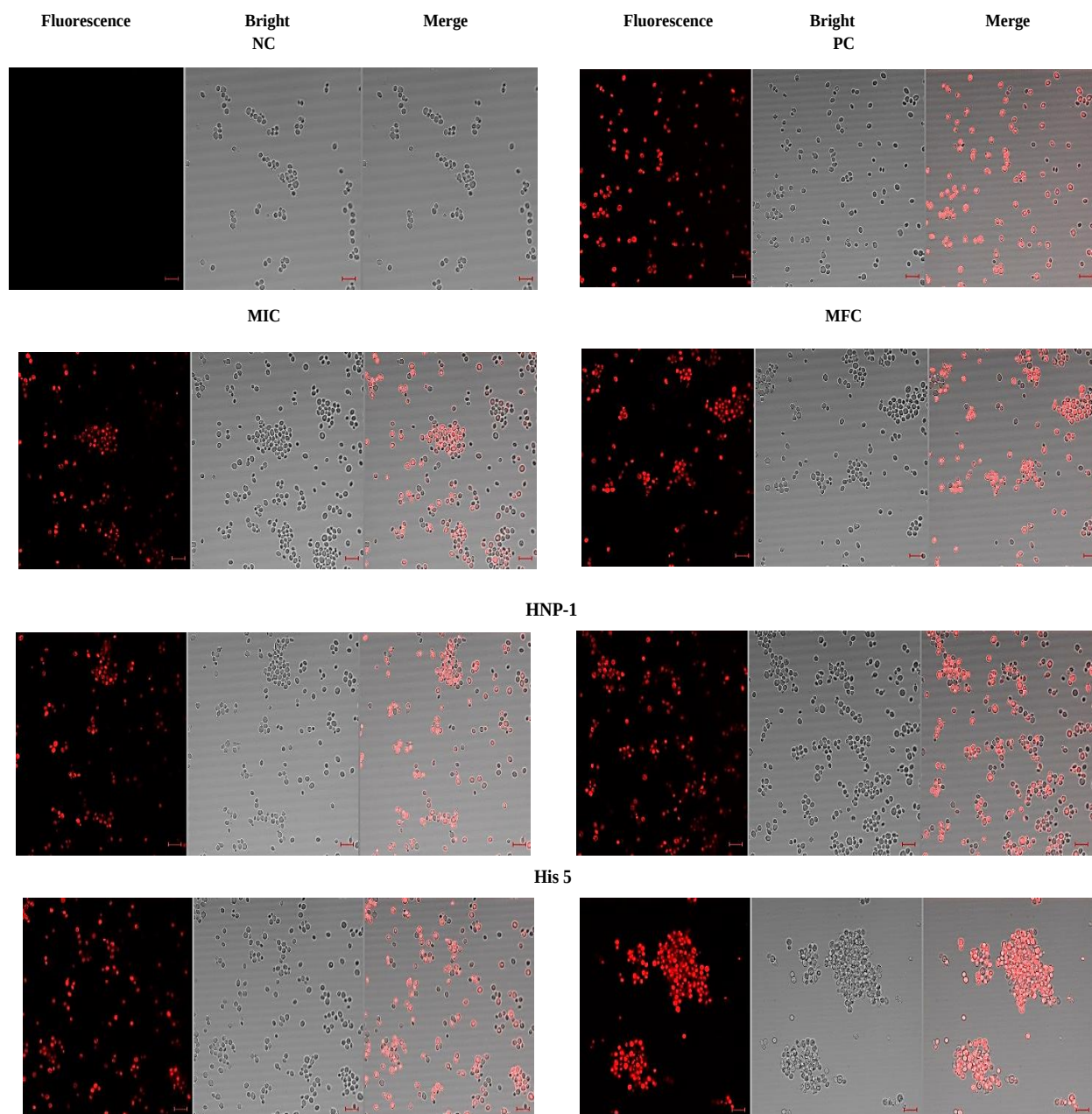


Figure 3.4: Confocal microscopy images of the effect of the tested peptides on *Candida auris* cells. The figures shown are representative data of three independent experiments. Permeabilization of the membrane of *C. auris* strains CAU-09 following exposure to the tested peptides (HNP-1, His 5 and hBD-3) at MIC/MFC concentrations for 4 hours. cells with membrane damage were stained with PI red; negative and positive and control represent untreated, and cells treated with 10 mM H₂O₂; Scale-bar: 10 μm. Images of the effect of the tested peptides (HNP-2, HNP-3, hBD-1 and hBD-2) on *C. auris* strains CAU-09 are available in C.3.

3.4. Discussion

The emergence of *C. auris*, the MDR species, emphasizes the need for new therapeutic strategies to control antifungal resistance (Lone and Ahmad 2019). Recently, new approaches influenced by the natural host immune defense against fungal pathogens represent potential alternatives to the classic antifungal therapy (Mercer and O'Neil 2020), and consequently the development of novel antifungal formulations based on natural AMPs has increased (Cesare et al. 2020). Our findings revealed the ability of selected human AMPs to exert fungicidal potential towards *C. auris*. Among them, hBD-3 showed the best activity with the lowest MIC/MFC of 6.25/12.5 µg/ml and the highest reduction in the percentage of cell viability by 68.4 and 99.4%. In addition, it showed 90 to 100% synergistic effect when combined with FLC, AmB and CAS. These results are encouraging in view of the high demand for developing alternative and/or potentiating antifungal therapies.

The management of *C. auris* infections has become challenging due to growing resistance to common antifungals. Additional concern is that the available therapeutics are relatively few and quite cytotoxic (Dahiya et al. 2020). Therefore, designing innovative and safe alternative antifungal compounds is desperately required. Several natural and synthetic AMPs from different sources have shown to cause growth inhibition or killing of different bacteria and fungi (Ciociola et al. 2016; do Nascimento Dias et al. 2020; Huang et al. 2014; Lin et al. 2015; Maiti et al. 2014), including *C. auris* (Dal Mas et al. 2019; Kubiczek et al. 2020; Pathirana et al. 2018; Raber et al. 2021; van Eijk et al. 2020; Vicente et al. 2019). In this study, the peptides based on the template of natural human AMPs, were examined for their antifungal potential towards *C. auris*. All the examined peptides (HNP-1, -2 and -3; hBD-1, -2 and -3; and His 5) exhibited fungicidal activity towards clinical *C. auris* isolates, including the MDR strains. The good susceptibility of *C. auris* to Histatin 5 has already been reported (Pathirana et al. 2018), but there are no reports, as far as we know, on the activity of human defensins against this yeast.

The fungicidal activity of the tested peptides was further interrogated by performing time kill assay, cell viability study and membrane permeability assay on *C. auris* susceptible *C. auris* CAU-02 and MDR CAU-09 strains. Analysis of time kill curves showed that peptides induced cell death which increase with time and concentration. The maximum fungicidal endpoints were almost identical in treatment with all peptides in both strains (susceptible and resistant), it seems that peptides at high concentration eradicated susceptible and resistant *C.*

auris cells with similar efficiencies. To further verify the fungicidal activity of AMPs tested against *C. auris* isolates, quantitative evaluation of the cell viability after 4 h incubation with the different peptides at their active concentrations were done using Muse Count & Viability kits. We found that all tested peptides significantly kill *C. auris* clinical strains ($p < 0.01$) regardless of their drug resistance-status, indicating potent fungicidal activity of these compounds.

Defensins represent a large family of AMPs contributing to the host defense response against invading pathogens. Human α - and β -defensins are predominantly produced by neutrophils and epithelial cells, respectively, and play a key role in protecting human skin and mucosal surfaces (Schneider et al. 2005). Many studies have been conducted to study the activity of human defensins against *C. albicans*, since these peptides are characterized by broad spectrum antimicrobial properties (Polesello et al. 2017). Our results showed that hBD-3 possesses high antifungal activity, which is comparable to the results described in previous studies showing that hBD-3 was more effective against *C. albicans* than hBD-1 and 2 (Krishnakumari et al. 2009; Vylkova et al. 2006). Since *C. auris* has shown good susceptibility to human β -defensins, future investigations of the potential fungicidal activity of these peptides against other different non-*albicans* *Candida* species will be beneficial to explore their potential use as antifungal lead compounds.

Our results also indicated that exposure of *C. auris* to histatin 5 had more potent antifungal activity against the drug resistant strain compared to the drug susceptible strain. Similar results have been reported in previous findings where treatment of *C. auris* isolates with histatin 5 at 45.54 $\mu\text{g/ml}$ (15 μM) for 1 h induced high level of killing (71 – 89%) in FLC- resistant strains compared to no or little killing (0 – 16.5%) towards the FLC- susceptible strains (Pathirana et al. 2018). Furthermore, the effect of histatin 5 on *C. auris* cell viability was greater when the exposure periods were prolonged for approximately 4 h. The mechanism of action by which histatin 5 reduces the viability of *C. albicans* is well researched. Recent studies points towards targeting multiple intracellular structures are most likely the cause of fungal cell death (Puri and Edgerton 2014). The significant fungicidal activity of histatin 5 against *C. auris* presented here encourages further studies to explore its specific target sites in this yeast.

Combination antifungal treatment is commonly used to treat invasive candidiasis that has not responded to monotherapy (Spitzer et al. 2017). For instance, combination of echinocandins with azoles or AmB has been proposed for invasive *C. auris* infection management. (Fakhim

et al. 2017; Jaggavarapu et al. 2020). The idea of combining two structurally and functionally different compounds appears to have strong potential in developing a successful therapeutic approach against multiple drug resistant infections (Zimmermann et al. 2007). Apparent synergism between different AMPs and antifungal drugs against clinical isolates of *C. albicans* has been reported *in vitro* (Bondaryk et al. 2017) and resulted in improved survival in animal models (MacCallum et al. 2013). Thus, we evaluated the combined action of AMPs with clinically approved antifungals against *C. auris*. We observed high levels of synergism in almost all the antifungal-peptides combinations. More pronounced synergy, however, was observed for CAS than for FLC and AmB. The mechanism of interaction, especially synergism, between AMPs and antifungals are unknown, further studies are needed to demonstrate the mechanism of these synergistic interactions. Additionally, the combinations improved the activity of antifungal drugs towards *C. auris*. In all tested combinations, remarkable decreases in the MIC values for all the AMPs and drugs in comparison with the active concentration of each AMP and drug alone were observed. These findings also highlight the importance of such approaches to decrease the toxicity and the likelihood of resistance developing against the classical antifungals. The results demand further *in vivo* investigation to prove these claims and to study the cytotoxicity effects of these AMPs on host cells. There are studies indicating that human peptides do not exhibit general cytotoxicity effect on the viability of human cells. These peptides have been shown to bind selectively with the microbial cells with no or minimal interaction with the host cells, and this selectivity is thought to be highly dependent on the cationic properties of these peptides, leading to high attraction towards negatively charged microbial membranes rather than the less negatively charged host cells membranes (Ebenhan et al. 2014; Helmerhorst et al. 1999).

The antimicrobial mode of action for defensins is still under investigation. AMPs have been reported to target the microbial cell membrane lipid structure resulting in the disruption of membrane integrity, followed by formation of unspecific membrane pores leading to cytoplasmic leakage and cell death (Rautenbach et al. 2016). The peptides in this study also affected *C. auris* membrane permeability at varying levels, as evident by confocal microscopy analysis revealing the red florescence resulting from cell membrane permeabilization. This indicated that the observed fungicidal effect of the tested peptides is related to an altered cell membrane permeability of *C. auris* which leads to cell death. These results are consistent with the recent studies where AMPs have been reported to cause physical disruption of fungal cell membranes, thereby causing cell leakage and cell death (Benfield and Henriques 2020; Struyfs

et al. 2021). Similarly, human AMP belongs to the cathelicidines family has also been reported to have fungicidal and membrane disruptive activity against *C. auris* (Rather et al. 2022; van Eijk et al. 2020). Hence, it can be concluded that the mode of actions of the tested peptides in *C. auris* involves the effect on its membrane integrity. However, different mechanisms cannot be excluded at this point, these remain to be discovered in future studies. Interestingly, while the interaction of the AMPs with the microbial membrane is believed to be a crucial step in their antimicrobial activity, some AMPs are known to combine multiple mechanisms and interact with different microbial structures, which therefore potentiate their killing activity (Fernández de Ullivarri et al. 2020). This could explain the different activity levels of the tested peptides as evident from the susceptibility results.

This study demonstrated strong fungicidal activity of selected AMPs towards a small number of *C. auris* clinical isolates from South Africa (African clade). It is also worth considering a larger number of isolates including strains from the other phylogenetic clusters (South American, East Asian, South Asian and Iranian clades) for future studies, since high genomic variations between these clades have already been proved by whole-genome sequences analysis (Chow et al. 2019; Lockhart et al. 2017b).

3.5. Conclusion

MDR *C. auris* has been associated with hospital-acquired invasive infections with high mortalities. Since the available treatment options are very limited and relatively toxic to host cells, this emphasizes the demand for the development of new alternatives to treat these infections. The results described in this work have shown that the tested AMPs, especially hBD-3, have fungicidal activity towards *C. auris* alone or when combined with known antifungal agents. Therefore, this study suggests the potential of human β -defensins and other human AMPs to be used as novel antifungal agents or in combination therapies.

3.6. References

- Ahmad A, Wani MY, Khan A, Manzoor N, Molepo J (2015) Synergistic interactions of eugenol-tosylate and its congeners with fluconazole against *Candida albicans*. Plos one 10(12):e0145053. <https://doi.org/10.1371/journal.pone.0145053>
- Baddley JW, Pappas PG (2005) Antifungal combination therapy. Drugs 65(11):1461-1480. <https://doi.org/10.2165/00003495-200565110-00002>
- Benfield AH, Henriques ST (2020) Mode-of-action of antimicrobial peptides: membrane disruption vs. intracellular mechanisms. Front Med Technol 2:610997. <https://doi.org/10.3389/fmedt.2020.610997>
- 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 3(3):46. <https://doi.org/10.3390/jof3030046>
- Buda De Cesare G, Cristy SA, Garsin DA, Lorenz MC (2020) Antimicrobial peptides: a new frontier in antifungal therapy. mBio 11(6):e02123-20. <https://doi.org/10.1128/mBio.02123-20>
- Candoni A, Caira M, Cesaro S, Busca A, Giacchino M, Fanci R, Delia M, Nosari A, Bonini A, Cattaneo C, Melillo L, Caramatti C, Milone G, Scime' R, Picardi M, Fanin R, Pagano L (2014) Multicentre surveillance study on feasibility, safety and efficacy of antifungal combination therapy for proven or probable invasive fungal diseases in haematological patients: the SEIFEM real-life combo study. Mycoses 57(6):342-350. <https://doi.org/10.1111/myc.12161>
- Candida auris*: Information for laboratorians and health professionals. Atlanta, Georgia: US department of health and human services, Centers for Disease Control and Prevention (2019) Available online: <https://www.cdc.gov/fungal/candida-auris/health-professionals.html>
- Choi KY, Chow LN, Mookherjee N (2012) Cationic host defence peptides: multifaceted role in immune modulation and inflammation. J Innate Immun 4(4):361-370. <https://doi.org/10.1159/000336630>

- Chow NA, de Groot T, Badali H, Abastabar M, Chiller TM, Meis JF (2019) Potential fifth clade of *Candida auris*, Iran, 2018. *Emerg Infect Dis* 25(9):1780-1781. <https://doi.org/10.3201/eid2509.190686>
- Ciociola T, Giovati L, Conti S, Magliani W, Santinoli C, Polonelli L (2016) Natural and synthetic peptides with antifungal activity. *Future Med Chem* 8(12):1413-1433. <https://doi.org/10.4155/fmc-2016-0035>
- Clinical and Laboratory Standards Institute (CLSI) Reference method for broth dilution antifungal susceptibility testing of yeasts. informat. Wayne, Pennsylvania 19087 USA (2008)
- Dahiya S, Chhillar AK, Sharma N, Choudhary P, Punia A, Balhara M, Kaushik K, Parmar VS (2020) *Candida auris* and nosocomial infection. *Curr drug targets* 21(4): 365-373. <https://doi.org/10.2174/1389450120666190924155631>
- Dal Mas C, Rossato L, Shimizu T, Oliveira EB, da Silva Junior PI, Meis JF, Colombo AL, Hayashi MAF (2019) Effects of the natural peptide crotamine from a South American rattlesnake on *Candida auris*, an emergent multidrug antifungal resistant human pathogen. *Biomolecules* 9(6):205. <https://doi.org/10.3390/biom9060205>
- do Nascimento Dias J, de Souza Silva C, de Araújo AR, Souza JMT, de Holanda Veloso Júnior PH, Cabral WF, da Glória da Silva M, Eaton P, de Souza de Almeida Leite JR, Nicola AM, Albuquerque P, Silva-Pereira I (2020) Mechanisms of action of antimicrobial peptides ToAP2 and NDBP-5.7 against *Candida albicans* planktonic and biofilm cells. *Sci Rep* 10(1):10327. <https://doi.org/10.1038/s41598-020-67041-2>
- Ebenhan T, Gheysens O, Kruger HG, Zeevaart JR, Sathekge MM (2014) Antimicrobial peptides: their role as infection-selective tracers for molecular imaging. *Biomed Res Int* 2014:867381. <https://doi.org/10.1155/2014/867381>
- Fakhim H, Chowdhary A, Prakash A, Vaezi A, Dannaoui E, Meis JF, Badali H (2017) *In Vitro* Interactions of echinocandins with triazoles against multidrug-resistant *Candida auris*. *Antimicrob Agents Chemother* 61(11):e01056-17. <https://doi.org/10.1128/AAC.01056-17>

- Fernández de Ullivarri M, Arbulu S, Garcia-Gutierrez E, Cotter PD (2020) Antifungal peptides as therapeutic agents. *Front Cell Infect Microbiol* 10:105. <https://doi.org/10.3389/fcimb.2020.00105>
- Hancock RE, Chapple DS (1999) Peptide antibiotics. *Antimicrob Agents Chemother* 43(6):1317-1323. <https://doi.org/10.1128/AAC.43.6.1317>
- Helmerhorst EJ, Reijnders IM, van 't Hof W, Veerman EC, Nieuw Amerongen AV (1999) A critical comparison of the hemolytic and fungicidal activities of cationic antimicrobial peptides. *FEBS letters* 449(2-3):105-110. [https://doi.org/10.1016/s0014-5793\(99\)00411-1](https://doi.org/10.1016/s0014-5793(99)00411-1)
- Hou X, Lee A, Jiménez-Ortigosa C, Kordalewska M, Perlin DS, Zhao Y (2019) Rapid detection of ERG11-associated azole resistance and FKS-associated echinocandin resistance in *Candida auris*. *Antimicrob Agents Chemother* 63(1):e01811-18. <https://doi.org/10.1128/AAC.01811-18>
- Huang Q, Fei J, Yu HJ, Gou YB, Huang XK (2014) Effects of human β -defensin-3 on biofilm formation-regulating genes *dltB* and *icaA* in *Staphylococcus aureus*. *Mol Med Rep* 10(2):825-831. <https://doi.org/10.3892/mmr.2014.2309>
- Jaggavarapu S, Burd EM, Weiss DS (2020) Micafungin and amphotericin B synergy against *Candida auris*. *Lancet Microbe* 1(8):e314-e315. [https://doi.org/10.1016/S2666-5247\(20\)30194-4](https://doi.org/10.1016/S2666-5247(20)30194-4)
- Kang HK, Kim C, Seo CH, Park Y (2017) The therapeutic applications of antimicrobial peptides (AMPs): a patent review. *J Microbiol* 55(1):1-12. <https://doi.org/10.1007/s12275-017-6452-1>
- Klepser ME, Ernst EJ, Lewis RE, Ernst ME, Pfaller MA (1998) Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. *Antimicrob Agents Chemother* 42(5):1207-1212. <https://doi.org/10.1128/AAC.42.5.1207>
- Krishnakumari V, Rangaraj N, Nagaraj R (2008) Antifungal activity of human beta defensins HBD-1-3 and their C-terminal analogs Phd1-3. *Antimicrob Agents Chemother* 53(1):256-260. <https://doi.org/10.1128/AAC.00470-08>
- Kubiczek D, Raber H, Gonzalez-García M, Morales-Vicente F, Staendker L, Otero-Gonzalez AJ, Rosenau F (2020) Derivates of the antifungal peptide Cm-p5 inhibit

development of *Candida auris* biofilms *in vitro*. *Antibiotics* 9(7):363. <https://doi.org/10.3390/antibiotics9070363>

Lin P, Li Y, Dong K, Li Q (2015) The antibacterial effects of an antimicrobial peptide Human β -Defensin 3 fused with carbohydrate-binding domain on *Pseudomonas aeruginosa* PA14. *Curr Microbiol* 71(2):170-176. <https://doi.org/10.1007/s00284-015-0814-x>

Lockhart SR, Berkow EL, Chow N, Welsh RM (2017a) *Candida auris* for the clinical microbiology laboratory: not your grandfather's *Candida* species. *Clin Microbiol Newsl* 39(13):99-103. <https://doi.org/10.1016/j.clinmicnews.2017.06.003>

Lockhart SR, Etienne KA, Vallabhaneni S, Farooqi J, Chowdhary A, Govender NP, Colombo AL, Calvo B, Cuomo CA, Desjardins CA, Berkow EL, Castanheira M, Magobo RE, Jabeen K, Asghar RJ, Meis JF, Jackson B, Chiller T, Litvintseva AP (2017b) Simultaneous emergence of multidrug-resistant *Candida auris* on 3 continents confirmed by whole-genome sequencing and epidemiological analyses. *Clin Infect Dis* 64(2):134-140. <https://doi.org/10.1093/cid/ciw691>

Lone SA, Ahmad A (2019) *Candida auris*-the growing menace to global health. *Mycoses* 62(8):620-637. <https://doi.org/10.1111/myc.12904>

Lupetti A, Paulusma-Annema A, Welling MM, Dogterom-Ballering H, Brouwer CP, Senesi S, Van Dissel JT, Nibbering PH (2003) Synergistic activity of the N-terminal peptide of human lactoferrin and fluconazole against *Candida* species. *Antimicrob Agents Chemother* 47(1):262-267. <https://doi.org/10.1128/AAC.47.1.262-267.2003>

Lyman M, Forsberg K, Reuben J, Dang T, Free R, Seagle EE, Sexton DJ, Soda E, Jones H, Hawkins D, Anderson A, Bassett J, Lockhart SR, Merengwa E, Iyengar P, Jackson BR, Chiller T (2021) Notes from the field: transmission of pan-resistant and echinocandin-resistant *Candida auris* in health care facilities—Texas and the district of Columbia, January–April 2021. *MMWR Morb Mortal Wkly Rep* 70(29):1022-1023. <https://doi.org/10.15585/mmwr.mm7029a2>

MacCallum DM, Desbois AP, Coote PJ (2013) Enhanced efficacy of synergistic combinations of antimicrobial peptides with caspofungin versus *Candida albicans* in insect

and murine models of systemic infection. Eur J Clin Microbiol Infect Dis 32(8):1055-1062.
<https://doi.org/10.1007/s10096-013-1850-8>

Mahlapuu M, Håkansson J, Ringstad L, Björn C (2016) Antimicrobial peptides: an emerging category of therapeutic agents. Front Cell Infect Microbiol 6:194.
<https://doi.org/10.3389/fcimb.2016.00194>

Maiti S, Patro S, Purohit S, Jain S, Senapati S, Dey N (2014) Effective control of *Salmonella* infections by employing combinations of recombinant antimicrobial human β -defensins hBD-1 and hBD-2. Antimicrob Agents Chemother 58(11):6896-6903.
<https://doi.org/10.1128/AAC.03628-14>

Malmsten M (2014) Antimicrobial peptides. Ups J Med Sci 119(2):199-204.
<https://doi.org/10.3109/03009734.2014.899278>

Mercer DK, O'Neil DA (2020) Innate inspiration: Antifungal peptides and other immunotherapeutics from the host immune response. Front Immunol 11:2177.
<https://doi.org/10.3389/fimmu.2020.02177>

Morales-López SE, Parra-Giraldo CM, Ceballos-Garzón A, Martínez HP, Rodríguez GJ, Álvarez-Moreno CA, Rodríguez JY (2017) Invasive infections with multidrug-resistant yeast *Candida auris*, Colombia. Emerg Infect Dis 23(1):162-164.
<https://doi.org/10.3201/eid2301.161497>

Nguyen LT, Haney EF, Vogel HJ (2011) The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol 29(9):464-72.
<https://doi.org/10.1016/j.tibtech.2011.05.001>

Osei Sekyere J (2018) *Candida auris*: A systematic review and meta-analysis of current updates on an emerging multidrug-resistant pathogen. Microbiologyopen 7(4):e00578.
<https://doi.org/10.1002/mbo3.578>

Ostrowsky B, Greenko J, Adams E, Quinn M, O'Brien B, Chaturvedi V, Berkow E, Vallabhaneni S, Forsberg K, Chaturvedi S, Lutterloh E, Blog D (2020) *Candida auris* isolates resistant to three classes of antifungal medications—New York, 2019. MMWR Morb Mortal Wkly Rep 69(1):6-9. <https://doi.org/10.15585/mmwr.mm6901a2>

- 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. *Antimicrob Agents Chemother* 62(2):e01872-17. <https://doi.org/10.1128/AAC.01872-17>
- Polesello V, Segat L, Crovella S, Zupin L (2017) *Candida* infections and human defensins. *Protein Pept Lett* 24(8):747-756. <https://doi.org/10.2174/0929866524666170807125245>
- Puri S, Edgerton M (2014) How does it kill?: understanding the candidacidal mechanism of salivary histatin 5. *Eukaryot Cell* 13(8):958-964. <https://doi.org/10.1128/EC.00095-14>
- Raber HF, Sejfić J, Kissmann AK, Wittgens A, Gonzalez-Garcia M, Alba A, Vázquez AA, Morales Vicente FE, Erviti JP, Kubiczek D, Otero-González A, Rodríguez A, Ständker L, Rosenau F (2021) Antimicrobial Peptides Pom-1 and Pom-2 from *Pomacea pomeliana* Are Active against *Candida auris*, *C. parapsilosis* and *C. albicans* Biofilms. *Pathogens* 10(4):496. <https://doi.org/10.3390/pathogens10040496>
- Rather IA, Sabir JSM, Asseri AH, Ali S (2022) Antifungal activity of human cathelicidin LL-37, a membrane disrupting peptide, by triggering oxidative stress and cell cycle arrest in *Candida auris*. *J Fungi (Basel)* 8(2):204. <https://doi.org/10.3390/jof8020204>
- Rautenbach M, Troskie AM, Vosloo JA (2016) Antifungal peptides: to be or not to be membrane active. *Biochimie* 130:132-145. <https://doi.org/10.1016/j.biochi.2016.05.013>
- Satoh K, Makimura K, Hasumi Y, Nishiyama Y, Uchida K, Yamaguchi H (2009) *Candida auris* sp. nov., a novel ascomycetous yeast isolated from the external ear canal of an inpatient in a Japanese hospital. *Microbiol Immunol* 53(1):41–44. <https://doi.org/10.1111/j.1348-0421.2008.00083>
- Schneider JJ, Unholzer A, Schaller M, Schäfer-Korting M, Korting HC (2005) Human defensins. *J Mol Med* 83(8):587-595. <https://doi.org/10.1007/s00109-005-0657-1>
- Scorzoni L, de Paula E Silva AC, Marcos CM, Assato PA, de Melo WC, de Oliveira HC, Costa-Orlandi CB, Mendes-Giannini MJ, Fusco-Almeida AM (2017) Antifungal therapy: new advances in the understanding and treatment of mycosis. *Front Microbiol* 8:36. <https://doi.org/10.3389/fmicb.2017.00036>

- Singh K, Shekhar S, Yadav Y, Xess I, Dey S (2017) DS6: anticandidal, antibiofilm peptide against *Candida tropicalis* and exhibit synergy with commercial drug. J Pept Sci 23(3):228-235. <https://doi.org/10.1002/psc.2973>
- Spitzer M, Robbins N, Wright GD (2017) Combinatorial strategies for combating invasive fungal infections. Virulence 8(2):169-185. <https://doi.org/10.1080/21505594.2016.1196300>
- Struyfs C, Cammue BPA, Thevissen K (2021) Membrane-Interacting Antifungal Peptides. Front Cell Dev Biol 9:649875. <https://doi.org/10.3389/fcell.2021.649875>
- Suchodolski J, Feder-Kubis J, Krasowska A (2017) Antifungal activity of ionic liquids based on (-)-menthol: a mechanism study. Microbiol Res 197:56-64. <https://doi.org/10.1016/j.micres.2016.12.008>
- van Eijk M, Boerefijn S, Cen L, Rosa M, Morren MJH, van der Ent CK, Kraak B, Dijksterhuis J, Valdes ID, Haagsman HP, de Cock H (2020) Cathelicidin-inspired antimicrobial peptides as novel antifungal compounds. Med Mycol 58(8):1073-1084. <https://doi.org/10.1093/mmy/myaa014>
- Vicente FEM, González-García M, Díaz Pico E, Moreno-Castillo E, Garay HE, Rosi PE, Jimenez AM, Campos-Delgado JA, Rivera DG, Chinae G, Pietro RCLR, Stenger S, Spellerberg B, Kubiczek D, Bodenberger N, Dietz S, Rosenau F, Paixão MW, Ständker L, Otero-González AJ (2019) Design of a helical-stabilized, cyclic, and nontoxic analogue of the peptide Cm-p5 with improved antifungal activity. ACS omega 4(21):19081-19095. <https://doi.org/10.1021/acsomega.9b02201>
- Vylkova S, Li XS, Berner JC, Edgerton M (2006) Distinct antifungal mechanisms: β -defensins require *Candida albicans* Ssa1 protein, while Trk1p mediates activity of cysteine-free cationic peptides. Antimicrob Agents Chemother 50(1):324-331. <https://doi.org/10.1128/AAC.50.1.324-331.2006>
- Wei GX, Bobek LA (2004) In vitro synergic antifungal effect of MUC7 12-mer with histatin-5 12-mer or miconazole. J Antimicrob Chemother 53(5):750-758. <https://doi.org/10.1093/jac/dkh181>

Yu G, Baeder DY, Regoes RR, Rolff J (2018) Predicting drug resistance evolution: insights from antimicrobial peptides and antibiotics. *Proc Biol Sci* 285(1874):20172687. <https://doi.org/10.1098/rspb.2017.2687>

Zhu Y, O'Brien B, Leach L, Clarke A, Bates M, Adams E, Ostrowsky B, Quinn M, Dufort E, Southwick K, Erazo R, Haley VB, Bucher C, Chaturvedi V, Limberger RJ, Blog D, Lutterloh E, Chaturvedi S (2020) Laboratory analysis of an outbreak of *Candida auris* in New York from 2016 to 2018: impact and lessons learned. *J Clin Microbiol* 58(4):e01503-19. <https://doi.org/10.1128/JCM.01503-19>

Zimmermann GR, Lehár J, Keith CT (2007) Multi-target therapeutics: when the whole is greater than the sum of the parts. *Drug Discov Today* 12(1-2):34-42. <https://doi.org/10.1016/j.drudis.2006.11.008>

Chapter 4: Antifungal activity of human antimicrobial peptides targeting apoptosis in *Candida auris*

4.1. Introduction

Candida auris is a highly threatening yeast that causes severe disseminated infections associated with substantial mortality (1). It is considered a multi-drug resistant (MDR) pathogen that showed resistance to many commonly used antifungal drugs, managing *C. auris* infections has therefore become a major healthcare concern (2). The high rates of resistance to the azoles and polyenes have prompted the use of echinocandins as an initial therapy. However, frequent susceptibility testing has been recommended as echinocandins resistance might indeed develop (3). Of major concern, only few promising agents are currently being developed as future therapeutic options to combat evolving resistance to antifungals (4). Thus, more prospective compounds, particularly those with new mechanism of action targeting novel antifungal pathways, are required to control infections caused by this MDR species.

Antimicrobial peptides (AMPs) constitute a diverse class of unique biomolecules within the innate defence system in a variety of unicellular and multicellular living organisms (5). They play a key role in defending the host against various microbial pathogens during the early stages of the infection process. In addition to their direct antimicrobial properties, they also exert indirect effects that modulate the host immune response, particularly in humans (6). Generally, these peptides demonstrate exceptional broad-spectrum antimicrobial action against common pathogenic microorganisms, likely contributed to their cationic and amphipathic properties which allow them to interact with the anionic lipid bilayer of the microbial membrane (7). In comparison to standard antimicrobial agents, most of them are less toxic to human cells and do not easily induce resistance (8). Consequently, AMPs represent the most promising alternative to antifungal drugs, which are often associated with resistance and serious adverse effects.

Different mechanisms of action for AMPs have been discovered, particularly membrane targeting by promoting its permeabilization (9). AMPs may also cross the membrane and bind to intracellular targets or inhibit metabolic pathways, which consequently leads to cell death in pathogens (10). Several studies have concluded that the key mechanisms for AMPs have been linked to the induction of apoptosis in pathogenic fungi (11).

Apoptosis is a type of regulated cell death resulted from ageing processes and triggered via several internal stimuli. (12). Apoptosis has been documented in yeasts where apoptotic cells display a characteristic series of morphological and biochemical alterations, in which plasma membrane integrity is maintained and intracellular components undergo degradation followed by secretion of some factors which stimulate phagocytosis (13). In contrast, necrosis, an unregulated type of cell death, is characterized by cell membrane rupture and release of intracellular contents which stimulate host immune reaction (13). Generally, apoptotic cells display characteristics markers which differentiate apoptosis from necrosis. These include the movement of phosphatidyl-serine (PS) to the outer cell membrane, release of cytochrome c from the mitochondria, production of reactive oxygen species (ROS) and condensation of chromatin inside the nuclei leading to nuclear fragmentation and DNA degradation (14). In yeast, apoptotic cell death can also be activated by different external triggers, for instance exposure to certain conditions such as acid or salt stress (15, 16). Moreover, distinctive classes of AMPs have been shown to induce apoptosis in *C. albicans* (11). Targeting apoptotic cell death in fungi has become an attractive aspect in developing novel antifungal agent.

Human defensins and human histatins peptides are among the most common AMPs with antifungal efficacy expressed in humans (17). The effectiveness of these peptides against many human pathogenic fungi, such as *Candida*, *Aspergillus* and *Cryptococcus* species has already been reported (18). Different antifungal mechanisms of such peptides have been reported in *C. albicans* (18). Previously, in our research, selected human defensins and histatin 5 (His 5) were shown to have remarkable fungicidal activity against *C. auris*. Moreover, they increased the cell membrane permeability as a part of their mechanism of action, nevertheless, this may not be sufficient to cause cell death (19).

In this study, the potential of the selected peptides to induce apoptosis and/or necrosis in *C. auris* cells as another antifungal mechanism was evaluated. we investigated some apoptotic hallmarks and triggers caused by three peptides involving mainly the phosphatidylserine externalization, DNA fragmentation, depolarization of mitochondrial membrane, cytochrome c release and production of intracellular ROS.

4.2. Materials and methods

4.2.1. *Candida* strains and media

Two clinical isolates of *C. auris* were utilized in this study, CAU-S (Strain susceptible to fluconazole, amphotericin B, and caspofungin) and CAU-R (Strain resistance to all three antifungals, MDR). The isolates were derived from different human clinical specimens, including blood, urine and body fluids. These isolates were obtained from the Division of Mycology, National Institute of Communicable Diseases, Johannesburg, South Africa, where they were confirmed as *C. auris*. The isolates were then transported to the Clinical Microbiology laboratory at the University of the Witwatersrand and stored in glycerol stock. For the experiments, they were grown at 37 °C on Sabouraud dextrose agar (SDA; Merck, Germany) plates for 24 h, and then a few colonies were suspended in 15 ml fresh Sabouraud dextrose Broth (SDB; Merck, Germany) medium with continuous shaking at 200 rpm for 18 h. Thereafter, a *Candida* suspension was made by diluting the cells to 1×10^6 CFU/ml after being washed three times with phosphate buffer solution (PBS; Sigma-Aldrich. St. Louis, MO, USA). Ethical approval for the use of isolates was granted by the Human Research Ethics Committee of the University of the Witwatersrand (M140159).

4.2.2. Antimicrobial peptides

Three peptides representing distinct classes of human AMPs, which are human β -defensin-3 (hBD-3), which belongs to β -defensins; Human neutrophil peptide-1 (HNP-1), which belongs to α -defensins; and His 5, which belongs to histidine, were obtained from Thermo Fisher Scientific, IL, USA. According to the supplier, peptides were synthesized utilizing the Fmoc solid phase technique. Mass spectrometric (MS) analyses using Applied Biosynthesis Voyager DE system was used to validate the peptide sequence, and purity of 95% was verified using analytical high-performance liquid chromatography (HPLC) system. Stock solutions (5 mg/ml) were prepared in sterile water and stored in the dark at -20°C for subsequent experiments.

4.2.3. Antifungal susceptibility testing

The minimum inhibitory concentrations (MICs) of the AMPs (hBD-3, HNP-1 and His 5) and the antifungals (fluconazole, amphotericin B and caspofungin; Sigma-Aldrich. St. Louis, MO, USA) towards *C. auris* isolates were determined following modified Clinical and Laboratory Standards Institute (CLSI) guidelines (20). In brief, 100 μl of media was placed in each well

of the microtiter plates. From the initial concentration of the test compounds, 100 µl were added to each well for two-fold dilutions, followed by 100 µl of the test culture. After 24 hours incubation at 35 °C, the MIC was recorded as the lowest concentration without visible growth. Wells without growth were then subcultured onto SDA plates and incubated for 24 hours to determine the minimum fungicidal concentrations (MFCs). MFC were regarded as concentration in the first well without any growth. To verify the culture viability and sterility of media, positive, negative, and culture controls were included in every set of experiments, and to validate the results, the experiment was performed in triplicate.

4.2.4. Preparation of protoplasts for cell death studies

To investigate the potential of the tested AMPs to induce apoptotic markers in *C. auris*, protoplasts were prepared by removing the cell wall using an enzymatic method (21). In brief, cells were subjected to various peptide concentrations (0.5 MIC, 1 MIC, and MFC) for 3 hours. Untreated negative and H₂O₂ (10 mM) treated positive controls were included as well. The cells were thereafter washed three times with protoplast buffer-A (30 mM DTT, 1 M sorbitol, 10 mM MgCl₂ and 50 mM Tris base; Sigma-Aldrich. St. Louis, MO, USA) for 5 min each time. Then, cells were incubated in protoplast buffer-B (1 mM DTT, 1 M sorbitol, 10 mM MgCl₂ and 50 mM tris base) enriched with 1 µg/ml lyticase enzyme (Sigma-Aldrich. St. Louis, MO, USA) at 25 °C for 2 hours. The cells were subsequently harvested and incubated in protoplast buffer-C (1 M sorbitol, 10 mM MgCl₂ and 50 mM tris base) at 25 °C for 15 min. Finally, the protoplasts were washed once and resuspended in PBS for further use.

4.2.5. FITC Annexin V/PI double labelling assay

The FITC Annexin V/PI double labelling assay was employed to study the translocation of phosphatidylserine (PS) from the inner to the outer membrane during early apoptosis. The translocation was detected using the FITC annexin V Apoptosis Detection Kit I (BD Biosciences, CA, USA) as per manufacturer's recommendation. The prepared protoplasts, as described above, were washed with cold PBS and resuspended in 1X Annexin V binding buffer at a density of 1×10^6 cells/ml and 100 µl of this cell suspension were transferred to a 5 ml culture tube, 5 µl of FITC-Annexin V and 5 µl of propidium iodide (PI) were then added, the tube was gently mixed and incubated at 25 °C for 15 min in the dark. Then, 400 µl of 1X binding buffer were added to each tube. A flow cytometer (BD LSRFortessa™, Becton Dickinson, NJ, USA) was used to quantify the results and FlowJo™ v10.8 Software (BD

Biosciences) was used to analyze the data. The cell population was categorized into four quadrants based on FITC-Annexin V and PI staining: Q1: (FITC-Annexin V⁻/PI⁺ - necrosis), Q2: (FITC-Annexin V⁺/PI⁺ - late apoptosis), Q3: (FITC-Annexin V⁺/PI⁻ - early apoptosis) and Q4: (FITC-Annexin V⁻/PI⁻ - viable cells).

4.2.6. TUNEL (terminal deoxynucleotidyl transferase nick-end labeling) assay

To study the late stage of apoptosis, which is characterized by DNA fragmentation, the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Germany) was used following the manufacturer's guidelines. Briefly, protoplasts were permeabilized with 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) at 25 °C for 15 minutes and washed twice with deionized water. Protoplast were then labelled with 50 µl of TUNEL reaction solution (450 µl Label and 50 µl Enzyme Solutions), incubated for at 37 °C for 1 hour in dark condition, then washed twice with PBS. Imaging and quantification were performed using Confocal laser scanning microscope (Zeiss LSM 780) with Airyscan (excitation/emission Ex/Em: 495/519 nm).

4.2.7. Measurement of mitochondrial membrane potential/membrane depolarization assay

The depolarization of *C. auris* mitochondrial membranes following the exposure to the test AMPs was studied utilizing the JC-10 Mitochondrial Membrane Potential Assay Kit (Microplate; Abcam, UK) as per the manufacture's instructions. In brief, the protoplasts, as stated above, were plated in a 96-Well Black/Clear Bottom Microtiter Plates (Thermo Fisher Scientific, Germany) at 90 µL per well. First, we added 50 µL of JC-10 dye-loading solution (a mixture of 50 µl 100X JC-10 and 5 ml assay buffer A) and incubated for 30 min at 37 °C. Then, we added 50 µl of JC-10 assay buffer B and fluorescence intensity was immediately measured utilizing a SpectraMax[®] iD3 Microplate Readers (Molecular Devices, USA). Fluorescence was determined at excitation/emission Ex/Em = 540/590 nm for JC-10 aggregates (red fluorescence) and at 490/525 nm for JC-10 monomers (green fluorescence). The ratio of red to green fluorescence was calculated to assess alterations in mitochondrial membrane potential.

4.2.8. Evaluation of cytochrome c release

The cytochrome c release from the mitochondria in *C. auris* cells exposed to the test AMPs was examined using a spectrophotometric method as described (22). Briefly, the previously prepared protoplasts were harvested by centrifugation and homogenized in buffer-I, which contains 2 mM EDTA, 50 mM Tris (pH 7.5) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA), enriched with 2% glucose. The homogenized mixture was then centrifuged at 5000 rpm for 10 min to separate cell debris and collect the supernatant, which was further centrifuged at 50,000 rpm for 45 min and examined to quantify the levels of cytosolic cytochrome c. To determine the levels of mitochondrial cytochrome c, the resulting cell pellet was suspended in buffer-II, which contains 2 mM EDTA and 50 mM Tris (pH 5.0), and finally centrifuged at 10,000 rpm for 30 s to extract pure mitochondria. The extract was then treated with 500 mg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) to obtain reduced cytochrome c. The relative levels of the reduced form were measured by determining the absorbance using a spectrophotometer (UV-1800, Shimadzu, Japan) at 550 nm.

4.2.9. Evaluation of intracellular reactive oxygen species (ROS) accumulation

The effect of test AMPs on intracellular ROS production was evaluated utilizing the fluorometric technique with 2',7'-dichlorofluorescein diacetate (H₂DCFDA, Molecular Probes) as previously described (23). *C. auris* cells, pre-treated with different concentration of the tested AMPs for 3 hours, were washed and suspended in PBS. H₂DCFDA (10 µM) was added to the suspension and incubated for 30 min at 28 °C. Then, the cells were washed twice with PBS and analysed using flow cytometer (Becton Dickinson, NJ, USA).

4.2.10. Data analysis

All experiments were conducted in triplicate. Statistical analysis was performed using Graph Pad Prism 9.0 software. Data were presented as the mean ± standard deviation (mean ±SD) of three independent replicates. Differences were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's comparison test. *P* value of <0.05 was considered statistically significant.

4.3. Results

4.3.1. Susceptibility testing for peptides and antifungals

Table 4.1 illustrates the MIC and MFC results for AMPs and antifungals against two *C. auris* isolates which were determined in this research and in prior study (19). The antifungals (fluconazole, amphotericin B and caspofungin) were initially used to further confirm the susceptibility pattern of the tested strains and determine test concentration for further experiments. According to these results, the chosen peptides (hBD-3, HNP-1 and His 5) exhibited substantial antifungal activity against both the sensitive and MDR *C. auris* strains. Thereafter, to determine which type of cell death underlies the fungicidal effects of the tested AMPs against *C. auris*, the influence of each AMP at different concentrations on the induction of apoptotic markers were investigated.

4.3.2. Effect of AMPs on PS externalization in *C. auris*

The FITC Annexin V/PI double labelling technique is a commonly used approach to study apoptosis by flow cytometry. Annexin V-FITC exhibits a high affinity for the externalized PS in apoptotic cells, revealing the existence of both of early and late apoptotic cells. Whereas PI penetrates cells with damaged plasma membrane, confirming the existence of late apoptotic and necrotic cells. Both dyes, however, are impermeable to viable healthy cells with intact membrane (24). As shown by flow cytometry results (Table 4.2), when CAU-S and CAU-R were exposed to hBD-3 and HNP-1 (at either 0.5 MIC, 1 MIC, and MFC), cells exhibited significant increase in the percentage of early apoptosis (FITC-Annexin V⁺/PI⁻) and late apoptosis (FITC-Annexin V⁺/PI⁺), with no to minimal non-significant undergoing necrosis (FITC-Annexin V⁻/PI⁺), when compared to untreated cell population. On the other hand, a large proportion of necrotic cells was observed as a result of His 5 exposure (at 0.5 MIC, 1 MIC, and MFC), with some less significant early apoptotic and late apoptotic cells for both tested strains. Overall, the effects of the tested peptides were concentration-dependent and notably comparable in both the sensitive and resistant *C. auris* strains. Fig. 4.1 displays representative flowcytometric dot plots of *C. auris* exposed to various concentrations of the tested peptides, as well untreated negative and H₂O₂ (10 mM) treated positive controls.

Table 4.1: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of peptides and antifungals against *Candida auris* isolates (n=3)

AMPs / Antifungal		<i>C. auris</i> strains	
		CAU-S	CAU-R
hBD-3	MIC	3.125	6.25
	MFC	6.25	12.5
HNP-1	MIC	25	50
	MFC	50	100
His 5	MIC	25	25
	MFC	50	50
AMB	MIC	0.125 (S)	4 (R)
	MFC	0.25	8
CAS	MIC	0.25 (S)	2 (R)
	MFC	0.5	4
FLU	MIC	16 (S)	500 (R)

- The values are given in µg/ml, each value represents the median of 3 independent experiments.
- hBD-3, human β -Defensin-3; HNP-1, human α -Defensin-1; His 5, Histatin 5; AMB, amphotericin B; CAS, caspofungin; FLU, fluconazole. The values are given in µg/ml.
- S, sensitive; R, resistance. Classification based on CDC guidelines (Tentative MIC Breakpoints); FLU (S<32 µg/ml; R $\geq$ 32 µg/ml); AMP (S<2 µg/ml; R $\geq$ 2 µg/ml); CAS (S<2 µg/ml; R $\geq$ 2 µg/ml)

Table 4.2: The proportion of *Candida auris* cells showing early apoptosis, late apoptosis and necrosis after being exposed to 0.5 MIC, MIC and MFC of hBD-3, HNP-1 and His 5, as determined by FITC Annexin V/PI double labelling assay (n=3).

% of non-viable cells (mean $\pm$ SD)		Negative control	hBD-3			HNP-1			His 5		
			½ MIC	MIC	MFC	½ MIC	MIC	MFC	½ MIC	MIC	MFC
Early apoptosis	CAU-S	0.06 $\pm$ 0.02	22.87 $\pm$ 2.08***	49.27 $\pm$ 1.43****	69.47 $\pm$ 1.28****	15.20 $\pm$ 1.90**	40.00 $\pm$ 0.25****	45.30 $\pm$ 1.60****	0.03 $\pm$ 0.00	0.78 $\pm$ 0.28	1.94 $\pm$ 0.75
	CAU-R	0.08 $\pm$ 0.02	20.17 $\pm$ 2.13***	37.27 $\pm$ 0.33****	42.13 $\pm$ 1.63****	25.97 $\pm$ 2.43***	28.40 $\pm$ 1.35****	39.37 $\pm$ 1.53****	0.13 $\pm$ 0.01	4.13 $\pm$ 2.01	3.18 $\pm$ 1.25
Late apoptosis	CAU-S	0.04 $\pm$ 0.01	0.96 $\pm$ 0.31*	4.15 $\pm$ 0.70**	7.30 $\pm$ 0.21****	0.64 $\pm$ 0.08**	6.00 $\pm$ 0.26****	25.27 $\pm$ 0.92****	0.07 $\pm$ 0.00	4.62 $\pm$ 1.05*	5.07 $\pm$ 1.38*
	CAU-R	0.03 $\pm$ 0.01	2.38 $\pm$ 0.50**	23.83 $\pm$ 0.32****	31.67 $\pm$ 1.87****	3.22 $\pm$ 0.26***	19.00 $\pm$ 1.60****	32.47 $\pm$ 1.72****	2.01 $\pm$ 0.50*	4.47 $\pm$ 1.36*	3.05 $\pm$ 0.73*
Necrosis	CAU-S	0.09 $\pm$ 0.05	0.41 $\pm$ 0.30	0.03 $\pm$ 0.01	0.03 $\pm$ 0.02	0.09 $\pm$ 0.03	1.71 $\pm$ 0.85	1.09 $\pm$ 0.36	7.96 $\pm$ 0.81***	23.90 $\pm$ 1.20****	60.07 $\pm$ 0.42****
	CAU-R	0.05 $\pm$ 0.05	0.48 $\pm$ 0.46	1.48 $\pm$ 0.51	1.16 $\pm$ 0.58	0.00 $\pm$ 0.00	0.21 $\pm$ 0.04	1.31 $\pm$ 0.52	23.33 $\pm$ 1.53***	58.20 $\pm$ 1.70****	69.70 $\pm$ 1.20****

- Untreated *C. auris* cells were used as negative control.
- Data are shown as means $\pm$ SD from three independent experiments.
- Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparisons test. The asterisks denote a significant difference (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) compared with the negative control group.

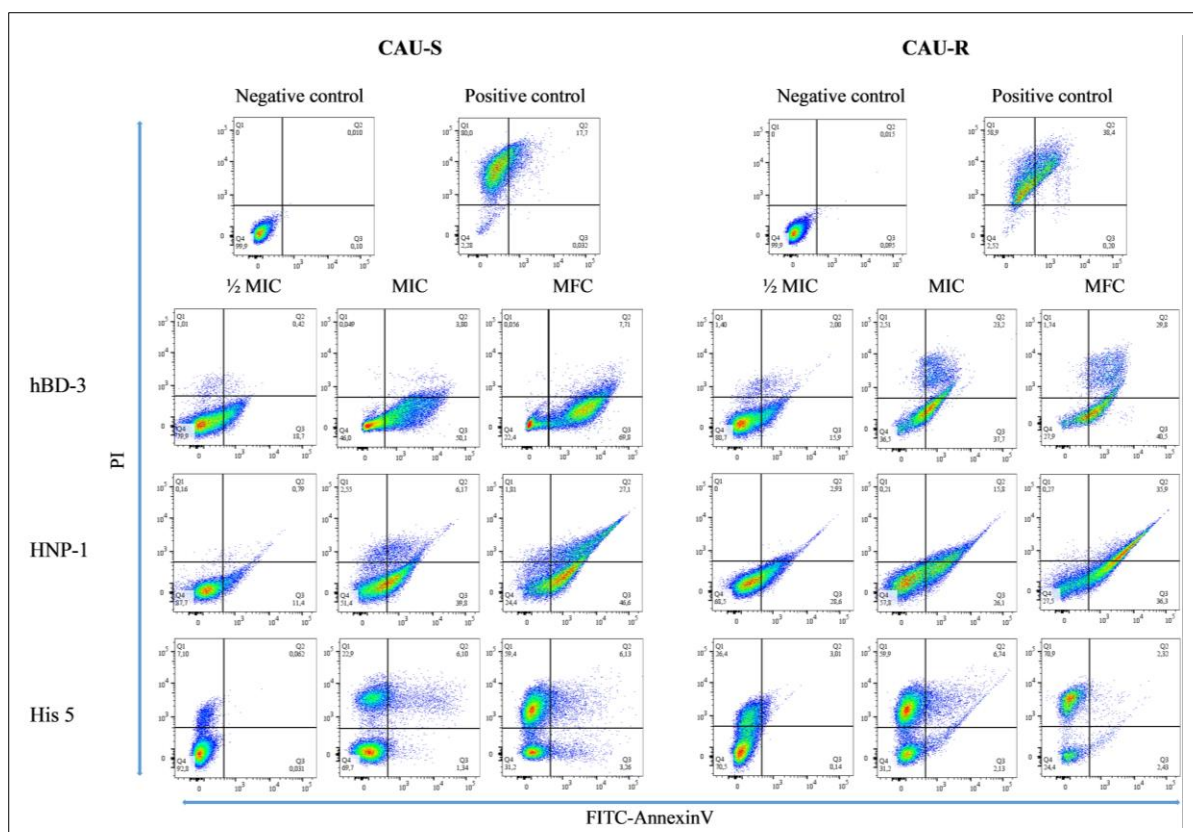


Figure 4.1: Flow cytometric analysis of phosphatidylserine exposure using FITC Annexin V/PI double labelling assay. *C. auris* cells were exposed to 0.5 MIC, MIC and MFC of hBD-3, HNP-1 and His 5. Untreated cells and cells exposed to H₂O₂ (10 mM) were used as negative and positive controls respectively. In each density plot quadrant Q1: shows necrotic cells (FITC-Annexin V–/PI+); Q2: late apoptotic cells (FITC-Annexin V+/PI+); Q3: early apoptotic cells (FITC-Annexin V+/PI–) and Q4: shows viable cells (FITC-Annexin V–/PI–). The figures shown are representative data of three independent experiments.

4.3.3. Effect of AMPs on DNA fragmentation in *C. auris*

The other important indicator in apoptosis research is DNA fragmentation, which was detected using TUNEL staining approach. This technique is based on the selective binding of modified fluorescein-dUTP with the DNA strand breaks, and its green fluorescence indicates DNA fragmentation in late apoptotic cells (25). In this assay, confocal microscopy imaging verified the existence of green fluorescence in cells exposed to the different concentration of hBD-3 and HNP-1, which confirms the presence of late of apoptotic cells. This is consistent with the results observed in positive control cells treated with 10 mM H₂O₂ (Fig. 4.2). As shown in Fig. 4.3, when CAU-S and CAU-R were subjected to three different concentrations of hBD-3 and HNP-1, samples revealed a significant increase in the number of TUNEL positive cells,

compared to negative control group. In contrast, green fluorescence spots were occasionally observed when cells were exposed to His 5 (at 0.5 MIC, 1 MIC, and MFC), in both *C. auris* strains. Furthermore, the results of this assay indicate that hBD-3 and HNP-1 exhibit a concentration-dependent impact on the overall percentages and the green fluorescence intensity of TUNEL positive cells in both the sensitive and resistant *C. auris* strains. While His 5 showed non-significant numbers of TUNEL positive cells, the fluorescence was also noticeably less intense than that seen in the positive control. Therefore, these findings verify that, hBD-3 and HNP-1, unlike His 5, produced characteristics\ DNA fragmentation associated with late apoptosis in *C. auris* which correlates well with the abovementioned flowcytometric results.

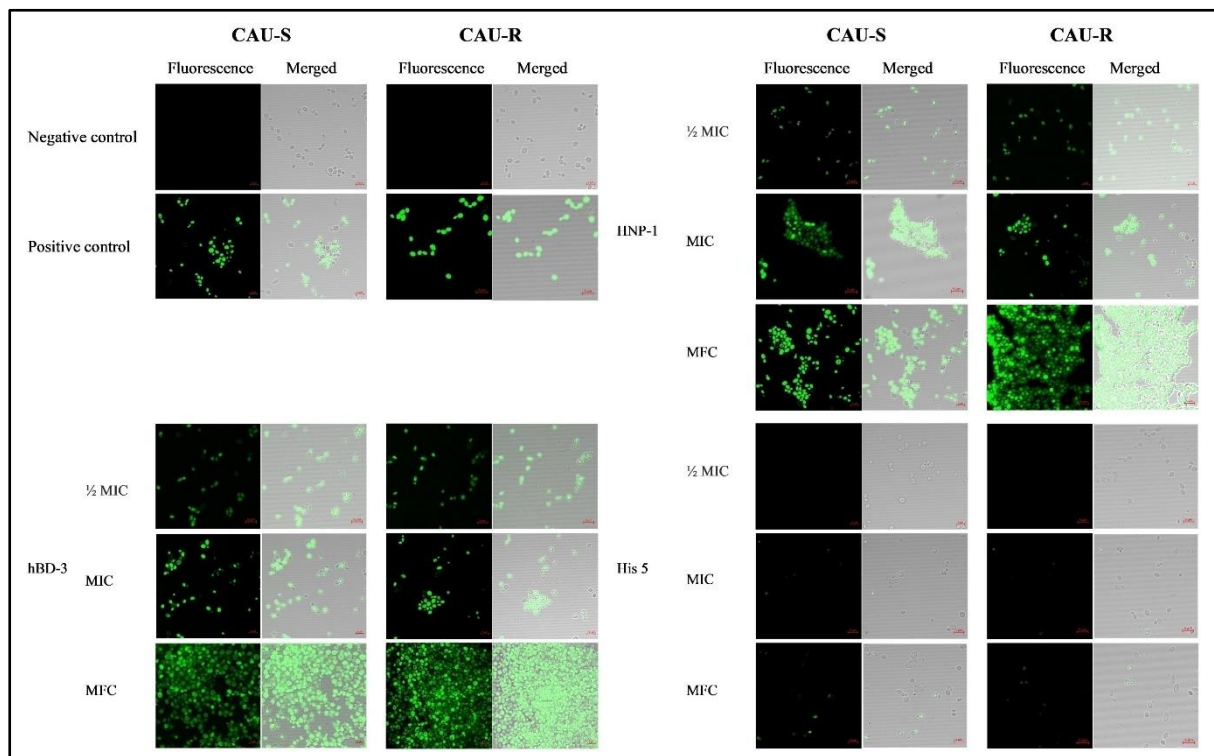


Figure 4.2: Confocal scanning fluorescence images represent DNA fragmentation caused by tested peptides in *Candida auris* cells as shown by TUNEL assay. Cells exposed to 0.5 MIC, MIC and MFC of hBD-3, HNP-1 and His 5. Untreated cells and cells exposed to H₂O₂ (10 mM) were used as negative and positive controls, respectively. TUNEL positive cells (green fluorescence) represent apoptotic cells. The figures shown are representative data of three independent experiments.

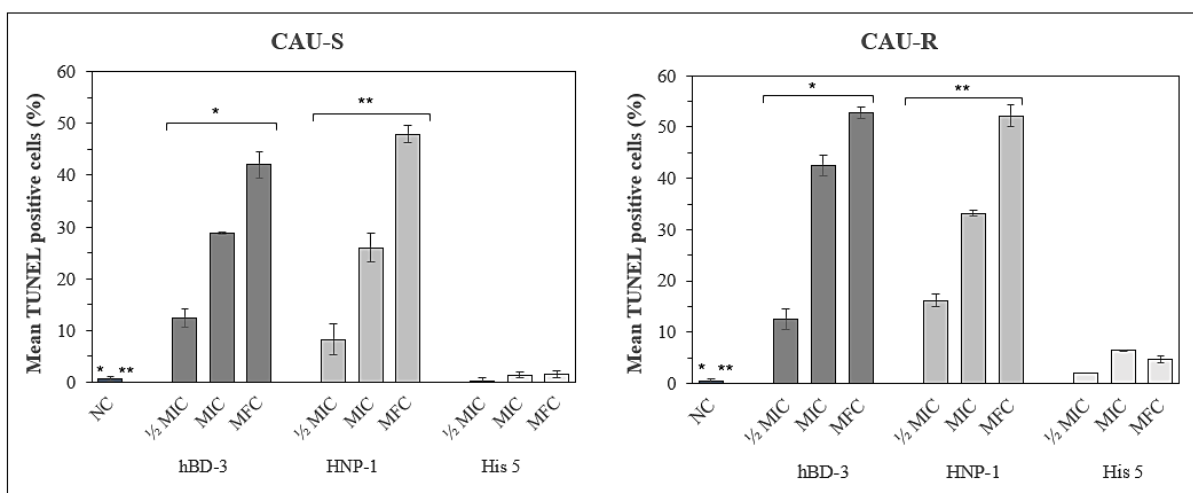


Figure 4.3: The proportion of TUNEL positive cells represent DNA fragmentation caused by tested peptides in *Candida. auris* cells. Cells exposed to 0.5 MIC, MIC and MFC of hBD-3, HNP-1 and His 5. Untreated cells were used as negative control (NC). Bar graphs represent the average percentage of three independent experiments (means $\pm$ SD), and statistical significance was determined using one-way ANOVA and Dunnett's test (*, ** $p < 0.01$).

4.3.4. Effect of AMPs on mitochondrial membrane potential

Since mitochondria have a crucial role in the induction of apoptosis, the influence of the tested AMPs on the mitochondrial membrane potential was studied using JC-10 dye. In healthy cells, this dye forms red fluorescent aggregates, while in apoptotic cells with low mitochondrial membrane potential, it spreads across the cytoplasm in green fluorescent monomers. The magnitude of mitochondrial membrane potential was assessed by determining the JC-10 aggregates to monomers ratio, the increase in JC-10 monomers associated with apoptosis results in decrease in this ratio, which ultimately indicates mitochondrial membrane depolarization (26). As shown in Fig. 4.4, the tested AMPs produced varying levels of mitochondrial membrane depolarization. Both hBD-3 and HNP-1 caused a significant, concentration-dependent drop in mitochondrial membrane potential in CAU-S and CAU-R at all tested concentrations, when compared to untreated cells. Regarding His 5 exposure, significant decrease in mitochondrial membrane potential was observed only at fungicidal concentrations in both strains.

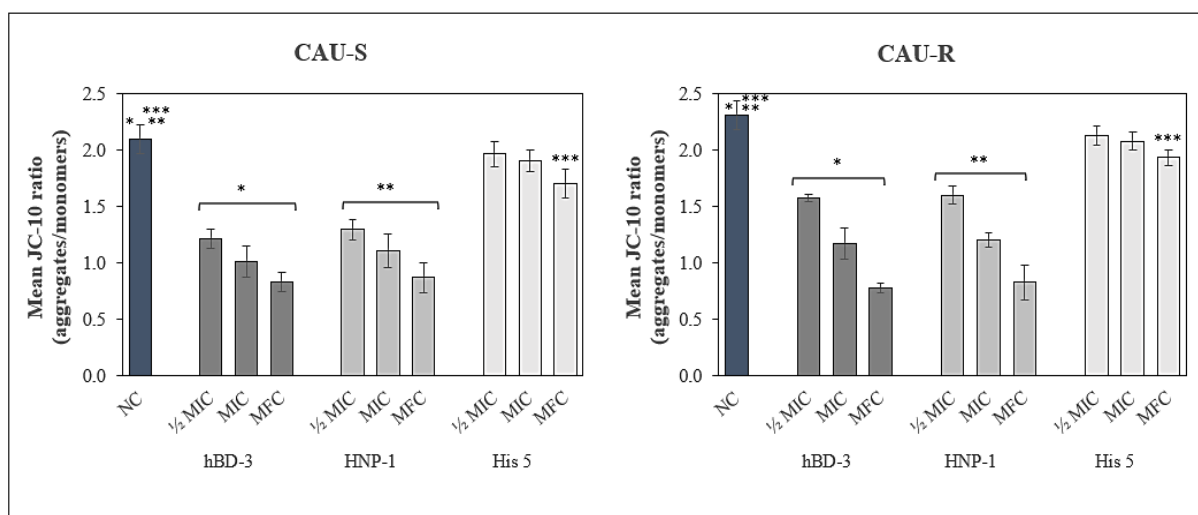


Figure 4.4: The effect of the tested peptides on mitochondrial membrane potential of *Candida auris* as determined by JC-10 staining. Cells exposed to 0.5 MIC, MIC and MFC of hBD-3, HNP-1 and His 5. Untreated cells were used as negative control (NC). Mitochondrial membrane potential was shown as the ratio of JC-10 aggregates to JC-10 monomers. A decrease in the ratio was considered as depolarization of mitochondrial membrane. Bar graphs represent the average percentage of three independent experiments (means $\pm$ SD), and statistical significance was determined using one-way ANOVA and Dunnett's test (*, **, *** $p < 0.05$).

4.3.5. Effect of AMPs on cytochrome c release

A further crucial indicator of apoptosis is the release of cytochrome c from the mitochondria. Thus, the effect of the tested peptides on cytochrome c release was investigated by measuring its levels in the mitochondria and the cytosol. In the untreated cells population, the relative levels of both cytosolic and mitochondrial cytochrome c considered to be 1.0. As shown in Fig.4.5, when CAU-S and CAU-R were exposed to different concentrations of hBD-3 and HNP-1, cells exhibited significant decrease in the relative level of mitochondrial cytochrome c, and substantial increased in the relative level of cytosolic cytochrome c, compared to untreated cells. On the other hand, His 5 exposure displayed non-significant changes in the relative levels of cytochrome c in the mitochondria and the cytosol in both strains. These results demonstrated that hBD-3 and HNP-1 exhibit an impact on cytochrome c release in a concentration-dependent manner, which supported the previous findings regarding the influence of the tested peptides on mitochondrial membrane potential.

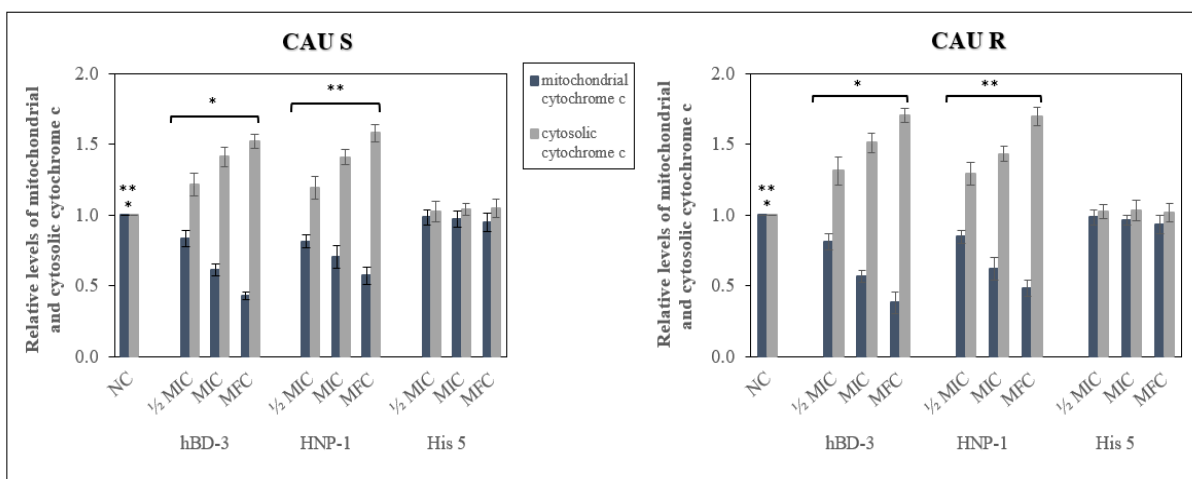


Figure 4.5: The effect of the tested peptides on cytochrome c release from the mitochondria to the cytoplasm in *Candida auris*. Cells exposed to 0.5 MIC, MIC and MFC of hBD-3, HNP-1 and His 5. Untreated cells were used as negative control (NC). Mitochondrial cytochrome c and cytosolic cytochrome c were analysed by measuring absorbance at 550 nm with a spectrophotometer. Data represent the average percentage of three independent experiments (means $\pm$ SD), and statistical significance was determined using one-way ANOVA and Dunnett's test (*, ** $p < 0.05$).

4.3.6. Effect of AMPs on intracellular ROS overproduction

Intracellular ROS have been recognized as important mediators of apoptosis in yeasts cells, as they stimulate and control apoptosis. Thus, to determine whether overproduction of ROS would be associated with the peptides fungicidal effects, intracellular ROS levels were determined using a fluorogenic dye (H_2DCFDA). The nonfluorescent H_2DCFDA is converted into the highly fluorescent 2',7'-dichlorofluorescein (DCF) via oxidation by intracellular ROS, and their levels can be determined by measuring the fluorescence of DCF by flowcytometry. As anticipated, flow cytometry findings verified the presence of excessive fluorescence in *C. auris* cells subjected to various concentrations of the tested peptides as compared to untreated cells, indicating intracellular ROS overproduction (Appendix D, Fig.D1). As shown in Fig. 4.6, hBD-3, HNP-1 and His 5, at all tested concentrations, demonstrated a significant increase in the percentage of fluorescent cells in CAU-S and CAU-R, compared to untreated populations. These findings suggested that peptides promote ROS production in *C. auris*.

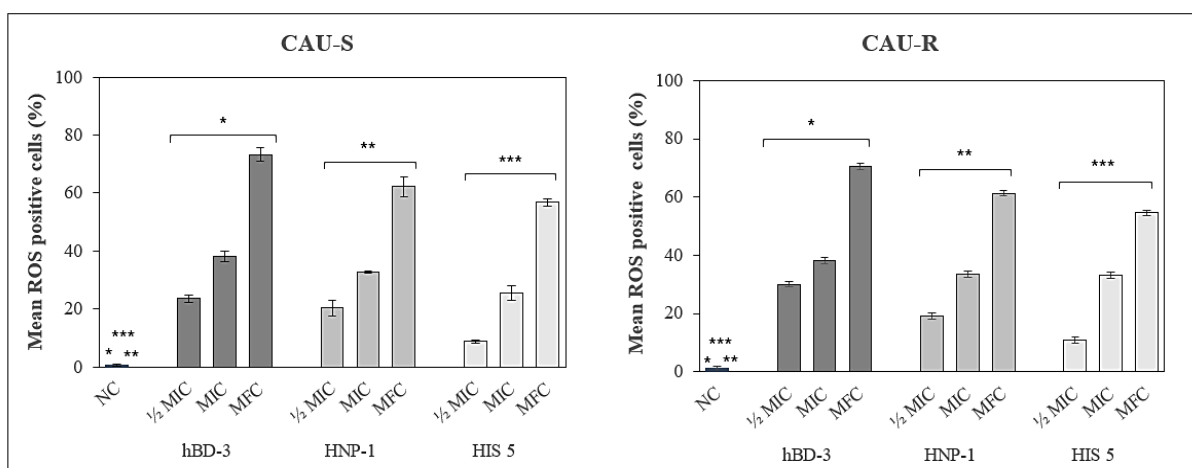


Figure 4.6: The effect of the tested peptides on ROS production in *Candida auris* as determined by H₂DCFDA staining. Cells exposed to 0.5 MIC, MIC and MFC of hBD-3, HNP-1 and His 5. Untreated cells were used as negative control (NC). Bar graphs represent the percentage of cells that produce ROS in the presence of different concentration of peptides. Data are presented from three independent experiments (means $\pm$ SD), and statistical significance was determined using one-way ANOVA and Dunnett's test (*, **, *** $p < 0.01$).

4.4. Discussion

The growing resistance to the main classes of antifungal agents in *C. auris* emphasizes an immediate demand for alternative therapies (27). Considering this, AMPs have been suggested as the most promising candidates for combating antifungal resistance (28). Previously, we have shown that particular human AMPs exhibited fungicidal activity against *C. auris* and altered its membrane permeability as a mechanism of action (19). Several studies, however, have revealed that many AMPs with membrane-interacting effects exhibit additional mechanisms such as induction of apoptosis (29). Understanding the mechanisms of these compound is essential for their prospective development as future therapeutics. Therefore, the present study assessed whether the antifungal effects of AMPs towards *C. auris* are mediated via apoptosis. Our findings demonstrated that the chosen peptides indeed trigger two different types of cell death in this pathogen. Whilst hBD-3 and HNP-1 promoted characteristic changes suggesting apoptotic cell death, His 5 caused necrosis as indicated by the lack of apoptotic hallmarks. The results of this study emphasize the potential of the tested human AMPs for developing innovative antifungal medications.

Apoptosis in yeasts has been studied thoroughly over the past years. Indeed, yeasts exhibit apoptotic signs similar to those found in human cells, such as PS externalization, DNA fragmentation and mitochondrial membrane depolarization (30). Furthermore, a variety of agents and stimuli have been identified as apoptotic inducers in *Candida albicans*, including acidic stress such as acetic acid and H₂O₂ and antifungals such as amphotericin B (31) and caspofungin (32). Interestingly, prolonged exposure to high doses of these compounds has been associated with accidental necrosis due to the severe cellular damage, features that might underestimate the level of apoptosis (33). Moreover, the overproduction of ROS which represents the primary trigger of apoptosis, could be a result of excessive cellular stress that occurs at a later stage (34). While both apoptosis and necrosis can contribute to antifungal activity, inducing a controlled cell death pathway like apoptosis might offer potential advantages in terms of reduced inflammation and potentially lower risk of resistance development. In contrast, necrosis might be less desirable due to its uncontrolled nature, which can trigger an inflammatory response in the host, potentially leading to tissue damage. However, it can still be a successful antifungal strategy if it leads to complete eradication of the fungus. Previously, we demonstrated that seven human AMPs at their MICs/MFCs significantly permeabilized *C. auris* membranes after 4 hours, suggesting potential induction of late apoptosis and/or necrosis (35). Here, to gain more insight into the type of cell death underlying peptide's antifungal activities against *C. auris*, we investigated several apoptotic markers in response to the most effective peptides (hBD-3, HNP-1 and His 5) at different concentrations, focusing on a shorter exposure time of 3 hours.

To further investigate peptide's antifungal effects, the early apoptotic change at the level of *C. auris* membrane was first observed. The plasma membrane plays a crucial role in cellular homeostasis and acts as a physical barrier to the extracellular surroundings. It contains protein transporters and channels for the movement of nutrients, inorganic and organic molecules. Hence, membrane disruption would certainly result in cellular death (36). The plasma membrane of yeast cells has asymmetrical distributions of phospholipids, with most of the PS directing toward the cytoplasm. During apoptosis, however, PS moves to the outer layer of plasma membrane (37). Following hBD-3 and HNP-1 treatment, FITC Annexin V/PI double staining assay showed that PS was located on the outer layer of membrane (FITC-Annexin V⁺/PI⁻), which indicate early apoptosis. While cells with double staining (FITC-Annexin V⁺/PI⁺) were mostly observed at higher concentrations suggesting late stage of apoptosis (Fig.4.1). Although both strains showed a significant percentage of early and late apoptotic

cells, a larger population arrested at the late apoptotic stage in CAU-R compared to CAU-S. It is possible that these peptides induced a stress response in the resistant strain, which might inadvertently activate distinct apoptotic pathways leading to a higher percentage of late apoptotic cells. Future studies are warranted to elucidate the underlying mechanisms behind this observation. This could involve exploring the activation of specific apoptotic signalling pathways or investigating potential differences in cell wall or membrane composition between the strains. PS externalization typically occurs before DNA fragmentation, which is a hallmark of late apoptosis (38). Confocal images of TUNEL staining further confirmed the existence of late apoptotic cells after hBD-3 and HNP-1 treatment (Fig.4.2).

Taken together, the above findings demonstrated that hBD-3 and HNP-1 caused early apoptosis which subsequently progressed to late apoptosis, indicating that both human defensins have the potential to trigger all apoptotic phases in *C. auris* clinical isolates, including the MDR strain. These results go beyond the previous study, which showed that hBD-3 induced killing activity and significantly increased membrane permeabilization with no gross disruption in *C. albicans* (39). Others have shown that a number of AMPs induced early (PS externalization) and late (DNA fragmentation) apoptotic signs in *C. albicans* including human defensin hepcidin 25 (40) human lactoferrin (41) insect defensin coprisin (42), plant defensin raphanus sativus antifungal peptide 2 (43).

Furthermore, mitochondria are key cellular structure that play a major role in biological functions such as energy production and are also involved in the biological processes of cell death. (44). Given that, as in mammals, mitochondrial malfunction results in depolarization of the mitochondrial membrane along with the release of proapoptotic substances are known to be involved during early stages of apoptosis in yeast. One of the many crucial and extensively studied proapoptotic molecules is cytochrome c (45). Constant mitochondrial membrane potential is important for the ATP synthesis by mediating the transfer of hydrogen ions in healthy cells. Prolonged decrease in the membrane potential and ATP levels leads to inhibition of the mitochondrial respiratory chain, which eventually initiates apoptosis (46). Additionally, cytochrome c release to the cytosol is the key process that activates the yeast metacaspase YCA1, which in turn induces apoptosis (38).

We therefore studied these mitochondrial apoptotic indicators in *C. auris* following the exposure to the tested peptides. Our results showed that hBD-3 and HNP-1 prompted significant mitochondrial membrane depolarization and cytochrome c release from the

mitochondria into the cytosol, as per to the JC-10 and cytochrome c assays. This is consistent with previous finding showing that the release of cytochrome c as a consequence of mitochondrial membrane depolarization has been observed in yeast cells undergoing apoptosis upon exposure to many agents (47). According to these findings, it can be suggested that the tested human defensins induce apoptotic cell death via the mitochondria-dependent pathway.

His 5 displayed an encouraging killing activity against different resistant *Candida* species including *C. auris*, which is attributed to several mechanisms that are still not fully explained (48). Our results provide evidence that His 5 promotes necrosis in *C. auris*, as shown by the non-significant TUNEL staining and the flow cytometric analysis (FITC-Annexin V⁻/PI⁺ cells). A previous study demonstrated that His 5 has no primary effect towards the membrane of *C. albicans* but caused cell death via non-lytic activities. Loss of membrane integrity, however, was observed with prolonged exposure to high concentrations. It has been suggested that membrane disruption was a consequence following cell death rather than the initial effect of His 5 (49). Additionally, our findings revealed that His 5 has a minor effect on *C. auris* mitochondrial functions by slightly decreasing the membrane potential with non-significant release of cytochrome c. This is consistent with what has been found in previous study which has shown that this peptide exerted a similar effect on mammalian mitochondria (50). Recent studies have shown that His 5 induced an osmotic imbalance resulting in ions leakage from the cells via an energy-dependent pathway and decreased the production of ATP within the mitochondria (51). A more recent study has demonstrated that *C. auris* was able to take up His 5, leading to its intracellular buildup in a way comparable to *C. albicans* (48). In line with these studies, it can be proposed that membrane disruption is not involved in His 5-mediated necrosis against *C. auris*, yet rather His 5 targets intracellular structure(s) following its uptake and ultimately causes cell death. However, further studies to understand its antimicrobial mechanisms are still needed.

Moreover, the detection of ROS has been identified as one of the main triggers of apoptosis, as well as one of the crucial elements underlying other forms of cell death in yeasts, such as necrosis (52). Under natural circumstances, cells produce ROS as outcomes of biological metabolism. and the cellular defence system have the capacity to prevent ROS-induced physical damages. However, this physiological balance is disturbed when cells are exposed to different external circumstances, such as oxidative and thermal stress. Excessive ROS levels results in direct protein, lipid and DNA damage, which results in cell death (53). Therefore,

ROS production was studied to determine whether they are involved in *C. auris* cell death caused by the tested peptides. Our results indicated that hBD-3, HNP-1 and His 5 treatments resulted in a significant increase in ROS levels. Although for many AMPs the generation of ROS has been identified as a direct trigger for apoptotic cell death in *C. albicans* (40, 54). It has been considered as an indirect consequence for the killing mechanisms of His 5 (55). Thus, it can be deduced that ROS play different roles in the fungicidal activities of the tested AMPs.

Mitochondria have been recognized as a major generator of ROS, which are primarily produced during cellular respiration (56). Several studies reveal that ROS play crucial role in all forms of cell death as they determine and regulate the type of underlying cell death. However, the interaction between intracellular ROS accumulation and mitochondrial malfunction is not fully explained. (57). Taken together, our findings show that high ROS levels associated with decreased mitochondrial membrane potential and cytochrome c release resulted in *C. auris* apoptosis after human defensins treatment. On the other hand, His 5-treatment showed excessive ROS production with minimal effect on the mitochondrial membrane potential and no cytochrome c release leading to *C. auris* necrosis. More research will be required to establish the role of ROS in the distinct forms of cell death.

AMPs have been proposed as future generation of antifungal, considering that they are nontoxic to human cells and do not trigger antifungal resistance development (28). In addition, attenuation of antimicrobial activity of AMPs in the presence of physiological concentrations of salts and degradation by human and *C. auris* proteases has been addressed through formulation, creation of peptidomimetics equivalent to AMPs and the use of D- or non-natural amino acids (58-60). Nevertheless, to the best of our knowledge, this work is the first to provide a specific understanding into the underlying basic mechanisms of human AMPs against *C. auris*, which might promote the development of novel therapies to combat antifungal resistant fungi. It should be highlighted that the studied peptides could possibly trigger cell death in *C. auris* through additional mechanisms. Therefore, further research is necessary to fully clarify the mechanisms of action of these peptides in combating *C. auris* and other pathogenic *Candida* species.

4.5. Conclusion

The results of this study demonstrated that hBD-3, HNP-1 and His 5 have strong fungicidal activity against both sensitive and MDR *C. auris* strains. Both hBD-3 and HNP-1 trigger

apoptosis in *C. auris* via mitochondria-dependent pathway and ROS production. Unlike His 5 which induce necrosis associated with ROS production but no cytochrome c release. Overall, our findings support the concept that these AMPs demonstrate excellent templates for future design and development as a novel antifungal therapy for resistance fungal pathogens.

4.6. References

1. Ahmad S, Alfouzan W. *Candida auris*: epidemiology, diagnosis, pathogenesis, antifungal susceptibility, and infection control measures to combat the spread of infections in healthcare facilities. *Microorganisms*. 2021;9(4):807.
2. Chen J, Tian S, Han X, Chu Y, Wang Q, Zhou B, *et al*. Is the superbug fungus really so scary? A systematic review and meta-analysis of global epidemiology and mortality of *Candida auris*. *BMC Infect Dis*. 2020;20(1):827.
3. Biagi MJ, Wiederhold NP, Gibas C, Wickes BL, Lozano V, Bleasdale SC, *et al*. Development of high-level echinocandin resistance in a patient with recurrent *Candida auris* candidemia secondary to chronic candiduria. *Open Forum Infect Dis*. 2019;6(7):ofz262.
4. Lamoth F, Lewis RE, Kontoyiannis DP. Investigational antifungal agents for invasive mycoses: a clinical perspective. *Clin Infect Dis*. 2022;75(3):534-544.
5. Swidergall M, Ernst JF. Interplay between *Candida albicans* and the antimicrobial peptide armory. *Eukaryot cell*. 2014;13(8):950-957.
6. Bowdish DM, Davidson DJ, Hancock R. A re-evaluation of the role of host defence peptides in mammalian immunity. *Curr Protein Pept Sci*. 2005;6(1):35-51.
7. Boto A, Pérez de la Lastra JM, González CC. The road from host-defense peptides to a new generation of antimicrobial drugs. *Molecules (Basel, Switzerland)*. 2018;23(2):11.
8. Kumar P, Kizhakkedathu JN, Straus SK. Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility *in vivo*. *Biomolecules*. 2018;8(1):04.
9. Teixeira V, Feio MJ, Bastos M. Role of lipids in the interaction of antimicrobial peptides with membranes. *Prog Lipid Res*. 2012;51(2):149-177.
10. Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol*. 2005;3(3):238-250.
11. Yeaman MR, Büttner S, Thevissen K. Regulated cell death as a therapeutic target for novel antifungal peptides and biologics. *Oxid Med Cell Longev*. 2018;2018:5473817.

12. Rockenfeller P, Madeo F. Apoptotic death of ageing yeast. *Exp Gerontol.* 2008;43(10):876-881.
13. Strich R. Programmed cell death initiation and execution in budding yeast. *Genetics.* 2015;200(4):1003-1014.
14. Herker E, Jungwirth H, Lehmann KA, Maldener C, Fröhlich K-U, Wissing S, *et al.* Chronological aging leads to apoptosis in yeast. *J Cell Biol.* 2004;164(4):501-507.
15. Ludovico P, Sousa MJ, Silva MT, Leão CI, Côrte-Real M. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. *Microbiology.* 2001;147(9):2409-2415.
16. Huh GH, Damsz B, Matsumoto TK, Reddy MP, Rus AM, Ibeas JI, *et al.* Salt causes ion disequilibrium-induced programmed cell death in yeast and plants. *Plant J.* 2002;29(5):649-659.
17. Wang G. Human antimicrobial peptides and proteins. *Pharmaceuticals (Basel, Switzerland).* 2014;7(5):545-594.
18. Fazly Bazzaz BS, Seyedi S, Hoseini Goki N, Khameneh B. Human Antimicrobial peptides: Spectrum, mode of action and resistance mechanisms. *Int J Pept Res Ther.* 2021;27(1):801-816.
19. Shaban S, Patel M, Ahmad A. Fungicidal activity of human antimicrobial peptides and their synergistic interaction with common antifungals against multidrug-resistant *Candida auris*. *Int Microbiol.* 2023;26(2):165-177.
20. CLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts. 4th ed. CLSI standard M27.: Wayne, PA: Clinical and Laboratory Standards Institute 2017.
21. Khan A, Ahmad A, Khan LA, Manzoor N. *Ocimum sanctum* (L.) essential oil and its lead molecules induce apoptosis in *Candida albicans*. *Res Microbiol.* 2014;165(6):411-419.
22. Wu X-Z, Chang W-Q, Cheng A-X, Sun L-M, Lou H-X. Plagiochin E, an antifungal active macrocyclic bis(bibenzyl), induced apoptosis in *Candida albicans* through a metacaspase-dependent apoptotic pathway. *Biochim Biophys Acta.* 2010;1800(4):439-447.

23. Kobayashi D, Kondo K, Uehara N, Otokozawa S, Tsuji N, Yagihashi A, *et al.* Endogenous reactive oxygen species is an important mediator of miconazole antifungal effect. *Antimicrob Agents Chemother.* 2002;46(10):3113-3117.
24. Kari S, Subramanian K, Altomonte IA, Murugesan A, Yli-Harja O, Kandhavelu M. Programmed cell death detection methods: a systematic review and a categorical comparison. *Apoptosis.* 2022;27(7-8):482-508.
25. Kyrylkova K, Kyryachenko S, Leid M, Kioussi C. Detection of apoptosis by TUNEL assay. *Methods Mol Biol.* 2012;887:41-47.
26. Younes N, Alsahan BS, Al-Mesaifri AJ, Da'as SI, Pintus G, Majdalawieh AF, *et al.* JC-10 probe as a novel method for analyzing the mitochondrial membrane potential and cell stress in whole zebrafish embryos. *Toxicol Res (Camb).* 2022;11(1):77-87.
27. Jacobs SE, Jacobs JL, Dennis EK, Taimur S, Rana M, Patel D, *et al.* *Candida auris* pan-drug-resistant to four classes of antifungal agents. *Antimicrob Agents Chemother.* 2022;66(7):e0005322.
28. Buda De Cesare G, Cristy SA, Garsin DA, Lorenz MC. Antimicrobial peptides: a new frontier in antifungal therapy. *mBio.* 2020;11(6):20.
29. Struyfs C, Cammue BPA, Thevissen K. Membrane-interacting antifungal peptides. *Front Cell Dev Biol.* 2021;9:649875.
30. Grosfeld EV, Bidiuk VA, Mitkevich OV, Ghazy E, Kushnirov VV, Alexandrov AI. A systematic survey of characteristic features of yeast cell death triggered by external factors. *J Fungi (Basel).* 2021;7(11):886.
31. Phillips AJ, Sudbery I, Ramsdale M. Apoptosis induced by environmental stresses and amphotericin B in *Candida albicans*. *Proc Natl Acad Sci U S A.* 2003;100(24):14327-14332.
32. Hao B, Cheng S, Clancy CJ, Nguyen MH. Caspofungin kills *Candida albicans* by causing both cellular apoptosis and necrosis. *Antimicrob Agents Chemother.* 2013;57(1):326-332.

33. Ludovico P, Sousa MJ, Silva MT, Leao CL, Corte-Real M. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. *Microbiology*. 2001;147(Pt 9):2409-2415.
34. Perrone GG, Tan SX, Dawes IW. Reactive oxygen species and yeast apoptosis. *Biochim Biophys Acta*. 2008;1783(7):1354-1368.
35. Poon IK, Hulett MD, Parish CR. Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death Differ*. 2010;17(3):381-397.
36. Zhang Y, Chen X, Gueydan C, Han J. Plasma membrane changes during programmed cell deaths. *Cell Res*. 2018;28(1):9-21.
37. Chaurio RA, Janko C, Munoz LE, Frey B, Herrmann M, Gaipf US. Phospholipids: key players in apoptosis and immune regulation. *Molecules*. 2009;14(12):4892-4914.
38. Carmona-Gutierrez D, Eisenberg T, Buttner S, Meisinger C, Kroemer G, Madeo F. Apoptosis in yeast: triggers, pathways, subroutines. *Cell Death Differ*. 2010;17(5):763-773.
39. Vylkova S, Nayyar N, Li W, Edgerton M. Human beta-defensins kill *Candida albicans* in an energy-dependent and salt-sensitive manner without causing membrane disruption. *Antimicrob Agents Chemother*. 2007;51(1):154-161.
40. Chen RC, Lan CY. Human antimicrobial peptide hepcidin 25-induced apoptosis in *Candida albicans*. *Microorganisms*. 2020;8(4):585.
41. Andres MT, Viejo-Diaz M, Fierro JF. Human lactoferrin induces apoptosis-like cell death in *Candida albicans*: critical role of K⁺-channel-mediated K⁺ efflux. *Antimicrob Agents Chemother*. 2008;52(11):4081-4088.
42. Lee J, Hwang JS, Hwang IS, Cho J, Lee E, Kim Y, *et al*. Coprisin-induced antifungal effects in *Candida albicans* correlate with apoptotic mechanisms. *Free Radic Biol Med*. 2012;52(11-12):2302-2311.
43. Aerts AM, Francois IE, Meert EM, Li QT, Cammue BP, Thevissen K. The antifungal activity of RsAFP2, a plant defensin from *raphanus sativus*, involves the induction of reactive oxygen species in *Candida albicans*. *J Mol Microbiol Biotechnol*. 2007;13(4):243-247.

44. Pereira C, Silva RD, Saraiva L, Johansson B, Sousa MJ, Corte-Real M. Mitochondria-dependent apoptosis in yeast. *Biochim Biophys Acta*. 2008;1783(7):1286-1302.
45. Ludovico P, Rodrigues F, Almeida A, Silva MT, Barrientos A, Corte-Real M. Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in *Saccharomyces cerevisiae*. *Mol Biol Cell*. 2002;13(8):2598-2606.
46. Zorova LD, Popkov VA, Plotnikov EY, Silachev DN, Pevzner IB, Jankauskas SS, *et al*. Mitochondrial membrane potential. *Anal Biochem*. 2018;552:50-59.
47. Guaragnella N, Zdravlevic M, Antonacci L, Passarella S, Marra E, Giannattasio S. The role of mitochondria in yeast programmed cell death. *Front Oncol*. 2012;2:70.
48. Pathirana RU, Friedman J, Norris HL, Salvatori O, McCall AD, Kay J, *et al*. Fluconazole-resistant *Candida auris* is susceptible to salivary histatin 5 killing and to intrinsic host defenses. *Antimicrob Agents Chemother*. 2018;62(2):17.
49. Edgerton M, Koshlukova SE, Lo TE, Chrzan BG, Straubinger RM, Raj PA. Candidacidal activity of salivary histatins. Identification of a histatin 5-binding protein on *Candida albicans*. *J Biol Chem*. 1998;273(32):20438-20447.
50. Petruzzelli R, Clementi ME, Marini S, Coletta M, Di Stasio E, Giardina B, *et al*. Respiratory inhibition of isolated mammalian mitochondria by salivary antifungal peptide histatin-5. *Biochem Biophys Res Commun*. 2003;311(4):1034-1040.
51. Komatsu T, Salih E, Helmerhorst EJ, Offner GD, Oppenheim FG. Influence of histatin 5 on *Candida albicans* mitochondrial protein expression assessed by quantitative mass spectrometry. *J Proteome Res*. 2011;10(2):646-655.
52. Farrugia G, Balzan R. Oxidative stress and programmed cell death in yeast. *Front Oncol*. 2012;2:64:64.
53. Costa V, Moradas-Ferreira P. Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: insights into ageing, apoptosis and diseases. *Mol Aspects Med*. 2001;22(4-5):217-246.
54. Cho J, Lee DG. Oxidative stress by antimicrobial peptide pleurocidin triggers apoptosis in *Candida albicans*. *Biochimie*. 2011;93(10):1873-1879.

55. Veerman EC, Nazmi K, Van't Hof W, Bolscher JG, Den Hertog AL, Nieuw Amerongen AV. Reactive oxygen species play no role in the candidacidal activity of the salivary antimicrobial peptide histatin 5. *Biochem J.* 2004;381(Pt 2):447-452.
56. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, oxidative stress and cell death. *Apoptosis.* 2007;12(5):913-922.
57. Villalpando-Rodriguez GE, Gibson SB. Reactive oxygen species (ROS) regulates different types of cell death by acting as a rheostat. *Oxid Med Cell Longev.* 2021;9912436.
58. Papo N, Shai Y. Effect of drastic sequence alteration and D-amino acid incorporation on the membrane binding behavior of lytic peptides. *Biochemistry.* 2004;43(21):6393-6403.
59. Svenson J, Stensen W, Brandsdal BO, Haug BE, Monrad J, Svendsen JS. Antimicrobial peptides with stability toward tryptic degradation. *Biochemistry.* 2008;47(12):3777-3788.
60. Ikonomova SP, Moghaddam-Taaheri P, Wang Y, Doolin MT, Stroka KM, Hube B, *et al.* Effects of histatin 5 modifications on antifungal activity and kinetics of proteolysis. *Protein Sci.* 2020;29(2):480-493.

Chapter 5: Anti-virulence and anti-efflux pump activity of synthetic defensins and histatin in *Candida auris*

5.1. Introduction

Candida auris, a multidrug-resistant yeast emerging as a global menace, has become a significant healthcare concern in the last decade (1). It has caused serious outbreaks, widespread infections, and significant risk of morbidity and mortality in healthcare facilities (2). One of the current challenges with *C. auris* is its resistance to the majority of the available antifungals (3). While the existing antifungal armamentarium remains limited, there is a renewed focus on developing new antifungal drugs (4). Therefore, innovative and improved therapeutics with novel mechanistic targets are imperative to combat *C. auris* infections.

The emergence of *C. auris* strains with resistance to all groups of antifungal drugs, which include polyenes, azoles and echinocandins, has been linked to several factors. One major factor is its capacity to develop biofilms, which are complex populations of organized sessile cells integrated in an extracellular matrix (5). Sessile cells tend to be less sensitive to the treatment than free-floating planktonic cells, due to reduced penetration of antifungal drugs into the dense biofilm structure (6). Biofilm development has been demonstrated to be a crucial virulence component in *C. auris*, and biofilm-forming isolates have been linked to increased morbidity and mortality rates (7). In addition to its tendency to develop biofilms, numerous researchers have demonstrated that the pathogenicity of *C. auris* is mediated by various virulence factors (8). As per our previous research, the investigated *C. auris* isolates displayed different levels of adherence to host cells and produced hydrolytic enzymes, particularly proteinases, which facilitate fungal penetration into the host tissue (9). The expression of each virulence factor is modulated by specific genes, and many virulence genes, such as those responsible for biofilm formation and hydrolytic enzyme secretion, have been identified in *C. auris* (10, 11).

Targeting virulence factors has emerged as a promising alternative to traditional antifungal therapies (12). Antifungal activity indicates the capacity of a drug to either kill or impede the growth of fungi, while the anti-virulence approach refers to the potential of a drug to inhibit the fungal pathogenicity (13). This approach act by reducing *Candida* virulence, which

attenuates the fungus thereby preventing infection, without killing the fungal cells. In addition, such a method exerts less selective pressure on fungal existence, which is responsible for resistance emergence (14). Various natural compounds have been shown to exhibit anti-virulence activity by suppressing the expression of virulence factors in *C. albicans* (15).

Antimicrobial peptides (AMPs) are various classes of natural compounds with rapid potent antimicrobial properties against bacteria, viruses and fungi (16). They are produced by humans and other living entities and serve as the initial line of defence for the host towards a variety of pathogens (17). Human-derived AMPs, such as defensins and histatins, have demonstrated strong antifungal activity against *Candida* species (18). They have a variety of mechanisms of action against *Candida* cells, such as disrupting cell wall integrity, permeabilizing cell membrane, and interacting with intracellular targets (19). Our previous study demonstrated the fungicidal activity and the membrane permeabilization effect of selected AMPs against *C. auris* (20). Furthermore, they have also been shown to suppress virulence factors expression in *C. albicans*, such as adhesion and biofilm formation, at subinhibitory concentrations (18).

Another major mechanism contributing to *C. auris* antifungal resistance is overexpression of efflux pumps (21). Efflux pumps are transmembrane proteins that actively transport a variety of substrates out of cells, including antifungal drugs, which leads to reduced intracellular drug concentrations and decreased antifungal efficacy (22). It has been established that *C. auris* has different kinds of efflux pumps that correlate with resistance to antifungal agents. The most important efflux pumps are the ATP-binding cassette (ABC) transporters and the major facilitator superfamily (MFS) transporter, which have been associated with azoles resistance (23). The importance of these pumps in *C. auris* drug resistance highlights the necessity of developing new approaches to conquer efflux pump-mediated resistance.

Considering these facts, this current research aims to examine the influence of selected human AMPs on virulence factors in *C. auris*, including adhesion, proteinase activity, forming and mature biofilm, as well as their effect on the activity of efflux pump, and the expression of related genes.

5.2. Materials & Methods

5.2.1 *Candida* strains, media and antimicrobial peptides

In the current study, two clinical isolates of *C. auris* were used. Both isolates were retrieved from the glycerol stock in the Clinical Microbiology laboratory at the University of the Witwatersrand. Based on our previous findings, the first isolate, CAU-S, was susceptible to fluconazole, amphotericin B, and caspofungin, and the second isolate, CAU-R, was resistant to the three antifungals (multi-drug resistant, MDR), in their planktonic form. They were grown at 37 °C on Sabouraud dextrose agar plates for 24 hours, then a few colonies were incubated at 37 °C in 10 ml fresh Sabouraud dextrose Broth medium for further 18 h with continuous shaking at 200 rpm. A *candida* suspension was made by washing the cells three times with phosphate buffer solution (PBS) and diluted to about 1×10^6 CFU/ml before being used for the subsequent experiments. The ethical waiver for the collection and use of isolates was given by the Human Research Ethics Committee of the University of the Witwatersrand (M140159). Three peptides belong to the various families of AMPs, including: human β -Defensin-3 (hBD-3), human neutrophil peptide-1 (HNP-1) and histatin 5 (His 5) were purchased from Thermo Fisher Scientific and utilized in this study. Peptides were mixed with purified water to get a stock solution of 5 mg/ml, which was then maintained at -20°C in the dark.

5.2.2. Effect of peptides on the virulence factors of *C. auris*

To examine the impact of the peptides on the main virulence factors of *C. auris*, the isolates were treated with the tested AMP at various sub-minimum inhibitory concentrations (sub-MICs) and minimum inhibitory concentrations (MICs), which were determined in our previous study. MICs of hBD-3, HNP-1 and His 5 were determined to be 3.125, 25 and 25 $\mu\text{g/ml}$, respectively, for CAU-S strain, and 6.25, 50 and 25 $\mu\text{g/ml}$, respectively, for CAU-R strain (20).

5.2.2.1. Viability assay

To quantify the extent of cell death caused by each peptide, *C. auris* cells viability was assessed using a Muse™ cell analyzer (EMD Millipore) following the manufacturer's instructions. Cells were treated with the tested peptides at their 0.25 MICs, 0.5 MICs and MICs for 2 hours at 37°C . Negative controls with untreated cells and positive controls with cells treated with hydrogen peroxide (20 mM H_2O_2) were included for comparison. After treatment, cells were

washed, resuspended in PBS, and mixed with the provided count & viability reagent. Following a brief incubation, the analyzer quantified both total cell count and viability of the treated cells.

5.2.2.2. Adherence to epithelial cells assay

To evaluate the effect of tested AMPs on *C. auris* adhesion to epithelial cells, an adherence assay was carried out utilizing the method by Patel and coauthors (24), with some modifications. In brief, 5 ml of SDB supplemented with the tested AMPs at 0.25 MICs, 0.5 MICs and MICs were inoculated with standardized cell suspension and incubated at 37 °C for 2 hours. *Candida* cells were then washed three times and resuspended in 2 ml of distilled water. Negative controls which contained untreated cells were included. Then, using a sterile swab, the investigator (myself) collected buccal epithelial cells (BECs), which were washed three times and resuspended in 2 ml of sterile distilled water. Thereafter, both yeast cells and BECs suspensions were combined and cultured for 2 hours with continuous shaking at 200 rpm at 37 °C. Post incubation, the BECs and non-adherent yeast cells were separated using a nylon filter with a 20 µm pore size. Slides were prepared, stained using the Gram stain procedure and examined using Leica DM 500 microscope under X1000 magnification. The adherence ability was expressed as the number of adherent *Candida* cells per 100 BECs. Every sample consist of three slides in total and the results were checked three times.

5.2.2.3. Proteinase assay

The impact of the tested peptides on proteinase secretion was investigated using a BSA (bovine serum albumin) agar plate assay (25). Briefly, standardized cell suspension was subcultured in 5 ml SDB supplemented with the tested peptides at 0.25 MICs, 0.5 MICs and MICs and incubated at 37 °C for 2 hours. Negative controls which contained untreated cells were included. Then, aliquots of 2 µl were positioned at equal distances on BSA agar plates (agar 2%, 1.45 g yeast nitrogen base without amino acids, 1.45 g ammonium sulphate, 20 g glucose, 1000 ml distilled water supplemented with 2 g BSA). The plates were incubated at 37 °C for 4 to 5 days. Post incubation, the proteinase secretion was observed as a halo surrounding the yeast colonies, which indicate BSA degradation by the enzyme, and the following equation was used to determine the relative proteinase secretion (Pz):

$$Pz = \frac{\text{diameter of the colony}}{\text{diameter of the colony} + \text{the halo around the colony}}$$

Results were interpreted as follows: Pz of 1.0 indicated no proteinase production and Pz of < 1.0 indicated positive proteinase production.

5.2.2.4. Biofilm formation assay

To examine the impact of tested peptides on *C. auris* biofilm formation, a method described by Patel and coauthors was used (26), with a few minor modifications. This technique involves the development of biofilms on microtiter plates followed by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reduction test to assess the metabolic activity of sessile cells as an indication of their viability. In short, 96-well flat bottom microtiter plates were inoculated with 100 µl of standardized cell suspension and incubated at 37°C for 2 hours to promote cell adhesion to the microtiter plate wells. Planktonic cells were then aspirated, and wells were cleaned with sterile PBS before 100 µl of fresh media with the tested AMPs at different concentrations (0.25 MICs, 0.5 MICs and MICs) was added to each well. Then the plates were incubated for 24 hours at 37°C. Thereafter, 50 µl of MTT (5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to each well of the microtiter plates, and the plates were incubated for 5 hours at 37°C. Then, a MTT solvent (100 µl of DMSO) was added and the absorbance was measured at 490 nm using an iMark, BioRad microplate reader. The experiments were performed in triplicate as well as keeping both sterility and culture controls. The percentage of biofilm inhibition was calculated using the following equation.

$$\text{Biofilm inhibition \%} = \frac{(\text{mean OD}_{490} \text{ control} - \text{mean OD}_{490} \text{ test})}{\text{mean OD}_{490} \text{ control}} \times 100$$

5.2.2.5. Mature biofilms assay

To examine the impact of tested AMPs on eradicating mature biofilm, *C. auris* cells were allowed to grow at ideal biofilm-forming circumstances for 24 hours, as described above. Then, after removing the grow medium, the biofilm was carefully rinsed using sterile PBS. Microtiter plate wells were filled with media supplemented with the different concentrations of the tested peptides, except for the culture control wells which contained untreated cells. and then the plates were incubated for another 24 hours at 37 °C. Thereafter, the MTT reduction assay, as previously mentioned, was used to measure the metabolic activity of the biofilms. The experiments were performed in triplicates and media and growth controls were included.

5.2.2.6. Confocal microscopy imaging of *C. auris* biofilms

CLSM was used to visually evaluate the impact of the tested peptides on biofilm formation, as described previously (27). At ideal biofilm-forming circumstances, *C. auris* strains were allowed to grow on glass coverslips in 6-well microtiter plates. The tested peptides at MICs were added to the specified wells and the plates were incubated for 24 hours at 37 °C. After the incubation period, the biofilms were twice washed with PBS and the coverslips were moved to a fresh 6-well microtiter plate. Then, the biofilms were stained with 10 µM of FUN-1 and 25 µg/ml of concanavalin A (ConA)-Alexa Fluor 488 conjugate (Invitrogen, ZA) for 45 minutes at 37°C in the dark. The coverslips were placed on glass plates and examined with a confocal laser scanning microscope (Carl Zeiss LSM 780) with Airyscan (excitation\emission: 543\ 560 nm for FUN-1, red fluorescence, and 488\ 505 nm for ConA, green fluorescence).

To visualize the impact of the tested AMPs on *C. auris* mature biofilms, a parallel experiment was established. *C. auris* strains were allowed to grow on glass coverslips in 6-well microtiter plates for 24 hours at 37 °C. Following the biofilm formation, the tested peptides at MICs were added to each well and the plates were incubated for another 24 hours at 37 °C. Then, the double staining approach with FUN-1 and ConA followed by observation with CLSM were carried out as described above.

5.2.3. Rhodamine-6G extracellular efflux assay

To determine the effect of the tested AMPs on the efflux pump activity of *C. auris*, the Rhodamine-6G (R6G) extracellular efflux assay was utilized (28). Briefly, standardized cell suspension of CAU-R was inoculated in 5 ml SDB supplemented with the tested peptides at 0.5 MICs and MICs and incubated at 37 °C for 2 hours. Negative control which contained untreated cells was included. Cells were then washed and resuspended in fresh glucose free PBS. 10 µM of R6G was added to cell suspension and incubated for 55 minutes to allow R6G uptake by the cells. Thereafter, cells were washed and resuspended in fresh PBS supplemented with 5% glucose to stimulate R6G efflux into the extracellular fluid. A total of 100 µl was taken every 10 minutes for 1 hour and centrifuged for 1 minute at 3,000 rpm. The absorption of the resulting supernatant was recorded by spectrophotometer at 527 nm and the concentration of R6G was determined using a standard concentration curve.

5.2.4. Rhodamine-6G intracellular accumulation assay

To further confirm the effect of the tested AMPs on the efflux pump activity of *C. auris*, fluorescence microscopy was used to assess the intracellular accumulation of R6G (29). CAU-R cells were treated with the tested AMPs for 2 hours were stained with R6G (10 μ M). The cells were then washed and resuspended in PBS supplemented with 5% glucose and R6G (4 μ M). After 30 minutes of incubation, the cells were pelleted, resuspended in 1 ml of PBS and examined using fluorescence microscopy (excitation\emission: 529\ 553 nm). The experiments were performed in triplicates and negative control which contained untreated cells was included.

5.2.5. Quantitative Reverse Transcription PCR (RT-qPCR) assay

RT-qPCR was used to assess the impact of peptides on the relative expression of genes related to efflux pump and virulence factors. In summary, standardized cell suspension of CAU-R was treated to peptides (at MICs) and then incubated for 2 hours at 37°C. Cells without treatment served as the negative control. Using the RNA MiniPrep kit (Inqaba Biotechnical Industries Ltd.), total RNA was extracted, according to the manufacturer's instructions. The nanodrop 2000 spectrophotometer (Thermo Scientific, MA, USA) was used to quantify the concentration of total RNA. The A260/A280 ratio was used to determine the purity of the RNAs, and a ratio greater than two was utilized for the PCR experiment. Next, using the cDNA synthesis kit (Lasec South Africa Ltd.), cDNA was synthesized as recommended. Using a RocheLight® Cyclor Nano instrument Real-time PCR system (Roche, Basel, Switzerland), RT-qPCR was carried out using the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems). The primers used are listed in Table S1. The following thermal cycling conditions were used: 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 53 °C for 15 seconds, and extension at 72 °C for 1 minute. UDG activation was carried out at 50 °C for 2 minutes (Hold). Dual-lock DNA polymerase was also heated to 95 °C for 2 minutes (Hold). The following were the criteria of the dissociation curve (melt curve stage): pre-melting at 95 °C for 15 seconds, with a ramp velocity of 1.6 °C/sec; Melting at a rate of 0.15 °C/sec at 95 °C for 15 seconds; melting at a rate of 1.6°C/sec at 60 °C for 1 minute. The LightCycler Nano device was utilized to ascertain the dissociation curve and CT results. Using the $\Delta\Delta$ CT method, the fold change in gene expression was measured and examined in relation to the housekeeping gene *ACT1* and untreated controls.

5.2.6. Cytotoxicity assay

The haemolytic assay was carried out using horse red blood cells in order to ascertain the cytotoxicity of the test peptides. The blood cells were centrifuged for 10 minutes at 2000 rpm to extract the erythrocytes. To obtain a 10% erythrocyte suspension, pellets were cleaned and resuspended in PBS. 100 µl of this suspension were mixed with 100 µl of a different test peptide dilution series. A positive control of 1% Triton X-100, which result in complete haemolysis, and a negative control of PBS were included. After 1 hour incubation period at 37 °C, the tubes were centrifuged for 10 minutes at 2000 rpm. 150 µl of the supernatant were added to a 96-well microtiter plate, and the absorbance at 450 nm was measured. The following formula was used to get the haemolysis percentage:

$$\text{Haemolysis \%} = \frac{(\text{A 450 of peptide treated sample} - \text{A 450 of negative control sample})}{(\text{A 450 of positive control sample} - \text{A 450 of negative control sample})} \times 100$$

5.2.7. Statistics

GraphPad Prism version 9.01 was used to create and perform statistical analysis on all the data and graphs. Every experiment was conducted in triplicate, and the results were reported as means ± standard deviation. To compare the treated and untreated control groups, a two-way ANOVA was employed, with a P value of less than 0.05 being deemed significant.

5.3. Results

5.3.1. Effect of peptides on *C. auris* cell viability

Exposing *C. auris* cell to sub-MICs and MICs of the tested peptides for 2 hours showed minimal non-significant reduction in cell viability (Fig 5.1). This was confirmed by measuring the total cell count and percentage of viable cells using Muse™ Count & Viability kit, which differentiate between live and dead cells through selective dye permeability.

5.3.2. Inhibition of *C. auris* adherence to BECs

Adhesion is an important initial stage of fungal infection, preceding tissue invasion and production of hydrolytic enzymes. Hence, the effect of the peptides on the adherence ability of *C. auris* to epithelial cells was studied. Results showed significant concentration-dependent reduction in the adherence when *C. auris* cells were exposed to hBD-3, HNP-1 and His 5 at 0.25 MIC, 0.5 MIC, and MIC (Fig. 5.2). Both sensitive (CAU-S) and resistant (CAU-R) *C.*

auris strains behaved similarly ($p < 0.001$). Notably, the resistant strain has shown a 2× higher adherence ability than the sensitive strain.

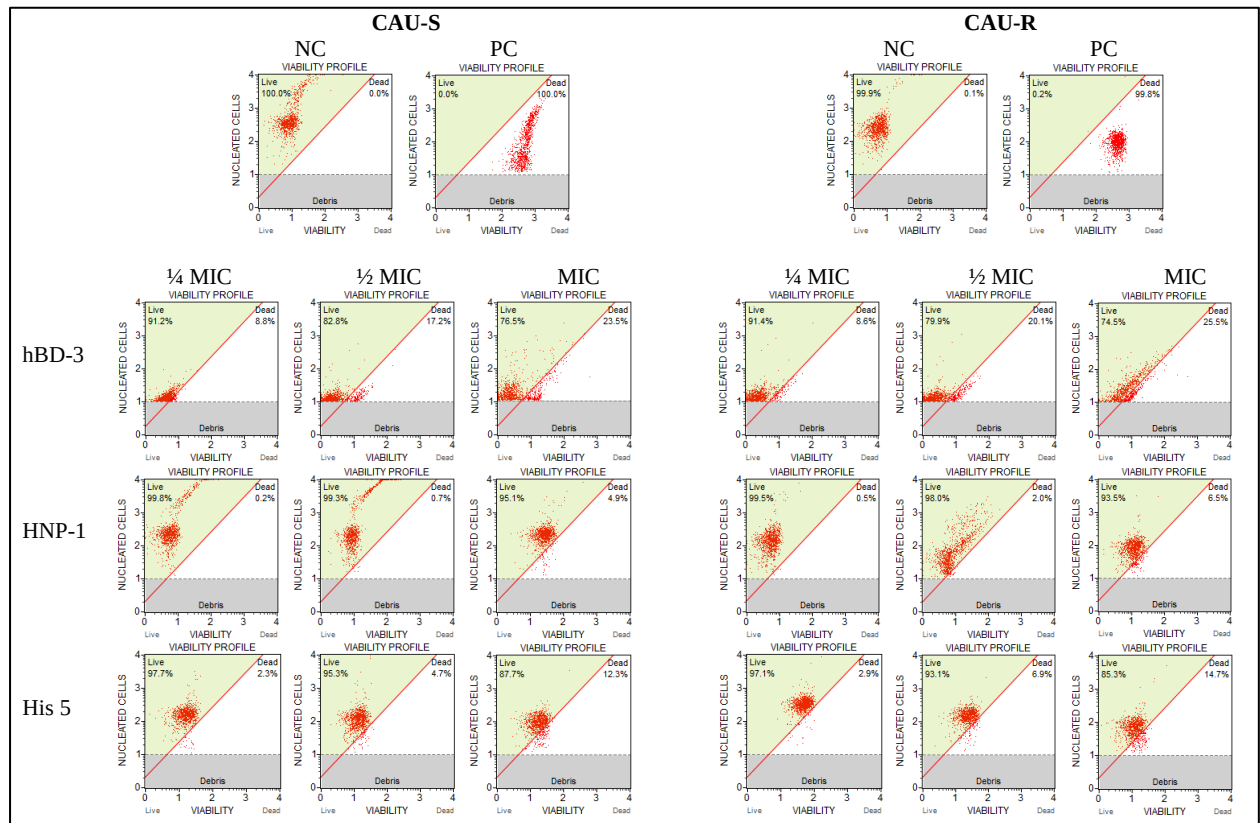


Figure 5.1: The effect of various concentrations of the tested peptides on cell count and viability of *Candida auris*. The figures shown are representative data of three independent experiments. Cells were exposed to 0.25 MIC, 0.5 MIC, and MIC of hBD-3, HNP-1 and His 5, in both sensitive (CAU-S) and resistant (CAU-R) *C. auris* strains, Untreated cells were used as negative control (NC) and positive control (PC) represent cells treated with 20 mM H₂O₂.

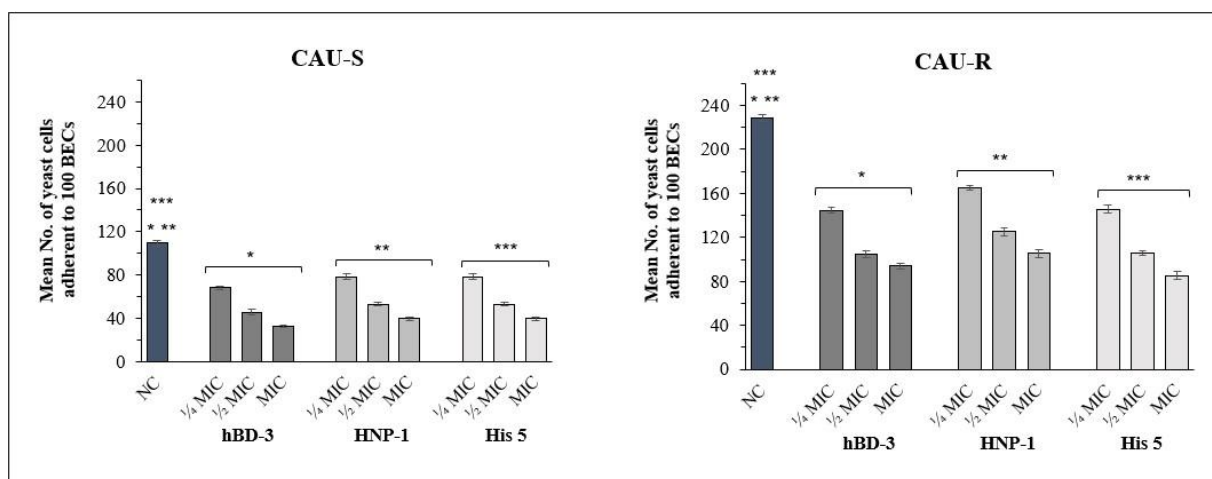


Figure 5.2: The effect of various concentrations of the tested peptides on *Candida auris* adherence to BECs. Quantification of number of yeast cells adherent to 100 BECs showing significant concentration-dependent reduction in adherence when cells were exposed to 0.25 MIC, 0.5 MIC, and MIC of hBD-3, HNP-1 and His 5, in both sensitive (CAU-S) and resistant (CAU-R) *C. auris* strains. Untreated cells were used as negative control (NC). Bar graphs represent the average of three independent experiments (means $\pm$ SD), and statistical significance was determined using one-way ANOVA and Dunnett's test (*, **, *** $p < 0.001$).

5.3.3. Inhibition of proteinase secretion in *C. auris*

The impact of peptides on proteinase secretion in *C. auris* was studied using BSA agar plates. Pz value was used as to measure the enzyme activity, whereas high Pz values indicate low proteinase secretion, low Pz values indicate high enzymatic secretion, and Pz of 1.0 indicated no proteinase activity. Fig. 5.3 shows that all three test peptides produced significant reduction in the relative amount of proteinase secretion, as evident from the increased Pz values. Both sensitive and resistant strains behaved similarly ($p < 0.05$). Additionally, the effect was concentration-dependent, with higher peptides concentrations having a greater inhibitory effect in both strains. Interestingly, *C. auris* strains produce different amounts of proteinase, as the enzyme activity of the resistant strain was higher (mean Pz = 0.37) than the sensitive strain (mean Pz = 0.70).

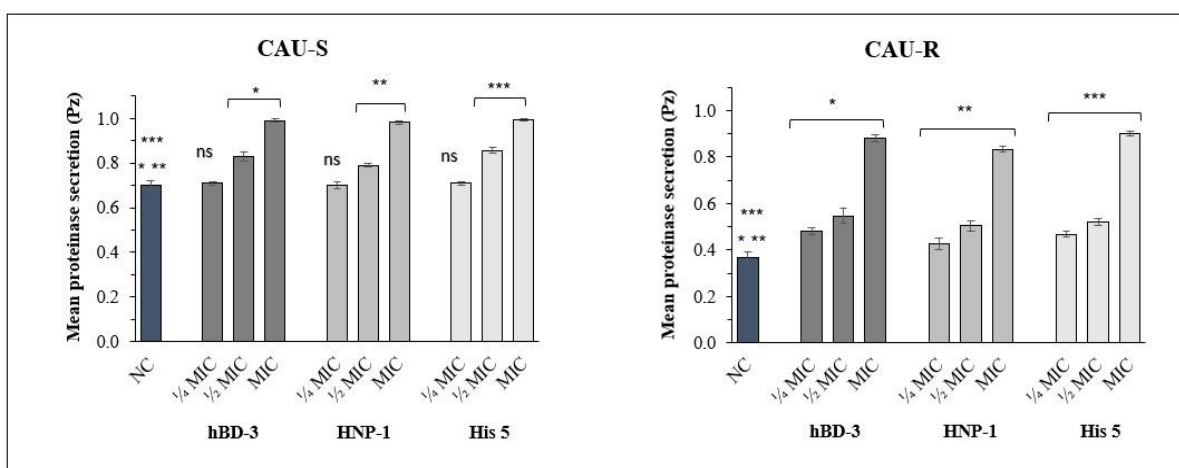


Figure 5.3: The effect of various concentration of the tested peptides on proteinase secretion of *Candida auris* using BSA plates. Quantification of the relative amount of proteinase secretion (Pz), a high Pz value indicates low enzyme secretion. Figures show significant concentration-dependent reduction in proteinase secretion when cells were exposed to 0.25 MIC, 0.5 MIC and MIC of hBD-3, HNP-1 and His 5, in both sensitive (CAU-S) and resistant (CAU-R) *C. auris* strains. Untreated cells were used as negative control (NC). Bar graphs represent the average of three independent experiments (means $\pm$ SD), and statistical significance was determined using one-way ANOVA and Dunnett's test (*, **, *** $p < 0.05$, ns; non-significant).

5.3.4. Inhibition of the metabolic activity of *C. auris* biofilm cells

When CAU-S and CAU-R were exposed to hBD-3, HNP-1 and His 5 (at 0.25 MIC, 0.5 MIC and MIC) at early stage of biofilm formation, samples exhibited significant concentration-dependent inhibition in biofilm metabolic activity with p values of < 0.01 , compared to negative control group (Fig. 5.4 A). Likewise, when 24 hours mature biofilms were exposed to the tested peptides at the same concentrations, significant concentration-dependent inhibition was observed in both strains as well (Fig. 5.4 B). Notably, the metabolic activity of the mature biofilm of resistant strain was 3 $\times$ higher (mean OD = 1.65) compared to the biofilm of sensitive strain (mean OD = 0.54).

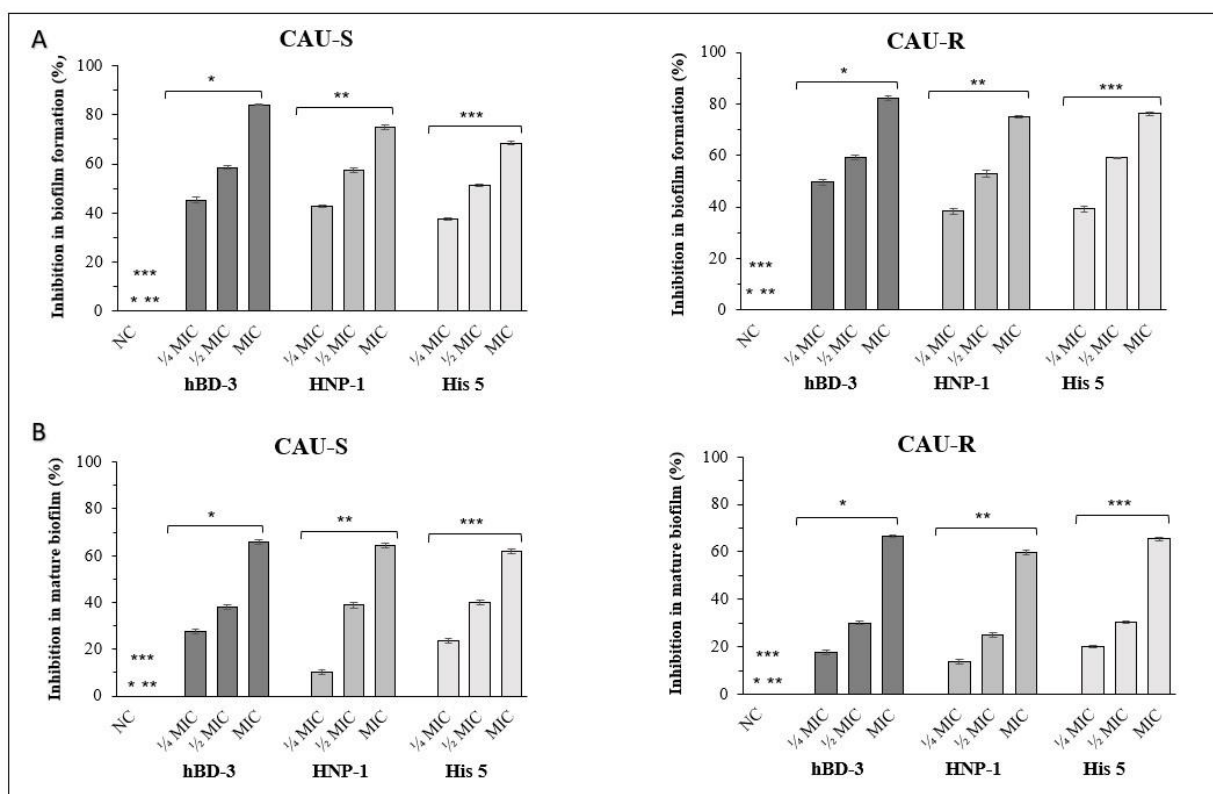


Figure 5.4: The effect of various concentration of the tested peptides on *Candida auris* biofilm metabolic activity using MTT reduction assay. (A) Figures show significant concentration-dependent inhibition in metabolic activity when cells at early stage of biofilm formation were exposed to 0.25 MIC, 0.5 MIC and MIC of hBD-3, HNP-1 and His 5, in both sensitive (CAU-S) and resistant (CAU-R) *C. auris* strains. (B) Figures show significant concentration-dependent inhibition in metabolic activity when mature biofilms cells were exposed to the different concentrations of the tested peptides, in both *C. auris* strains. Untreated cells were used as negative control (NC). Bar graphs represent the average of three independent experiments (means $\pm$ SD), and statistical significance was determined using one-way ANOVA and Dunnett's test (*, **, *** $P < 0.0001$).

5.3.5. CLSM examination of biofilm

To further confirm the inhibitory effect of the tested peptides, additional CLSM analysis on the form and architecture of both treated and untreated *C. auris* biofilms using ConA and FUN-1 staining was performed. While FUN-1 labelled the metabolically active cells and fluoresces orange-red, it remains in the cytoplasm of dead cells and fluoresces yellow-green. Conversely, Con-A fluoresces green upon binding to the polysaccharides in the biofilm matrix and cell walls. As shown in Fig. 5.5, the architecture of the untreated 24 hours biofilms appeared to be a complicated dense architecture of metabolically active cells, which is readily visible as an

orange-red fluorescence in a green fluorescence biofilm matrix (Fig. 5.5A). Likewise, untreated 48 hours biofilms showed the same architecture but less metabolically active cells throughout the biofilm, as evident by decreased orange-red fluorescence intensity (Fig. 5.5B). In contrast, early exposure to hBD-3, HNP-1 and His 5 at MIC demonstrated a decrease in biofilm density and killed most yeast cells. Hence, yellow-green fluorescence indicative of dead cells was noticed (Fig. 5.5A). Similarly, when mature biofilms were exposed to the tested peptides for 24 hours, biofilms underwent damage, and aggregation of dead cells were observed (Fig. 5.5B).

5.3.6. Inhibition of efflux pump activity in *C. auris*

Given the major role of the efflux pumps in drug resistance in *C. auris*, the effect of the tested AMPs on efflux pump activity was demonstrated using R6G extracellular efflux assay. As shown in Fig. 5.6, the negative control untreated cells show a consistent rise in the extracellular level of R6G. In contrast, R6G efflux was inhibited when in *C. auris* cells were exposed hBD-3, HNP-1 and His 5 (at 0.5 MIC, and MIC), which was evident from the low levels of the extracellular R6G. Then, we further confirmed the results by detecting the intracellular accumulation of the dye. Fig. 5.7A shows representative fluorescence images of the intracellular accumulation of R6G dye when *C. auris* cells were exposed to the three test AMPs. Fig. 5.7B shows significant increase in the fluorescence intensity due to intracellular accumulation of R6G when cells were exposed to the tested AMPs at the same concentrations, compared to negative control cells ($p < 0.0001$).

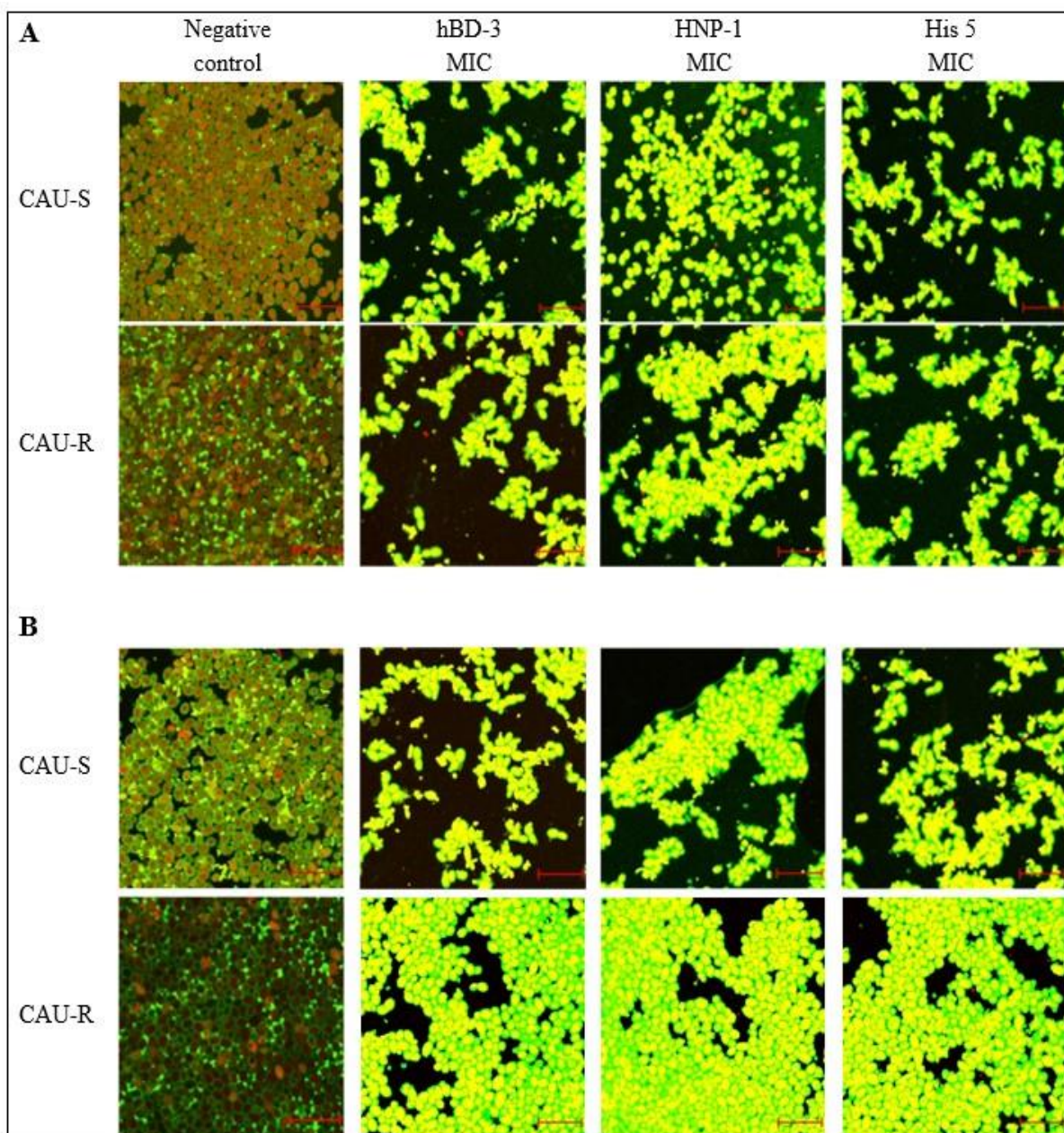


Figure 5.5: Confocal microscopy images of the effect of the tested peptides on forming and mature biofilm of *Candida auris*. Confocal laser scanning microscopy (CLSM) analysis of 24 hours biofilm (A) and 48 hours biofilm (B), which were treated with MIC of hBD-3, HNP-1 and His 5 at early stage of biofilm formation and after 24 hours, respectively. Untreated cells were used as negative control. The biofilms were stained using ConA and FUN-1 dye, the images obtained with a 63X oil immersion objective with magnification, X2.0. Viable cells appeared orange-red (FUN-1), fungal cell wall and biofilm matrix appeared green (ConA) and metabolically inactive and dead cells appeared yellow-green (FUN-1). The figures shown are representative data of three independent experiments. Scale-bar: 20 μm.

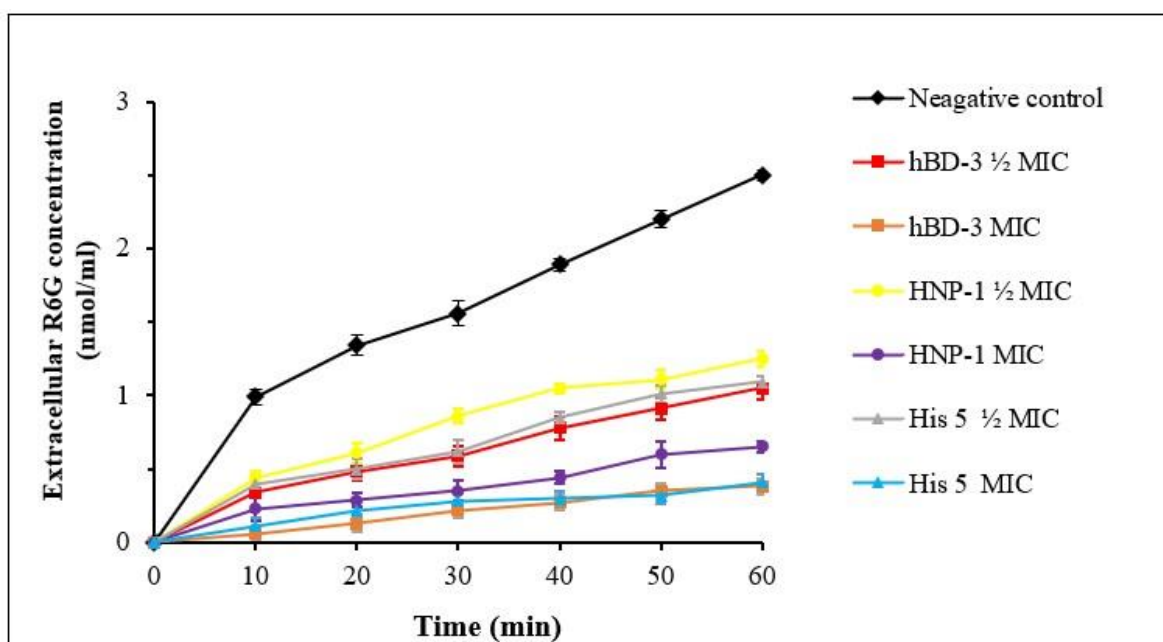


Figure 5.6: The effect of various concentration of the tested AMPs on efflux pump activity of *Candida auris* using Rhodamine-6G (R6G) efflux assay. The extracellular efflux of R6G was inhibited when cells were exposed to 0.5 MIC, and MIC of hBD-3, HNP-1 and His 5, in resistant (CAU-R) *C. auris* strains. Untreated cells were used as negative control. The values presented here is the average of three independent experiments (means $\pm$ SD).

5.3.7. Peptides downregulate the expression of genes linked to virulence and resistance of *C. auris*

Given the inhibitory effect of the tested AMPs against adherence ability, proteinase production, biofilm formation, mature biofilm and efflux pump activity of *C. auris*, we then assessed their effect on the expression of related genes including: *CDR1*, *CDR2*, *MDR1*, *SAP3*, *SNQ2*, *PGA26*, *PGA7* and *PGA52*. Using RT-qPCR, significant decrease in the relative expression of the abovementioned genes was observed when cells were pretreated with the tested peptides at MICs (Fig.5.8).

5.3.8. Cytotoxicity assay

Lastly, the toxicity of the peptides was also examined using horse red blood cells in a haemolytic experiment. After isolating the horse RBCs, AMPs at the different concentrations were added. Compared to the 100% haemolytic activity caused by Triton-X, which was utilized as a positive control, minimal non-significant haemolysis was observed for the three test peptides (Fig. 5.9).

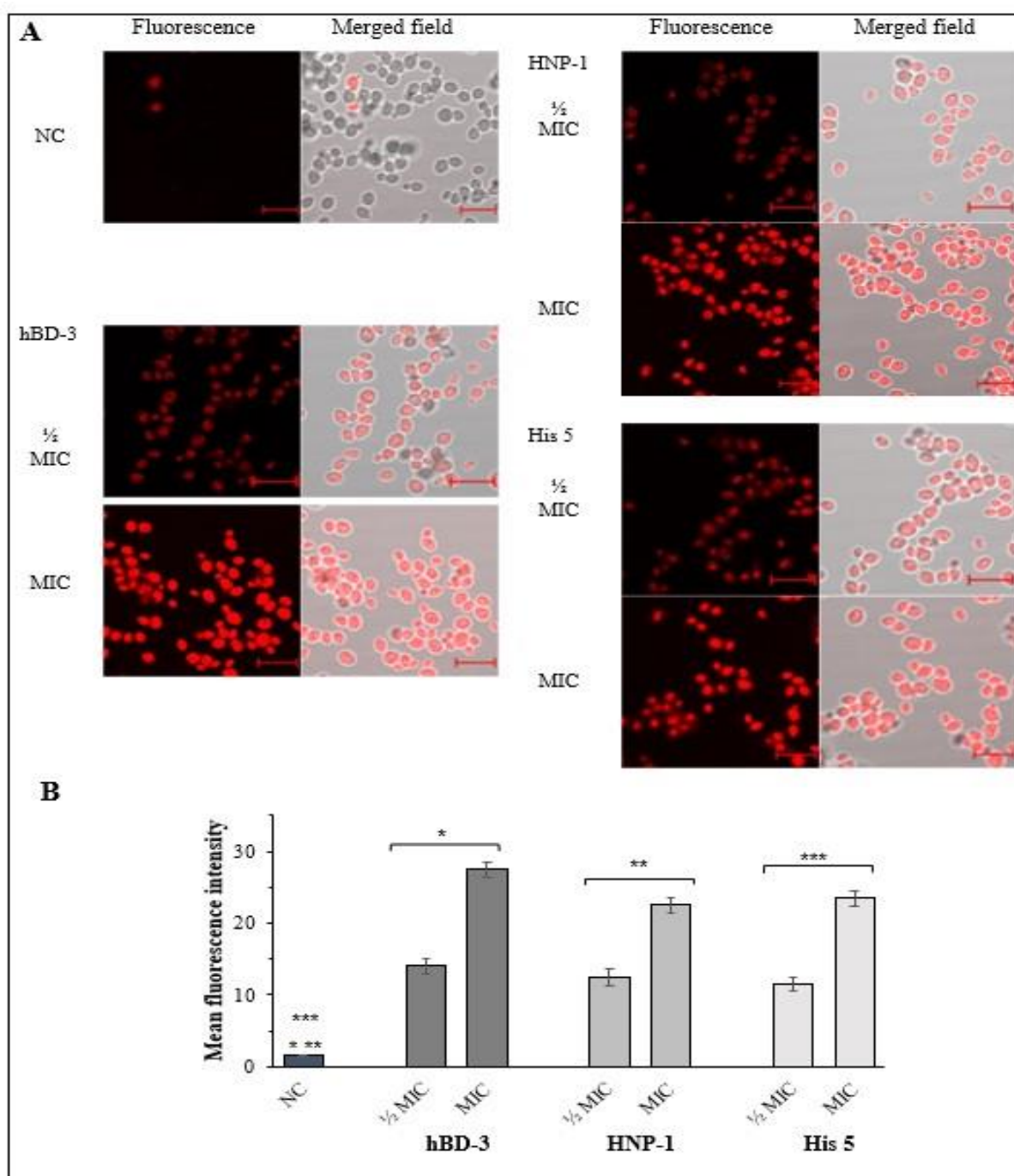


Figure 5.7: The effect of various concentration of the tested peptides on efflux pump activity of *Candida auris* CAU-R strain using Fluorescence microscopy. (A) Fluorescence images of R6G stained *C. auris* cells, showing the intracellular accumulation of R6G dye when cells were exposed to 0.5 MIC and MIC of hBD-3, HNP-1 and His 5. Untreated cells were used as negative control (NC). The figures shown are representative data of three independent experiments. Scale-bar: 5 μ m. (B) Quantification of the fluorescence intensity, showing significant concentration-dependent increase in the fluorescence intensity when cells were exposed to the different concentrations of the tested peptides. Bar graphs represent the average of three independent experiments (means $\pm$ SD), and statistical significance was determined using one-way ANOVA and Dunnett's test (*, **, *** P < 0.0001).

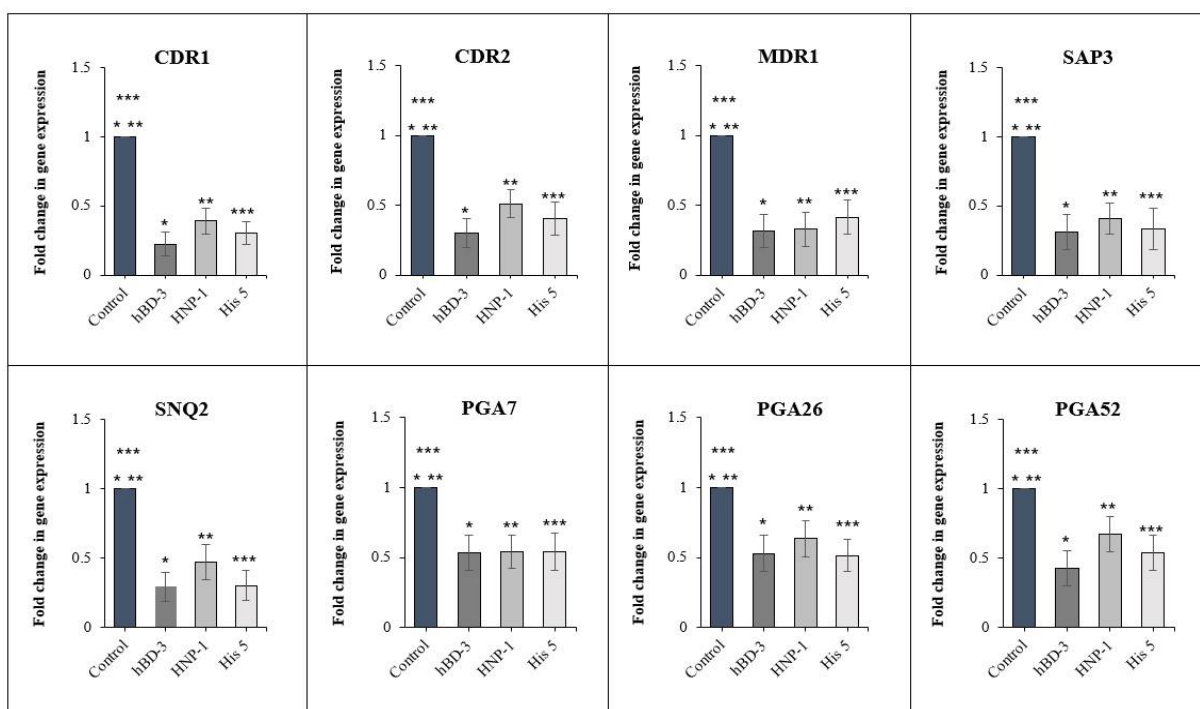


Figure 5.8: The effect of the tested peptides on gene expression of *Candida auris* CAU-R strain, *CDR1*, *CDR2*, *MDR1*, *SAP3*, *SNQ2*, *PGA26*, *PGA7* and *PGA52* using RT-qPCR. Data represents fold change in gene expression by adopting comparative Ct method. The gene expression between control untreated and cells exposed to MIC of hBD-3, HNP-1 and His 5 were compared and was normalized to a value of 1 and *ACT1* was used as housekeeping gene. Values represent the average of three independent experiments (means $\pm$ SD), and statistical significance was determined using one-way ANOVA and Dunnett's test (*, **, *** $P < 0.01$).

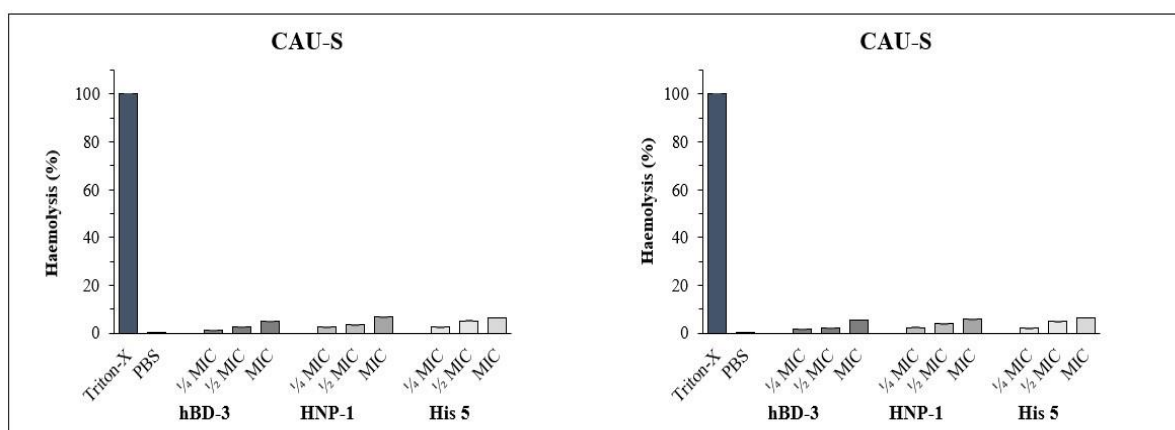


Figure 5.9: Cytotoxicity assay of the tested peptides by haemolytic activity. The tested peptides at varying concentrations showing the differences in haemolysis of red blood cells compared to negative control phosphate buffered saline (PBS). Positive control 1% Triton X (100% haemolysis). Values represent the average of three independent experiments (means $\pm$ SD), and statistical significance was determined using one-way ANOVA and Dunnett's test.

5.4. Discussion

The emergence of multidrug-resistant fungal pathogens, such as *C. auris*, necessitates the development of new antifungals with novel mechanisms of action. Targeting fungal virulence and efflux pump activity which are responsible for the pathogenicity and drug resistance respectively, has been suggested as an alternative approach for preventing fungal infections (15, 30). Recently, AMPs have been identified as attractive candidates with antifungal activity (19). Their effect on virulence and efflux pump was not known and therefore it was studied here. The results showed that hBD-3, HNP-1 and His 5 can significantly inhibit adherence to epithelial cells, production of proteinase, the mature biofilm and its formation and efflux pump in *C. auris*. In addition, our findings suggest that these effects likely occur through mechanisms independent of their direct killing effects. By using sub-MICs and MICs for a short 2-hour incubation, we ensured cell viability remained unaffected, allowing us to isolate the peptides' specific influence on virulence and efflux pumps.

Adhesion of *Candida* including *C. auris* to host cells is the first essential step in the infection process, that precedes tissue invasion and production of hydrolytic enzymes (31). Our data reveal that pretreatment of *C. auris* cells with subinhibitory concentrations of the three test AMPs significantly inhibited its adherence to epithelial cells, thus preventing one of the initial stages of fungal infection. At the concentration of 50 µg/ml His 5 has been shown to decrease *C. albicans* adherence to reconstructed human buccal epithelium (32). Similarly, hBD-3 (at 10 µM) has been shown to reduce *C. albicans* adherence to plastic surfaces by increasing the activity of exoglucanase enzyme, which degrades the β-glucan in the cell wall and hence disrupts its integrity (33). Here, we found that even at lower concentrations, His 5 (at 6.25 to 25 µg/ml) and hBD-3 (at 0.78 to 6.25 µg/ml) substantially inhibited *C. auris* adherence to BECs. Since the *C. auris* cells were exposed to the AMPs and then applied to the epithelial cells, AMPs may have affected the β-glucan component in the cell wall or they may have affected the adhesins protein component responsible for the adherence to the host cells (34).

The second step in the pathogenicity is the invasion of the host cells which is facilitated by the hyphae formation and the production of hydrolytic enzymes (35). Although *C. auris* is not capable of producing hyphae and phospholipases, despite the significant heterogenicity, the majority of them have been shown to produce proteinase enzyme (9). Again, the results showed that all three test AMPs can inhibit proteinase production, intercepting the second step in the disease process. Multifunctional molecules secreted aspartyl proteases (SAPs) are enzymes that

are secreted by *Candida* species and are encoded by the *SAP* gene family (*SAP1-SAP10*). *SAP* production by fungi increases colonization and penetration of the host tissue (36). *SAP-3* which is also involved in the adherence of *C. auris*, which was studied here and showed that the gene expression of *SAP-3* was down regulated as well as the phenotypic production was inhibited by the AMPs.

In addition, the resistant strain displayed higher baseline adherence and proteinase activity compared to the sensitive strain, suggesting a potential role of proteinase secretion in antifungal resistance. This highlights the additional challenge posed by resistant strains and emphasizes the importance of finding effective interventions.

Biofilm formation has been recognized as a crucial factor in *C. auris* virulence and its high levels of resistance (37). It also facilitates the persistent colonization of environmental surfaces and host tissues (38). Studies have shown that AMPs can inhibit biofilm formation and affect mature biofilms of *C. albicans* (39). Our findings revealed that the three test AMPs can significantly inhibit biofilm formation and affect mature biofilm of *C. auris*. This is important because mature biofilms consist of sticky extracellular matrix which promotes drug resistant and it is difficult to remove (40). The difference in biofilm formation observed between the two *C. auris* isolates emphasizes the inherent variability among strains and highlights the challenges associated with managing resistant isolates. However, the ability of these peptides to inhibit even the highly metabolically active resistant biofilms underscores their potential as promising antifungal agents in the fight against *C. auris* infections.

Beta defensins are known to have antimicrobial effect against bacterial and fungal biofilms. For example, hBD-3 (at 50 µg/ml) was found to inhibit mixed bacterial biofilm (41, 42). Likewise, three HNP-1 analogues have been shown to inhibit MDR *Escherichia coli* biofilm formation and mature biofilm (43). Here, we found that, at lower concentrations, hBD-3 (at 0.78 to 6.25 µg/ml) and HNP-1 (at 6.25 to 50 µg/ml) significantly reduced *C. auris* biofilm formation and mature biofilms. Contrary to our findings, another study has found that hBD-3 (at 0.6 µM to 1.2 µM) did not cause a significant decrease in *C. albicans* biofilm development (44). Our findings indicate that *C. auris* biofilms are more susceptible to the tested peptides than *C. albicans*. This can be explained by the earlier findings that *C. auris* formed less dense and thinner biofilm compared to *C. albicans* (45). His 5 has a potent activity against *C. albicans* and *C. glabrata* biofilm (46-48). The present study demonstrated that His 5 at the highest concentration tested (25 µg/ml) inhibited *C. auris* biofilm formation as well as reduced the

metabolic activity of mature biofilms by up to 76 and 66 %, respectively. The effect on biofilm formation may have been due to the effect on *C. auris* adherence to the plastic surface and the effect on the mature biofilm may have been due to the antimicrobial effect of the AMPs.

In addition, CLSM analysis confirmed the inhibitory effects of the tested AMPs against *C. auris* biofilm, with an apparent morphological difference between the treated and untreated biofilms (Fig. 4). When combined with fluorescent probes approach, CSLM is currently regarded as the preferred technique to study microbial biofilms, since it gives extensive morphological and architectural details of the biofilms (49). The current study showed that the tested AMPs disrupted the biofilm architecture and reduced the viability of the biofilm cells. Regarding their anti-virulence mechanisms, it is well known that AMPs interact with various intracellular targets (50). Herein to explore possible mechanisms, we have investigated the effect of the tested peptides on virulence genes expression. In *C. auris*, it has been reported that the genes *PGA7*, *PGA26* and *PGA52* were actively involved in initial adhesion and biofilm formation (51), Results of this study showed that the three tested AMPs significantly reduced the expression of these genes in *C. auris*. Therefore, the inhibitory effects of the tested AMPs on *C. auris* biofilms can be largely explained by the downregulation of related genes. However, the anti-biofilm efficacy of the tested AMPs could potentially be attained by additional multi-target mechanism.

Another important factor in *C. auris* antifungal drug resistance is the overexpression of efflux pumps, which has been primarily linked to resistance to azoles (5). In addition to contributing to antifungal drug resistance, efflux pumps have also been shown to play a role in the virulence of *C. auris*. A *de novo* transcriptomic analysis found that *C. auris* isolates that overexpress genes related to efflux pump, both ABC and MFS transporters, were more likely to form biofilms and to cause invasive infections (51). Therefore, the effect of the AMPs against the efflux pump activity in *C. auris* was studied. Our data, utilizing the R6G dye assay, demonstrated that all the test AMPs act as inhibitors of efflux pump activity in *C. auris* (Fig. 5). To understand the molecular basis of efflux pumps inhibition, we examined the effect of peptides on gene expression in the yeast. The genes *CDR1*, *CDR2* and *SNQ2* (belong to ABC transporters) and *MDR1* (belongs to MFS transporters) were functionally linked to the overexpression of efflux pumps in *C. auris* (51, 52). The three tested AMPs significantly downregulate the expression of all these genes. Previously, His 5 has found to reduce the efflux pump activity in *Saccharomyces cerevisiae* by inhibiting the Pdr5p transporter, which is

equivalent to ABC transporters in *C. albicans* (53). Our previous study demonstrated the strong synergistic activity when the tested peptides were combined with fluconazole (20). This study results suggest a possible mechanism for such synergism is the inhibition of efflux pump activity, which ultimately prevent fluconazole efflux and increase its intracellular levels.

AMP development has become an ongoing focus for the discovery of novel antimicrobial drugs with increased range of activity and lower cytotoxicity against human cells (54). While our previous work showed that the test peptides effectively kill *C. auris*, this study explored their ability to inhibit key virulence factors and efflux pumps. Importantly, these effects were observed at sub-MICs and MICs for a short incubation period, ensuring they were independent of any cell death caused by the AMPs. This suggests that AMPs have a more nuanced role, potentially targeting virulence and efflux pump mechanisms beyond simply killing the fungus. In addition, non-toxic characteristics of the tested peptides on mammalian red blood cells has also been established by their low haemolytic action. These results contribute to our understanding of the overall safety profile of the AMPs for potential therapeutic applications. Moreover, the effective anti-virulence activity of the tested peptides against both sensitive and resistant strains highlight their potential to overcome antifungal resistance. Several chemical changes, including substitutes and modifications to the amino acids sequence can be employed to increase their range of activity and avoid any drawbacks (54). Further research is needed to elucidate the specific mechanism of action of these peptides, to explore their potential for therapeutic development and to determine their efficacy in an animal model and subsequently clinical trials.

5.5. Conclusion

Our finding revealed that hBD-3, HNP-1, and His 5 inhibited key virulence factors in *C. auris*, which thereby reducing the pathogenicity of this yeast. The tested AMPs decreased adherence to epithelial cells, reduced the production of proteinases, inhibited biofilm formation and altered mature biofilm. AMPs also inhibited efflux pump activity and downregulated the expression of genes linked to virulence and efflux pumps in the MDR isolate. Overall, this study revealed a potential application of the tested AMPs in the development of effective anti-virulence and anti-efflux pump therapy to combat drug resistant *C. auris*.

5.6. References

1. Sanyaolu A, Okorie C, Marinkovic A, Abbasi AF, Prakash S, Mangat J, *et al.* *Candida auris*: An overview of the emerging drug-resistant fungal infection. *Infect Chemother.* 2022;54(2):236-246.
2. Sabino R, Verissimo C, Pereira AA, Antunes F. *Candida auris*, an agent of hospital-associated outbreaks: which challenging issues do we need to have in mind? *Microorganisms.* 2020;8(2):81.
3. Ademe M, Girma F. *Candida auris*: from multidrug resistance to pan-resistant strains. *Infect Drug Resist.* 2020;13:1287-1294.
4. Kim JH, Cheng LW, Land KM. Advances in antifungal development: discovery of new drugs and drug repurposing. *Pharmaceuticals (Basel).* 2022;15(7):87.
5. Chaabane F, Graf A, Jequier L, Coste AT. Review on Antifungal resistance mechanisms in the emerging pathogen *Candida auris*. *Front Microbiol.* 2019;10:2788.
6. Silva S, Rodrigues CF, Araujo D, Rodrigues ME, Henriques M. *Candida* Species biofilms' antifungal resistance. *J Fungi (Basel).* 2017;3(1):8.
7. Sherry L, Ramage G, Kean R, Borman A, Johnson EM, Richardson MD, *et al.* Biofilm-forming capability of highly virulent, multidrug-resistant *Candida auris*. *Emerg Infect Dis.* 2017;23(2):328-331.
8. Garcia-Bustos V, Cabanero-Navalon MD, Ruiz-Sauri A, Ruiz-Gaitan AC, Salavert M, Tormo MA, *et al.* What do we know about *Candida auris*? state of the art, knowledge gaps, and future directions. *Microorganisms.* 2021;9(10):177.
9. Shaban S, Patel M, Ahmad A. Improved efficacy of antifungal drugs in combination with monoterpene phenols against *Candida auris*. *Sci Rep.* 2020;10(1):1162.
10. Brown JL, Delaney C, Short B, Butcher MC, McKloud E, Williams C, *et al.* *Candida auris* phenotypic heterogeneity determines pathogenicity *in vitro*. *mSphere.* 2020;5(3):20.

11. Allert S, Schulz D, Kammer P, Grossmann P, Wolf T, Schauble S, *et al.* From environmental adaptation to host survival: Attributes that mediate pathogenicity of *Candida auris*. *Virulence*. 2022;13(1):191-214.
12. Gauwerky K, Borelli C, Korting HC. Targeting virulence: a new paradigm for antifungals. *Drug Discov Today*. 2009;14(3-4):214-222.
13. Ivanov M, Ciric A, Stojkovic D. Emerging antifungal targets and strategies. *Int J Mol Sci*. 2022;23(5):2756.
14. Pierce CG, Lopez-Ribot JL. Candidiasis drug discovery and development: new approaches targeting virulence for discovering and identifying new drugs. *Expert Opin Drug Discov*. 2013;8(9):1117-1126.
15. Ahmad Khan MS, Alshehrei F, Al-Ghamdi SB, Bamaga MA, Al-Thubiani AS, Alam MZ. Virulence and biofilms as promising targets in developing antipathogenic drugs against candidiasis. *Future Sci OA*. 2020;6(2):FSO440.
16. Lei J, Sun L, Huang S, Zhu C, Li P, He J, *et al.* The antimicrobial peptides and their potential clinical applications. *Am J Transl Res*. 2019;11(7):3919-3931.
17. Zhang LJ, Gallo RL. Antimicrobial peptides. *Curr Biol*. 2016;26(1):R14-19.
18. Perez-Rodriguez A, Eraso E, Quindos G, Mateo E. Antimicrobial peptides with anti-*Candida* activity. *Int J Mol Sci*. 2022;23(16):64.
19. Buda De Cesare G, Cristy SA, Garsin DA, Lorenz MC. Antimicrobial peptides: a new frontier in antifungal therapy. *mBio*. 2020;11(6):20.
20. Shaban S, Patel M, Ahmad A. Fungicidal activity of human antimicrobial peptides and their synergistic interaction with common antifungals against multidrug-resistant *Candida auris*. *Int Microbiol*. 2023;26(2):165-177.
21. Sharma C, Kumar N, Pandey R, Meis JF, Chowdhary A. Whole genome sequencing of emerging multidrug resistant *Candida auris* isolates in India demonstrates low genetic variation. *New Microbes New Infect*. 2016;13:77-82.

22. Cannon RD, Lamping E, Holmes AR, Niimi K, Baret PV, Keniya MV, *et al.* Efflux-mediated antifungal drug resistance. Clin Microbiol Rev. 2009;22(2):291-321.
23. Chatterjee S, Alampalli SV, Nageshan RK, Chettiar ST, Joshi S, Tatu US. Draft genome of a commonly misdiagnosed multidrug resistant pathogen *Candida auris*. BMC Genomics. 2015;16(1):686.
24. Patel M, Gulube Z, Dutton M. The effect of *Dodonaea viscosa var. angustifolia* on *Candida albicans* proteinase and phospholipase production and adherence to oral epithelial cells. J Ethnopharmacol. 2009;124(3):562-565.
25. Yousuf S, Ahmad A, Khan A, Manzoor N, Khan LA. Effect of garlic-derived allyl sulphides on morphogenesis and hydrolytic enzyme secretion in *Candida albicans*. Med Mycol. 2011;49(4):444-448.
26. Patel M, Srivastava V, Ahmad A. *Dodonaea viscosa var angustifolia* derived 5,6,8-trihydroxy-7,4' dimethoxy flavone inhibits ergosterol synthesis and the production of hyphae and biofilm in *Candida albicans*. Journal of Ethnopharmacology. 2020;259:112965.
27. Lattif AA, K. Mukherjee P, Chandra J, Swindell K, Lockhart SR, Diekema DJ, *et al.* Characterization of biofilms formed by *Candida parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis*. Int J Med Microbiol. 2010;300(4):265-270.
28. Xu J, Liu R, Sun F, An L, Shang Z, Kong L, *et al.* Eucalyptal D enhances the antifungal effect of fluconazole on fluconazole-resistant *Candida albicans* by competitively inhibiting efflux pump. Front Cell Infect Microbiol. 2019;9:00211.
29. Singh S, Fatima Z, Ahmad K, Hameed S. Fungicidal action of geraniol against *Candida albicans* is potentiated by abrogated CaCdr1p drug efflux and fluconazole synergism. PLoS One. 2018;13(8):e0203079.
30. Holmes AR, Cardno TS, Strouse JJ, Ivnitski-Steele I, Keniya MV, Lackovic K, *et al.* Targeting efflux pumps to overcome antifungal drug resistance. Future Med Chem. 2016;8(12):1485-1501.
31. Jacobsen ID. The role of host and fungal factors in the commensal-to-pathogen transition of *Candida albicans*. Curr Clin Microbiol Rep. 2023;10(2):55-65.

32. Moffa EB, Mussi MC, Xiao Y, Garrido SS, Machado MA, Giampaolo ET, *et al.* Histatin 5 inhibits adhesion of *C. albicans* to reconstructed human oral epithelium. *Front Microbiol.* 2015;6:885.
33. Chang HT, Tsai PW, Huang HH, Liu YS, Chien TS, Lan CY. LL37 and hBD-3 elevate the β -1,3-exoglucanase activity of *Candida albicans* Xog1p, resulting in reduced fungal adhesion to plastic. *Biochem J.* 2012;441(3):963-970.
34. Li T, Li L, Du F, Sun L, Shi J, Long M, *et al.* Activity and mechanism of action of antifungal peptides from microorganisms: a review. *Molecules.* 2021;26(11):3438.
35. Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev.* 2003;67(3):400-428.
36. Sobajo OA, Okiki PA, Ade-Ojo IP, Adelegan O, Adarabioyo MI. *Candida albicans* virulence genes SAP1 and SAP6 associated with vaginitis among pregnant women attending a Nigerian tertiary hospital. *New Microbes New Infect.* 2022;49-50:101029.
37. Singh R, Kaur M, Chakrabarti A, Shankarnarayan SA, Rudramurthy SM. Biofilm formation by *Candida auris* isolated from colonising sites and candidemia cases. *Mycoses.* 2019;62(8):706-709.
38. Cavaleiro M, Teixeira MC. *Candida* Biofilms: threats, challenges, and promising strategies. *Front Med.* 2018;5:00028.
39. Oshiro KGN, Rodrigues G, Monges BED, Cardoso MH, Franco OL. Bioactive peptides against fungal biofilms. *Front Microbiol.* 2019;10:2169.
40. Roy S, Gow NAR. The role of the *Candida* biofilm matrix in drug and immune protection. *Cell Surf.* 2023;10:100111.
41. Naaz T, Lahiri D, Pandit S, Nag M, Gupta PK, Al-Dayyan N, *et al.* Antimicrobial peptides against microbial biofilms: efficacy, challenges, and future prospect. *Int J Pept Res Ther.* 2023;29(3):48.
42. Lee J-K, Chang SW, Perinpanayagam H, Lim S-M, Park Y-J, Han SH, *et al.* Antibacterial efficacy of a human β -Defensin-3 peptide on multispecies biofilms. *J Endod.* 2013;39(12):1625-1629.

43. Moazzezy N, Asadi Karam MR, Rafati S, Bouzari S, Oloomi M. Inhibition and eradication activity of truncated α -defensin analogs against multidrug resistant uropathogenic *Escherichia coli* biofilm. PLoS One. 2020;15(7):e0235892.
44. Pusateri CR, Monaco EA, Edgerton M. Sensitivity of *Candida albicans* biofilm cells grown on denture acrylic to antifungal proteins and chlorhexidine. Arch Oral Biol. 2009;54(6):588-594.
45. Vila T, Montelongo-Jauregui D, Ahmed H, Puthran T, Sultan AS, Jabra-Rizk MA. Comparative evaluations of the pathogenesis of *Candida auris* phenotypes and *Candida albicans* using clinically relevant murine models of infections. mSphere. 2020;5(4):00760-20.
46. Curvelo JAR, Moraes DC, Anjos CAD, Portela MB, Soares RMA. Histatin 5 and human lactoferrin inhibit biofilm formation of a fluconazole resistant *Candida albicans* clinical isolate. An Acad Bras Cienc. 2019;91(1):e20180045.
47. Konopka K, Dorocka-Bobkowska B, Gebremedhin S, Düzgüneş N. Susceptibility of *Candida* biofilms to histatin 5 and fluconazole. Antonie Van Leeuwenhoek. 2010;97(4):413-417.
48. Moghaddam-Taaheri P, Leissa JA, Eppler HB, Jewell CM, Karlsson AJ. Histatin 5 variant reduces *Candida albicans* biofilm viability and inhibits biofilm formation. Fungal Genet Biol. 2021;149:103529.
49. Mukherjee PK, Zhou G, Munyon R, Ghannoum MA. *Candida* biofilm: a well-designed protected environment. Med Mycol. 2005;43(3):191-208.
50. Jorge P, Lourenço A, Pereira MO. New trends in peptide-based anti-biofilm strategies: a review of recent achievements and bioinformatic approaches. Biofouling. 2012;28(10):1033-1061.
51. Kean R, Delaney C, Sherry L, Borman A, Johnson EM, Richardson MD, *et al.* Transcriptome assembly and profiling of *Candida auris* reveals novel insights into biofilm-mediated resistance. mSphere. 2018;3(4):e00334-18.

52. Rybak JM, Doorley LA, Nishimoto AT, Barker KS, Palmer GE, Rogers PD. Abrogation of triazole resistance upon deletion of CDR1 in a clinical isolate of *Candida auris*. *Antimicrob Agents Chemother*. 2019;63(4):19.
53. da Rocha Curvelo JA, Reis de Sá LF, Moraes DC, Soares RM, Ferreira-Pereira A. Histatin-5 induces the reversal of Pdr5p mediated fluconazole resistance in *Saccharomyces cerevisiae*. *J Mycol Med*. 2018;28(1):137-142.
54. Botelho Sampaio de Oliveira K, Lopes Leite M, Albuquerque Cunha V, Brito da Cunha N, Luiz Franco O. Challenges and advances in antimicrobial peptide development. *Drug Discov Today*. 2023;28(8):103629.

Chapter 6: General discussion & clinical implications

6.1. General discussion

The emergence and escalating frequency of invasive fungal infections present a growing and substantial threat to global public health (1). Immunocompromised people, including those with chronic health conditions, such as diabetes mellitus, tuberculosis, AIDS or cancer are particularly vulnerable (2). Critically ill patients within intensive care units, those undergoing invasive procedures with broad-spectrum antibiotics, and those receiving immunosuppressive therapies also face heightened risk (3). Diagnostic challenges, limited treatment access, and emerging antifungal resistance further exacerbate the challenges associated with invasive fungal infections management (4).

Among these infections, invasive candidiasis represents a significant nosocomial infection contributing to an estimated 700,000 cases worldwide (5). This infection is associated with extended hospitalizations and elevated healthcare expenses (6). *Candida albicans* is the predominant pathogen, accounting for approximately 62% of all *Candida* infections. However, the non-*albicans* *Candida* species have increased worldwide recently, particularly in healthcare settings, and are often associated with greater resistance to antifungal drugs (7). Among them, *Candida auris* is a recently identified and rapidly spreading pathogen causing concern due to its multi-drug resistance (8, 9).

Furthermore, the COVID-19 pandemic has been linked to increased incidence of comorbid invasive fungal infections, including *C. auris* infections (10). Compounding this threat is the emergence of antifungal resistance, which prolongs treatment, hospitalization, and necessitates the use of expensive and potentially toxic second-line drugs that contribute to elevated mortality (11). The available therapeutic landscape for systemic fungal infections remains constrained, offering only three main classes, with new development efforts facing challenges (12). Though effective, these drugs present adverse effects, frequently exhibit drug to drug interactions and require specialized administration (13).

The multidrug-resistant (MDR) pathogen, *C. auris* exemplifies all these challenges, posing risks to patients while proving difficult to eradicate in healthcare settings (14). In 2022, the

World Health Organization (WHO) developed an inaugural listing of prioritized fungi (15). A total of nineteen fungal pathogens were ranked and classified into critical, high, and medium priority groups. With *C. auris* being in the first group, along with *C. neoformans*, *Aspergillus fumigatus*, and *C. albicans*. This is an innovative global initiative that emphasizes the need for sustainable investment in basic mycological research and enhance the response to these fungal pathogens (15). The alarming rise in resistance against major antifungal classes in *C. auris* necessitates the urgent development of alternative therapies. Therefore, the search for new antifungal agents with broader spectra and minimal toxicity is crucial to address this challenge.

The exploration of natural products as potential antifungal agents has yielded significant research, and numerous studies demonstrate their antifungal properties (16). Recent research investigates the potential of leveraging the innate antifungal defences of the human immune system as alternative therapeutic strategies against fungal infections (17). This has spurred the development of innovative antifungal therapies utilizing naturally occurring antimicrobial peptides (AMPs) (18). In this context, AMPs have emerged as particularly promising candidates to combat antifungal resistance. Notably, various studies highlight the effectiveness of both natural and synthetic AMPs from diverse sources against bacteria and fungi (19-22). These encouraging results warrant further investigation into AMPs and the development of novel antifungal agents specifically targeted towards MDR *C. auris*.

The intrinsic resistance to established antifungals is progressively complicating the therapeutic management of *C. auris* infections, coupled with the limited number and the cytotoxicity of available treatment options (23). As expected, antifungal susceptibility testing revealed high resistance levels for both fluconazole and amphotericin B, and good susceptibility to caspofungin in *C. auris* isolates (described in chapter 3). While echinocandins (e.g., caspofungin) remain the first-line choice due to their safety and efficacy in most *C. auris* infections, the emerging resistance and the high cost potentially limit their long-term use in some cases (24). Thus, continued research and development of innovative antifungal approaches are crucial to address the emerging resistance and provide optimal treatment options in the future.

Antifungal drug development is a complex and crucial endeavour in the battle against fungal infections, with the basic science level laying the groundwork for successful therapeutic candidates (25). This early-stage research contributes to the exploration of novel therapies by investigating the potential of AMPs against the concerning pathogen *C. auris*. Several crucial

aspects were meticulously evaluated to ensure the development of safe, effective, and long-lasting potential antifungal drug. These include low-concentration efficacy with minimal toxicity and cost-effectiveness, targeting multiple drug targets for rapid and sustained efficacy, overcoming drug resistance mechanisms, enhancing therapeutic outcomes through combination therapy and finally minimum cytotoxicity.

The results are discussed in detail, for a summary of the results refer to Figure 6.1, Page 142.

6.1.1. Effectiveness at low concentration for low toxicity and cost effectiveness

The susceptibility testing results are promising, indicating the potential of selected human defensins and His 5 as effective antifungal agents against *C. auris* (described in chapter 3). While previous report documented His 5 efficacy against *C. auris* (26), the antifungal effects of defensins towards this species has not been previously described. The observed MICs\MFCs values suggest that the peptides exhibit antifungal activity at relatively low concentrations. This is a significant advantage, as lower drug concentrations are generally associated with reduced risk of side effects and potentially lower treatment costs.

Furthermore, time-kill analyses demonstrated concentration and time-dependent cell death, which emphasizes their effectiveness at low concentrations and short duration. The comparable maximum fungicidal endpoints for all peptides across all strains suggest they are equally effective in eradicating both susceptible and resistant *C. auris* cells when used at sufficient concentrations. This is crucial because it indicates potential effectiveness against even drug-resistant *C. auris* strains, a major concern in healthcare settings. In addition, the viability studies demonstrated significant fungicidal activity after only 4 hours of exposure, further suggests potential for a shorter treatment duration. These findings confirm that the peptides have fungicidal activity against *C. auris*, irrespective of their existing resistance to antifungals, and reinforces their potential for overcoming resistance challenges and improving clinical outcomes.

These findings reinforce the established roles of defensins as potent antifungal agents against *Candida* species (27), and our study confirms their efficacy against *C. auris*. Among the tested peptides, hBD-3 exhibited the strongest activity, aligning with its previously reported superior efficacy against *C. albicans* compared to other beta defensins (28). Furthermore, these findings

further corroborate the well-documented antifungal activity of His 5 against various *Candida* species (29), and demonstrated its potent antifungal activity against *C. auris*.

It is important to acknowledge that AMP synthesis can be expensive and currently limits their widespread application (30). However, the observed low concentration efficacy of the test peptides offers a potential counterpoint to the cost concern. This means that a smaller amount of the peptide would be needed to achieve the desired therapeutic effect, potentially compensating the high production cost. Moreover, ongoing research and development efforts focused on optimizing peptide production and purification methods could significantly reduce the cost of peptides synthesis in the future, making them more commercially available (31).

6.1.2. Multiple drug targets to maximise benefit and for fast effectiveness

The potent antifungal efficacy of the test AMPs encouraged further investigation to explore their targets and mechanisms. The restricted number of available antifungal drug targets has impeded the progress of new antifungal therapy development (32). This limitation also contributes to the emergence of multidrug-resistant fungal pathogens (33). Thus, identifying novel fungal targets is crucial to overcome this challenge (34). In addition, targeting multiple fungal metabolic pathways or cellular processes simultaneously improves the efficacy of the agent and reduces resistance emergence (35). This study demonstrated the exceptional capacity of the tested AMPs to target multiple aspects of *C. auris* (cell membrane, apoptosis, biofilm and virulence factors), potentially leading to improved efficacy, faster fungal eradication and slow resistance development. This could ultimately reduce treatment duration and minimize potential complications.

6.1.2.1. Targeting cell membrane

Generally, AMPs are known to disrupt microbial cell membranes, causing pore formation, cytoplasmic leakage, and ultimately cell death (36). Similarly, the AMPs studied here, at high concentrations and prolonged exposure, induced varying degrees of membrane permeability in *C. auris*, as visualized using confocal microscopy (PI-positive cells). This indicates that the fungicidal activity observed is likely attributable to their ability to compromise membrane integrity (described in chapter 3). These findings align with earlier studies demonstrating the membrane-disruptive antifungal activity of different AMPs (37). Although membrane

interaction was recognized as a primary mechanism, other potential modes of action for these AMPs cannot be ruled out and warrant future investigation. Moreover, the differential inhibitory concentrations observed among the tested peptides may reflect a combination of mechanisms and interactions with various microbial structures, enhancing overall killing potential. Therefore, the three most active peptides (hBD-3, HNP-1 and His 5) were chosen for further studies focusing on elucidating the specific mechanisms of action of AMPs towards *C. auris*.

6.1.2.2. Targeting apoptosis

To elucidate the mechanism underlying the fungicidal effects of the tested AMPs, this study explored their potential to induce apoptosis (described in chapter 4). Yeast apoptosis models are widely used to screen promising antifungal agents that target specific regulators with minimal toxicity to human cells (38). Prior studies have demonstrated the ability of AMPs to induce apoptosis in yeast (37). Our findings indicate that hBD-3 and HNP-1 trigger both early and late hallmarks of yeast apoptosis in *C. auris*, including externalization of phosphatidylserine, DNA fragmentation, mitochondrial membrane depolarization, and cytochrome c release. These observations are aligned with prior studies reporting the ability of AMPs to induce apoptosis in *Candida* species (39, 40). In contrast, His 5 was observed to induce necrosis, as indicated by the absence of classical apoptotic markers and the presence of necrotic cells. Recent evidence suggests His 5 disrupts cellular homeostasis in *C. albicans* by inducing an osmotic imbalance, leading to ion leakage and decreased ATP production within mitochondria (41). Furthermore, His 5 uptake by *C. auris* has been observed, similar to *C. albicans* (26).

Importantly, both defensins significantly impacted mitochondrial membrane potential and cytochrome c activity in *C. auris* cells, indicating intrinsic apoptosis pathway involvement. The intrinsic mitochondrial pathway, involving cytochrome c release activating yeast metacaspase and initiating a pro-apoptotic cascade, is a well-established mechanism in yeast apoptosis. (42). Our results suggest that hBD-3 and HNP-1 potently induce apoptotic cell death in *C. auris*, likely mediated through mitochondrial disruption, cytochrome c release, and subsequent metacaspase-dependent pathway activation. On the other hand, His 5 minimally affected mitochondrial function in *C. auris*, with slight membrane depolarization and negligible cytochrome c release, further supporting a distinct necrotic mechanism. Our results align with

prior studies demonstrating minimal impact of His 5 on mitochondrial function in mammalian cells (43).

Our results also revealed increased reactive oxygen species (ROS) production upon treatment with all three AMPs. While ROS may directly trigger apoptosis induced by hBD-3 and HNP-1 (44), it is likely an indirect consequence of His 5-mediated necrosis (45). The precise interplay between ROS accumulation, mitochondrial dysfunction, and the type of cell death requires further elucidation. Collectively, the observed apoptotic and necrotic effects suggest the potential of these peptides for developing new antifungal therapies. However, further research is warranted to comprehensively elucidate their modes of action and identify additional cell death pathways that might be involved.

6.1.2.3. Targeting virulence factors

Candida species including *C. auris* cause disease by expressing various virulence factors. These factors allow the fungus to penetrate host tissues and establish infections. Drugs that target these virulence factors hold promise as a new and effective approach to combat fungal infections (46). The influence of the most potent peptides against key virulence factors of *C. auris* was assessed in this study (described in chapter 5). The results demonstrated that all three tested AMPs, even at subinhibitory concentrations, reduced its capacity to adhere to epithelial cells and secrete proteinase. This dual targeting could weaken *C. auris*, impeding its colonization and preventing damage to host tissues, potentially hindering the initial stages in the pathogenic process. Their anti-adherence activity might involve targeting either the adhesin proteins or the β -glucan of the cell wall, similar to the observed effect of hBD-3 on *C. albicans* (47). Alternatively, the AMPs downregulated the expression of *SAP3*, a gene crucial for *C. auris* adhesion, which was observed here, could play a role. This further strengthens their ability to disrupt the initial stages of fungal infection. These findings highlight the promising potential of AMPs as anti-virulence agents against *C. auris*. By targeting both adhesion and proteinase secretion, AMPs offer a novel strategy to combat fungal infections, alongside their direct antifungal activity.

6.1.2.4. Targeting biofilm

C. auris biofilm is a key factor to its ability to cause infection and resist treatment (48). Our study revealed a promising finding that the test AMPs significantly inhibited biofilm formation

and demonstrated effectiveness against already established biofilms (described in chapter 5). CLSM further confirmed the disruptive effects of AMPs on biofilm architecture and viability. They also suppressed the genes essential for biofilm development (*PGA26*, *PGA7* and *PGA52*), suggesting that their anti-biofilm effects might be partly attributed to gene regulation. Other possible mechanisms that AMPs might directly exert antimicrobial activity against biofilm cells, contributing to their disruption or affect *C. auris* adherence to surfaces, hindering initial biofilm formation. These results suggest that the tested AMPs target biofilm in *C. auris*, thereby hindering associated challenges like drug resistance. This study presents the first evidence of the superior efficacy of AMPs in targeting *C. auris* biofilm. Previous reports have demonstrated that AMPs can reduce *C. albicans* biofilm formation (49, 50).

Our findings corroborate these observations and support the potential of targeting virulence factors for novel antifungal therapies. While the study explored potential mechanisms through gene expression analysis, further investigation is needed to comprehensively understand how AMPs counteract *C. auris* virulence and to evaluate their efficacy *in vivo*. Furthermore, this study revealed that the resistant *C. auris* strain exhibited higher adherence, proteinase and biofilm activity compared to the sensitive strain (by observation), highlighting the complexities associated with managing drug-resistant isolates. Despite this challenge, the observed efficacy of the tested AMPs in inhibiting virulence factors in both sensitive and resistant strains underscore their potential to circumvent antifungal resistance. These findings highlight the potential of AMPs as promising antifungal agents against *C. auris* infections, particularly considering their ability to target virulence mechanisms. However, further research is necessary to optimize their efficacy and explore their potential for *in vivo* applications.

6.1.3. Reversal of drug resistance mechanism

Efflux pumps are another significant factor contributing to *C. auris* antifungal resistance, particularly against azoles (51). Studies have shown that overexpression of efflux pump has been linked to enhanced biofilm formation and invasive infections (52). This study demonstrated the efficacy of all test peptides as efflux pump inhibitors (described in chapter 5). In addition, the AMPs significantly downregulated expression of genes (*CDR1*, *CDR2*, *MDR1* and *SNQ2*) associated with efflux pumps in *C. auris*. This suggests their potential to overcome existing antifungal resistance mechanisms, offering a promising avenue for future therapeutic development. Previous study showed that His 5 reduced efflux pump activity in *Saccharomyces cerevisiae* by inhibiting the Pdr5p transporter, functionally equivalent to ABC

transporters in *C. albicans* (53). Further research is warranted to understand the precise interactions between the AMPs and efflux pumps in *C. auris*.

6.1.4. Enhanced efficacy with combination therapy

These promising findings support the continued exploration of these naturally occurring antimicrobial agents as potential antifungal therapies against *C. auris*. Combining antifungals offers several advantages to overcome resistance and toxicity challenges associated with *C. auris* treatment. Combination therapy becomes the preferred therapeutic approach to treat invasive fungal infections that exhibit resistance to single-agent therapy, including *C. auris* infections (54). Strategically combining compounds with distinct mechanisms has the potential to combat multidrug resistance (55). Prior reports demonstrate *in vitro* synergism between AMPs and conventional antifungals against *C. albicans*, including improved survival in animal models (56, 57). Therefore, the synergistic potential of combining AMPs with clinically approved antifungals was investigated (described in chapter 3). Significant synergy was observed in nearly all antifungal-AMP combinations, with greater synergy observed for caspofungin (100 %) compared to fluconazole (70 to 90 %) and amphotericin B (90 to 100 %). While the exact mechanisms of synergistic interactions remain unclear, using these combinations notably decreased the MICs (by 4- and 8- fold) of both AMPs and antifungals, highlighting their potential to reduce toxicity and the risk of antifungal resistance. Further studies, including *in vivo* investigations and cytotoxicity assessments, are needed to substantiate these findings. In addition, the efflux pump inhibition by the AMPs could be a potential mechanism for the observed synergy between these peptides and fluconazole, allowing fluconazole to accumulate intracellularly and enhance its efficacy.

6.1.5. Minimum cytotoxicity

Assessing the potential safety of novel drug candidates is crucial for their further development. In this study, the cytotoxicity of the most active test AMPs was evaluated using an *in vitro* haemolytic assay with mammalian red blood cells. The results revealed low levels of haemolysis across all tested concentrations (described in chapter 5), indicating minimal to no cytotoxic effects. Generally, human AMPs exhibit relatively low toxicity and good tolerance, making them attractive candidates for therapeutic development (58). The findings presented here suggest the potential safety of the compounds for further *in vivo* studies, paving the way for preclinical development.

Overall, this research emphasizes the promise of AMPs as innovative molecules for developing antifungal drugs to combat *C. auris* infections. Their effectiveness at low concentrations, potential for targeting multiple fungal processes, possible ability to overcome resistance mechanisms and their lower toxicity profile, are encouraging findings.

6.2. Recommendations and Clinical implications

- AMPs offer the potential as novel candidates to combat the growing challenge posed by drug resistance fungal pathogens, especially against *C. auris*.
- AMPs act synergistically with existing antifungal drugs, potentially allowing for lower drug dosages and reduced side effects.
- AMPs demonstrate fungicidal activity against both susceptible and MDR *C. auris* strains, offering potential as a therapeutic option where existing drugs are ineffective.
- Certain AMPs trigger apoptosis in *C. auris*, leading to a controlled and non-inflammatory elimination of the fungal cells. This offers potential benefits over fungicidal agents that can cause cell lysis and release of harmful intracellular components.
- AMPs target various aspects of fungal virulence, including adherence, proteinase production, biofilm formation, and efflux pumps, potentially reducing the risk of resistance development and enhancing effectiveness compared to single-target drugs.
- Compared to some conventional antifungal drugs, AMPs exhibit lower cytotoxicity towards mammalian cells, suggesting their potential for improved safety profiles. This can be particularly beneficial for immunocompromised patients or those with limited treatment options.

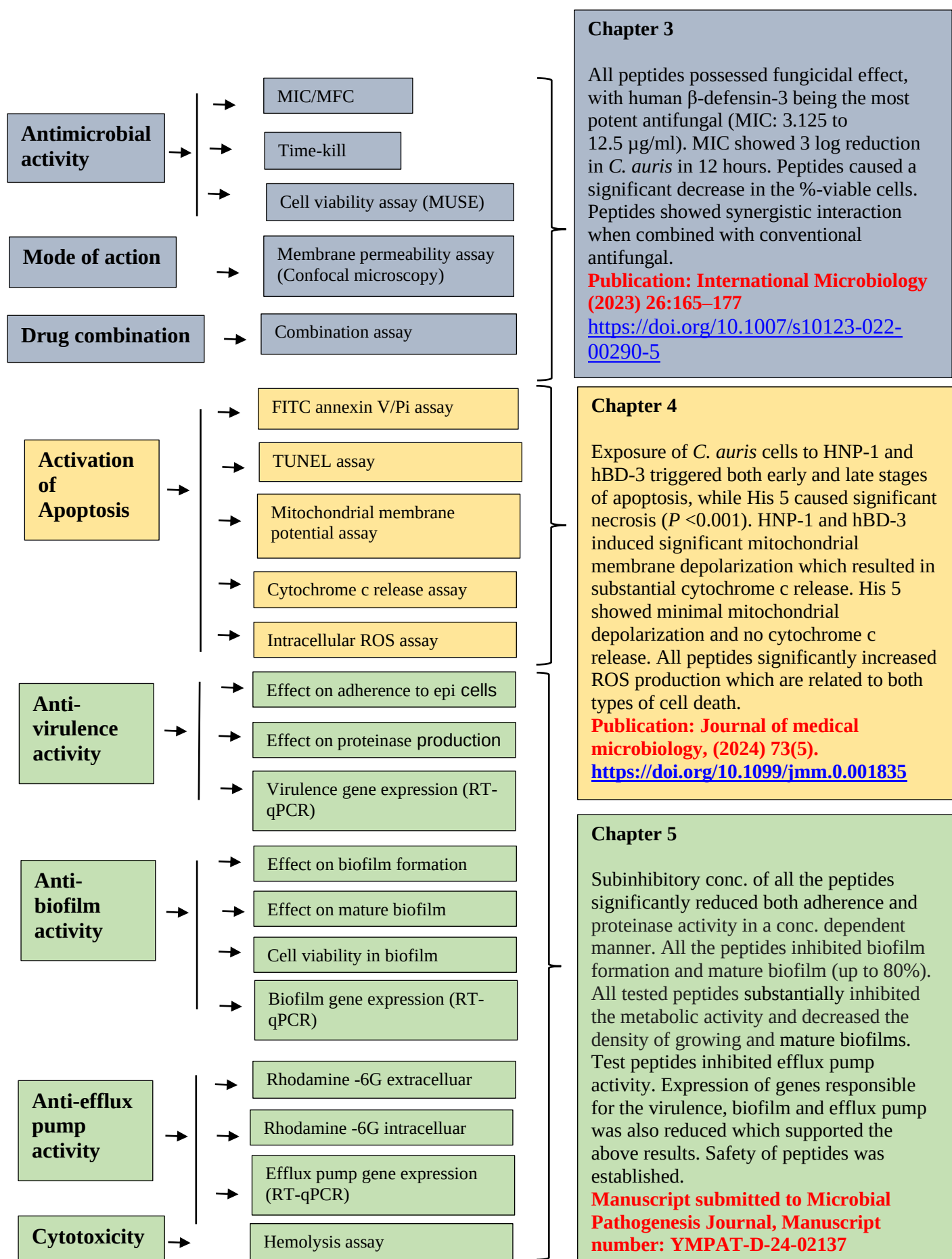


Figure 6.1: Flow diagram of study on anti-*C. auris* activity of Synthetic AMPs (hBD-3, HNP-1 and His 5)

6.3. References

1. Fisher MC, Gurr SJ, Cuomo CA, Blehert DS, Jin H, Stukenbrock EH, *et al.* Threats posed by the fungal kingdom to humans, wildlife, and agriculture. *mBio*. 2020;11(3).
2. Firacative C. Invasive fungal disease in humans: are we aware of the real impact? *Mem Inst Oswaldo Cruz*. 2020;115:e200430.
3. Bajwa S, Kulshrestha A. Fungal infections in intensive care unit: challenges in diagnosis and management. *Ann Med Health Sci Res*. 2013;3(2):238-244.
4. Fang W, Wu J, Cheng M, Zhu X, Du M, Chen C, *et al.* Diagnosis of invasive fungal infections: challenges and recent developments. *J Biomed Sci*. 2023;30(1):42.
5. Bongomin F, Gago S, Oladele RO, Denning DW. Global and multi-national prevalence of fungal diseases—estimate precision. *J Fungi (Basel)*. 2017;3(4):57.
6. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. *Nat Rev Dis Primers*. 2018;4(1):18026.
7. de Oliveira Santos GC, Vasconcelos CC, Lopes AJO, de Sousa Cartágenes MdS, Filho AKDB, do Nascimento FRF, *et al.* *Candida* Infections and therapeutic strategies: mechanisms of action for traditional and alternative agents. *Front Microbiol*. 2018;9.
8. Satoh K MK, Hasumi Y, Nishiyama Y, Uchida K, Yamaguchi H. *Candida auris* sp. nov., a novel ascomycetous yeast isolated from the external ear canal of an inpatient in a Japanese hospital. *Microbiol Immunol*. 2009 Jan;53(1):41-44.
9. Dahiya S, Chhillar AK, Sharma N, Choudhary P, Punia A, Balhara M, *et al.* *Candida auris* and nosocomial infection. *Curr Drug Targets*. 2020;21(4):365-373.
10. Tsai CS, Lee SS, Chen WC, Tseng CH, Lee NY, Chen PL, *et al.* COVID-19-associated candidiasis and the emerging concern of *Candida auris* infections. *J Microbiol Immunol Infect*. 2023;56(4):672-679.
11. Prestinaci F, Pezzotti P, Pantosti A. Antimicrobial resistance: a global multifaceted phenomenon. *Pathog Glob Health*. 2015;109(7):309-318.

12. Lamoth F, Lewis RE, Kontoyiannis DP. Investigational antifungal agents for invasive mycoses: a clinical perspective. *Clin Infect Dis.* 2022;75(3):534-544.
13. Houšť J, Spížek J, Havlíček V. Antifungal Drugs. *Metabolites.* 2020;10(3).
14. Morales-López SE P-GC, Ceballos-Garzón A, Martínez HP, Rodríguez GJ, Álvarez-Moreno CA, Rodríguez JY. Invasive infections with multidrug-resistant yeast *Candida auris*, Colombia. *Emerg Infect Dis.* 2017 Jan;23(1):162-164.
15. WHO. WHO fungal priority pathogens list to guide research, development and public health action. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO. 2022.
16. Vanreppelen G, Wuyts J, Van Dijck P, Vandecruys P. Sources of antifungal drugs. *J Fungi (Basel).* 2023;9(2).
17. Mercer DK, O'Neil DA. Innate inspiration: antifungal peptides and other immunotherapeutics from the host immune response. *Front Immunol.* 2020;11:2177.
18. Buda De Cesare G, Cristy SA, Garsin DA, Lorenz MC. Antimicrobial peptides: a new frontier in antifungal therapy. *mBio.* 2020;11(6).
19. do Nascimento Dias J, de Souza Silva C, de Araujo AR, Souza JMT, de Holanda Veloso Junior PH, et al. Mechanisms of action of antimicrobial peptides ToAP2 and NDBP-5.7 against *Candida albicans* planktonic and biofilm cells. *Sci Rep.* 2020;10(1):10327.
20. Ciociola T GL, Conti S, Magliani W, Santinoli C, Polonelli L. Natural and synthetic peptides with antifungal activity. *Future Med Chem.* 2016 Aug;8(12):1413-1433.
21. Huang Q FJ, Yu HJ, Gou YB, Huang XK. Effects of human β -defensin-3 on biofilm formation-regulating genes *dltB* and *icaA* in *Staphylococcus aureus*. *Mol Med Rep.* 2014 Aug;10(2):825-831.
22. Maiti S PS, Purohit S, Jain S, Senapati S, Dey N. Effective control of *Salmonella* infections by employing combinations of recombinant antimicrobial human β -defensins hBD-1 and hBD-2. *Antimicrob Agents Chemother.* 2014 Nov;58(11):6896-6903.

23. Zhu Y OBB, Leach L, Clarke A, Bates M, Adams E, Ostrowsky B, *et al.* Laboratory analysis of an outbreak of *Candida auris* in New York from 2016 to 2018: impact and lessons learned. J Clin Microbiol. 2020 Mar 25;58(4):e01503-1519.
24. Lyman M FK, Reuben J, Dang T, Free R, Seagle EE, Sexton DJ, *et al.* Notes from the field: transmission of pan-resistant and echinocandin-resistant *Candida auris* in health care facilities - Texas and the district of Columbia, January-April 2021. MMWR Morb Mortal Wkly Rep. 2021 Jul 23;70(29):1022-1023.
25. Bouz G, Doležal M. Advances in antifungal drug development: an up-to-date mini review. Pharmaceuticals (Basel). 2021;14(12).
26. Pathirana RU, Friedman J, Norris HL, Salvatori O, McCall AD, Kay J, *et al.* Fluconazole-resistant *Candida auris* is susceptible to salivary histatin 5 killing and to intrinsic host defenses. Antimicrob Agents Chemother. 2018;62(2).
27. Polesello V, Segat L, Crovella S, Zupin L. *Candida* infections and human defensins. Protein Pept Lett. 2017;24(8):747-756.
28. Vylkova S, Nayyar N, Li W, Edgerton M. Human beta-defensins kill *Candida albicans* in an energy-dependent and salt-sensitive manner without causing membrane disruption. Antimicrob Agents Chemother. 2007;51(1):154-161.
29. Puri S, Edgerton M. How does it kill?: understanding the candidacidal mechanism of salivary histatin 5. Eukaryot Cell. 2014;13(8):958-964.
30. Luo X, Chen H, Song Y, Qin Z, Xu L, He N, *et al.* Advancements, challenges and future perspectives on peptide-based drugs: Focus on antimicrobial peptides. Eur J Pharm Sci. 2023;181:106363.
31. George B, Pooja S, Suchithra TV, Sebastian D. Historical developments of antimicrobial peptide research. In: Ajesh K, Sreejith K, editors. Antimicrobial Peptides: Academic Press; 2023. p. 1-16.
32. Robbins N, Wright GD, Cowen LE. Antifungal Drugs: The Current armamentarium and development of new agents. Microbiol Spectr. 2016;4(5):10.

33. Chen L, Zhang L, Xie Y, Wang Y, Tian X, Fang W, *et al.* Confronting antifungal resistance, tolerance, and persistence: Advances in drug target discovery and delivery systems. *Adv Drug Deliv Rev.* 2023;200:115007.
34. Ahmad A, Molepo J, Patel M. Challenges in the development of antifungal agents against *Candida*: scope of phytochemical research. *Curr Pharm Des.* 2016;22(27):4135-4150.
35. Parente-Rocha JA, Bailão AM, Amaral AC, Taborda CP, Paccez JD, Borges CL, *et al.* Antifungal resistance, metabolic routes as drug targets, and new antifungal agents: an overview about endemic dimorphic fungi. *Mediators Inflamm.* 2017;2017:9870679.
36. Rautenbach M TA, Vosloo JA. Antifungal peptides: To be or not to be membrane active. *Biochimie.* 2016 Nov;130:132-145.
37. Struyfs C, Cammue BPA, Thevissen K. Membrane-interacting antifungal peptides. *Front Cell Dev Biol.* 2021;9:649875.
38. Almeida B, Silva A, Mesquita A, Sampaio-Marques B, Rodrigues F, Ludovico P. Drug-induced apoptosis in yeast. *Biochim Biophys Acta.* 2008;1783(7):1436-1448.
39. Chen RC, Lan CY. Human antimicrobial peptide Hecidin 25-induced apoptosis in *Candida albicans*. *Microorganisms.* 2020;8(4).
40. Andrés MT, Viejo-Díaz M, Fierro JF. Human lactoferrin induces apoptosis-like cell death in *Candida albicans*: critical role of K⁺-channel-mediated K⁺ efflux. *Antimicrob Agents Chemother.* 2008;52(11):4081-4088.
41. Edgerton M, Koshlukova SE, Lo TE, Chrzan BG, Straubinger RM, Raj PA. Candidacidal activity of salivary histatins. identification of a histatin 5-binding protein on *Candida albicans*. *J Biol Chem.* 1998;273(32):20438-20447.
42. Carmona-Gutierrez D, Eisenberg T, Buttner S, Meisinger C, Kroemer G, Madeo F. Apoptosis in yeast: triggers, pathways, subroutines. *Cell Death Differ.* 2010;17(5):763-773.
43. Petruzzelli R, Clementi ME, Marini S, Coletta M, Di Stasio E, Giardina B, *et al.* Respiratory inhibition of isolated mammalian mitochondria by salivary antifungal peptide histatin-5. *Biochem Biophys Res Commun.* 2003;311(4):1034-1040.

44. Cho J, Lee DG. Oxidative stress by antimicrobial peptide pleurocidin triggers apoptosis in *Candida albicans*. *Biochimie*. 2011;93(10):1873-1879.
45. Veerman EC, Nazmi K, Van't Hof W, Bolscher JG, Den Hertog AL, Nieuw Amerongen AV. Reactive oxygen species play no role in the candidacidal activity of the salivary antimicrobial peptide histatin 5. *Biochem J*. 2004;381(Pt 2):447-452.
46. Ahmad Khan MS, Alshehrei F, Al-Ghamdi SB, Bamaga MA, Al-Thubiani AS, Alam MZ. Virulence and biofilms as promising targets in developing antipathogenic drugs against candidiasis. *Future Sci OA*. 2020;6(2):Fso440.
47. Chang HT, Tsai PW, Huang HH, Liu YS, Chien TS, Lan CY. LL37 and hBD-3 elevate the β -1,3-exoglucanase activity of *Candida albicans* Xog1p, resulting in reduced fungal adhesion to plastic. *Biochem J*. 2012;441(3):963-970.
48. Singh R, Kaur M, Chakrabarti A, Shankarnarayan SA, Rudramurthy SM. Biofilm formation by *Candida auris* isolated from colonising sites and candidemia cases. *Mycoses*. 2019;62(8):706-709.
49. Moghaddam-Taaheri P, Leissa JA, Eppler HB, Jewell CM, Karlsson AJ. Histatin 5 variant reduces *Candida albicans* biofilm viability and inhibits biofilm formation. *Fungal Genet Biol*. 2021;149:103529.
50. Oshiro KGN, Rodrigues G, Monges BED, Cardoso MH, Franco OL. Bioactive peptides against fungal biofilms. *Front Microbiol*. 2019;10.
51. Chaabane F, Graf A, Jequier L, Coste AT. Review on antifungal resistance mechanisms in the emerging pathogen *Candida auris*. *Front Microbiol*. 2019;10:2788.
52. Kean R, Delaney C, Sherry L, Borman A, Johnson EM, Richardson MD, *et al*. Transcriptome assembly and profiling of *Candida auris* reveals novel insights into biofilm-mediated resistance. *mSphere*. 2018;3(4):10.1128/msphere.00334-18.
53. da Rocha Curvelo JA, Reis de Sá LF, Moraes DC, Soares RM, Ferreira-Pereira A. Histatin-5 induces the reversal of Pdr5p mediated fluconazole resistance in *Saccharomyces cerevisiae*. *J Mycol Med*. 2018;28(1):137-142.

54. Scorzoni L dPESA, Marcos CM, Assato PA, de Melo WC, de Oliveira HC, Costa-Orlandi CB, et al. Antifungal Therapy: new advances in the understanding and treatment of mycosis. *Front Microbiol.* 2017 Jan 23;8:36.
55. Candoni A CM, Cesaro S, Busca A, Giacchino M, Fanci R, Delia M, *et al.* SEIFEM GROUP (Sorveglianza Epidemiologica Infezioni Fungine nelle Emopatie Maligne). Multicentre surveillance study on feasibility, safety and efficacy of antifungal combination therapy for proven or probable invasive fungal diseases in haematological patients: the SEIFEM real-life combo study. *Mycoses.* 2014 Jun;57(6):342-350..
56. Lupetti A P-AA, Welling MM, Dogterom-Ballering H, Brouwer CP, Senesi S, Van Dissel JT, et al. Synergistic activity of the N-terminal peptide of human lactoferrin and fluconazole against *Candida* species. *Antimicrob Agents Chemother.* 2003 Jan;47(1):262-267.
57. MacCallum DM, Desbois AP, Coote PJ. Enhanced efficacy of synergistic combinations of antimicrobial peptides with caspofungin versus *Candida albicans* in insect and murine models of systemic infection. *Eur J Clin Microbiol Infect Dis.* 2013;32(8):1055-1062.
58. Ahmed A, Siman-Tov G, Hall G, Bhalla N, Narayanan A. Human antimicrobial peptides as therapeutics for viral infections. *Viruses.* 2019;11(8):704.

Chapter 7: Conclusions, limitations & future research

7.1. Conclusion

This study demonstrated the fungicidal activity of selected antimicrobial peptides (AMPs), particularly hBD-3, HNP-1 and His 5, both alone and in combination with existing antifungal drugs (fluconazole, amphotericin B and caspofungin) against *Candida auris*. Antimicrobial effect of these peptides was concentration and time-dependent effects on *C. auris* cells. At higher concentrations and prolonged exposure, all AMPs induced varying degrees of membrane permeabilization. Conversely, at lower concentrations and shorter exposure times, hBD-3 and HNP-1 triggered apoptosis through a mitochondria-dependent pathway and ROS production. In contrast, His 5 induced necrosis solely through reactive oxygen species (ROS) production, without affecting cytochrome c release. Furthermore, the AMPs, even at subinhibitory concentrations, significantly inhibited key virulence factors in *C. auris*, including adherence, proteinase production, and biofilm formation and mature biofilm. They also inhibited efflux pump activity and downregulated genes associated with virulence and efflux pumps in the multidrug resistant (MDR) isolate. In addition, the low cytotoxicity observed with these AMPs suggests their potential for further *in vivo* evaluation. Collectively, AMPs exhibit broad activity against both susceptible and MDR *C. auris* strains, highlighting their potential as novel antifungal agents alone or in combination to combat drug-resistant fungal pathogens, including *C. auris*. They offer potential not only for direct fungicidal activity but also as anti-virulence and anti-efflux pump agents, warranting further investigation for their therapeutic development and to optimize their use for clinical applications.

7.2. Limitations & future research

- The study was conducted in a controlled laboratory setting. Further studies are needed to evaluate the efficacy and safety of these AMPs *in vivo* (using animal models) and eventually in clinical trials.
- The study used a limited number of *C. auris* isolates, which might not represent the full diversity of strains and potential resistance mechanisms encountered in clinical settings. Further study including a larger sample size would provide broader insights into the potential and efficacy of the AMPs.
- While the study provides some insights into the mechanisms of action for each AMP (apoptosis vs. necrosis), a more comprehensive understanding of these mechanisms is required for further development and optimization.
- While the study briefly mentions the potential synergy between AMPs and existing antifungal drugs, further investigation is needed to optimize combination therapy strategies.
- While this preliminary study reports low cytotoxicity of the tested AMPs, a more comprehensive assessment with different cell lines and longer exposure times might be necessary.
- The study highlights the potential of AMPs, but further optimization of their properties, such as improving stability and delivery methods is crucial to ensure appropriate bioavailability and clinical applicability.
- The study only investigated selected AMPs. Further research is needed to explore the potential of other AMPs against *C. auris*.
- While the study demonstrated the effectiveness of AMPs against *C. auris*, investigating their activity against a wider range of *Candida* species and other fungal pathogens is crucial. This broader evaluation would provide valuable insights into the general applicability and overall efficacy of AMPs.

APPENDICES

Appendix A:

Figure A1: Ethical Clearance Certificate

 UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)	
28/04/2021	
Ref: W-CBP-210428-01	
TO WHOM IT MAY CONCERN	
Waiver:	This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)
Investigator:	Dr S Shaban Student No. (if appropriate): 1764671 Staff No. (if appropriate):
Supervisor:	Dr A Ahmad
School: Department:	Pathology Clinical Microbiology & Infectious Diseases Medical School University
Project title:	<i>Antifungal activity of synthetic peptides targeting apoptosis in Candida auris</i>
Reason:	In vitro laboratory study No human participants will be involved in the study
 _____	
Dr CB Penny Chairperson: Human Research Ethics Committee (Medical)	
Research Office Secretariat: Third Floor, Phillip Tobias Building, corner of St Andrews and York Roads, Parktown, Johannesburg 2193 Postal address: Private Bag 3, Wits 2050 Tel Nos: +27 (0)11 717 1234/1252/2656/2700 Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za Website: https://www.wits.ac.za/research/researcher-support/research-ethics/ethics-committees/	

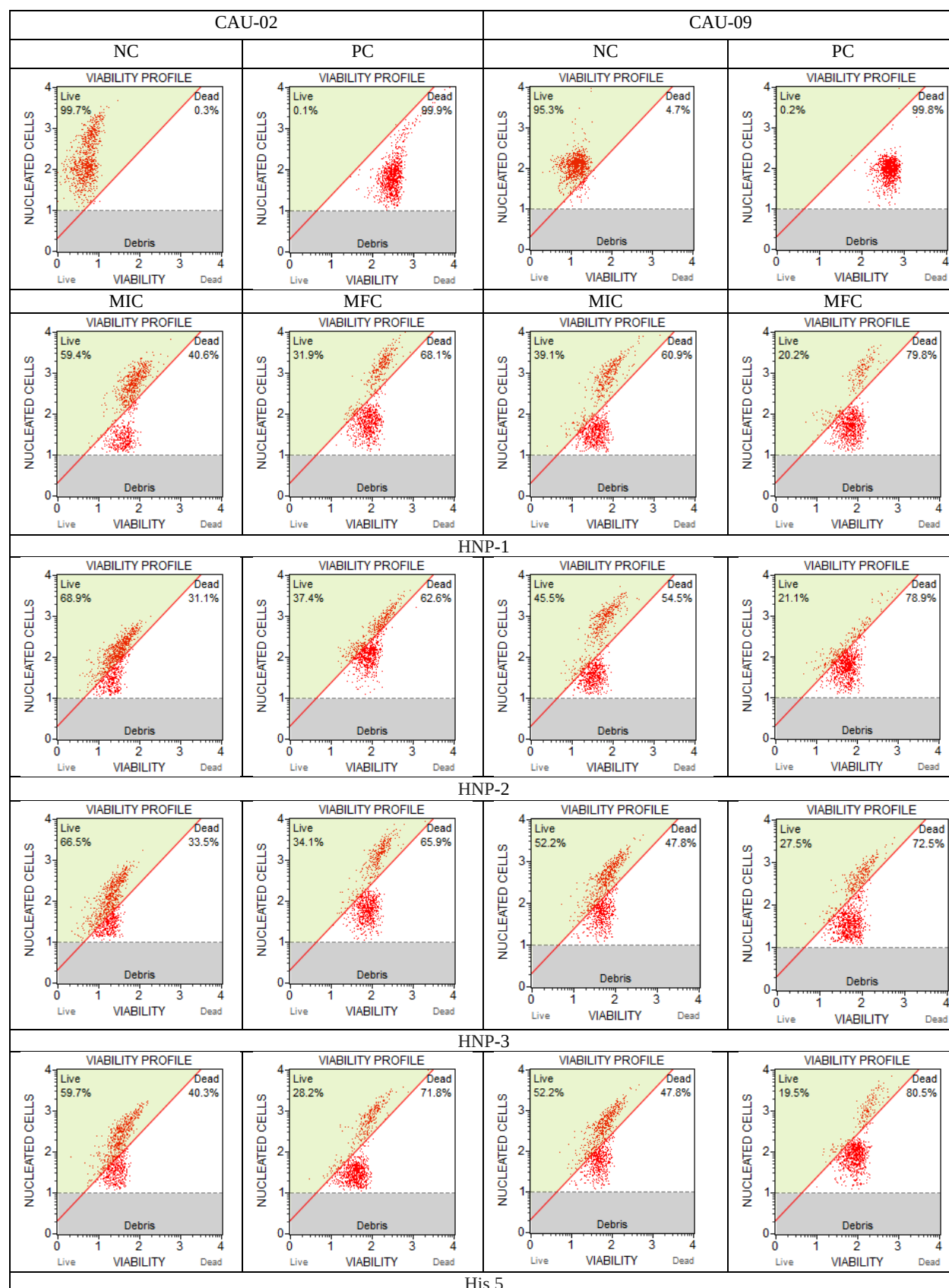
Appendix B:

Table B1: Clinical isolates used in this study.

No.	Strains	Species
CAU-01	MRL 3499	<i>Candida auris</i>
CAU-02	MRL3785	<i>Candida auris</i>
CAU-03	MRL4000	<i>Candida auris</i>
CAU-04	MRL2921	<i>Candida auris</i>
CAU-05	MRL5762	<i>Candida auris</i>
CAU-06	MRL5765	<i>Candida auris</i>
CAU-07	MRL6277	<i>Candida auris</i>
CAU-08	MRL6065	<i>Candida auris</i>
CAU-09	MRL6057	<i>Candida auris</i>
CAU-10	MRL6173	<i>Candida auris</i>

Appendix C:

Supplementary data for chapter 3



His 5

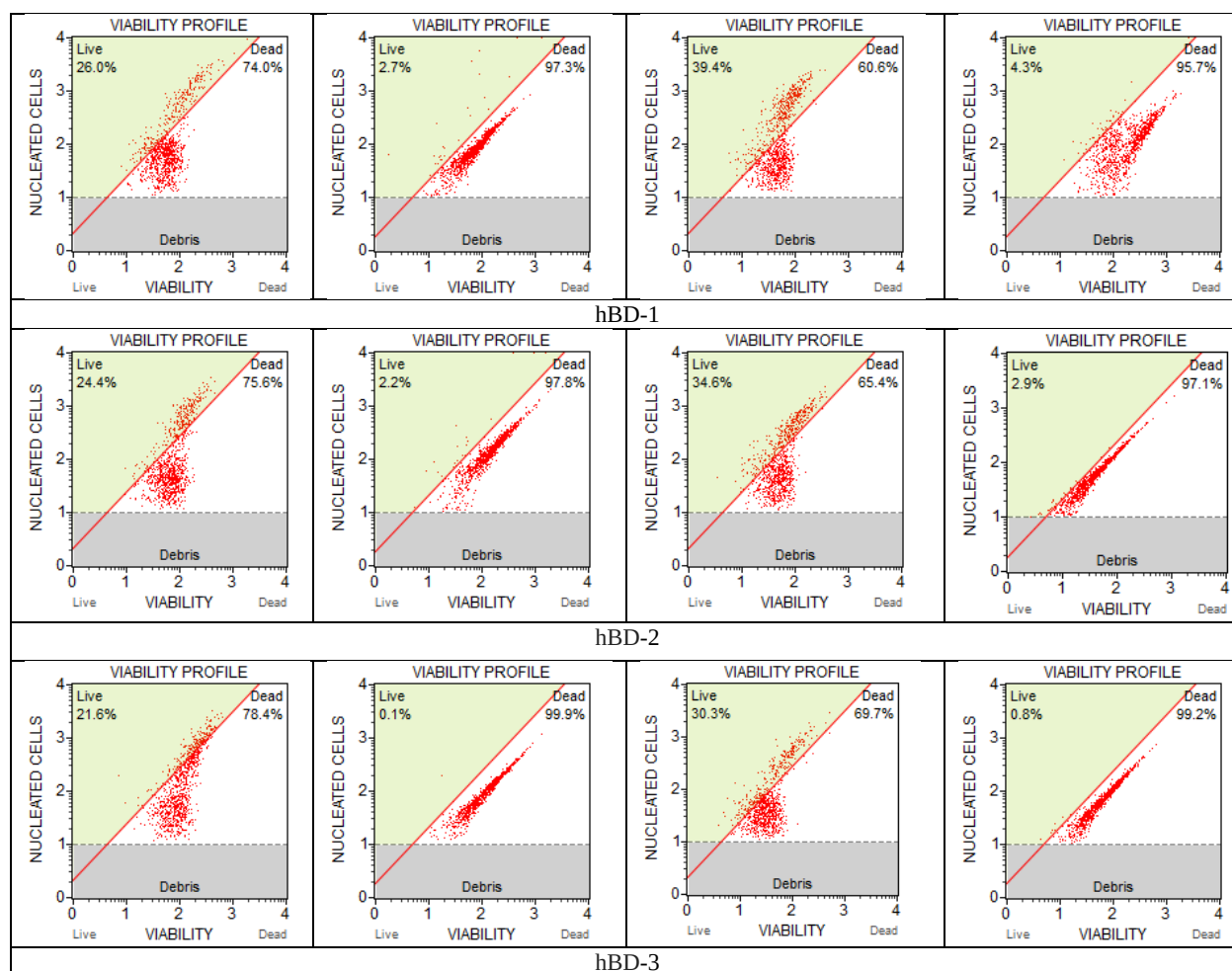


Figure C.1: Effect of the antimicrobial peptides on cell count and viability of *Candida auris*. The figures shown are representative data of three independent experiments. The viability of *C. auris* CAU-02 and CAU-09 cells; negative and positive and control represent untreated, and cells treated with 10 mM H₂O₂; *C. auris* CAU-02 and CAU-09 treated with HNP-1, HNP-2, HNP-3, His 5, hBD-1, hBD-2 and hBD-3 at different concentrations.

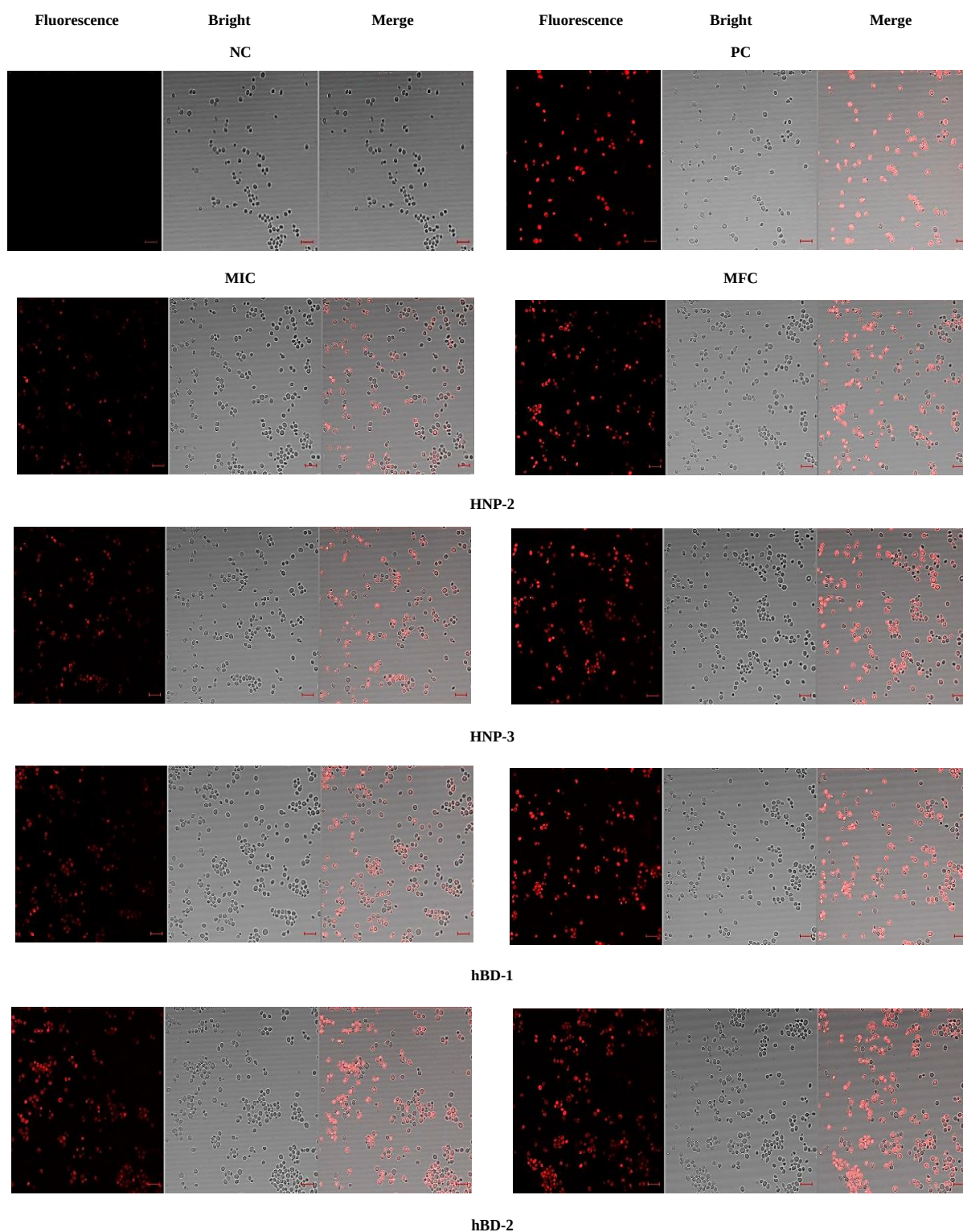


Figure C.2: Confocal microscopy images of the effect of the tested peptides on *Candida auris* cells. Permeabilization of the membrane of *C. auris* strains CAU-02 following exposure to the tested peptides (HNP-2, HNP-3, hBD-1 and hBD-2) at MIC/MFC concentrations for 4 hours. cells with membrane damage were stained with PI red; negative and positive control represent untreated, and cells treated with 10 mM H₂O₂ respectively. *Scale-bar:* 10 μm

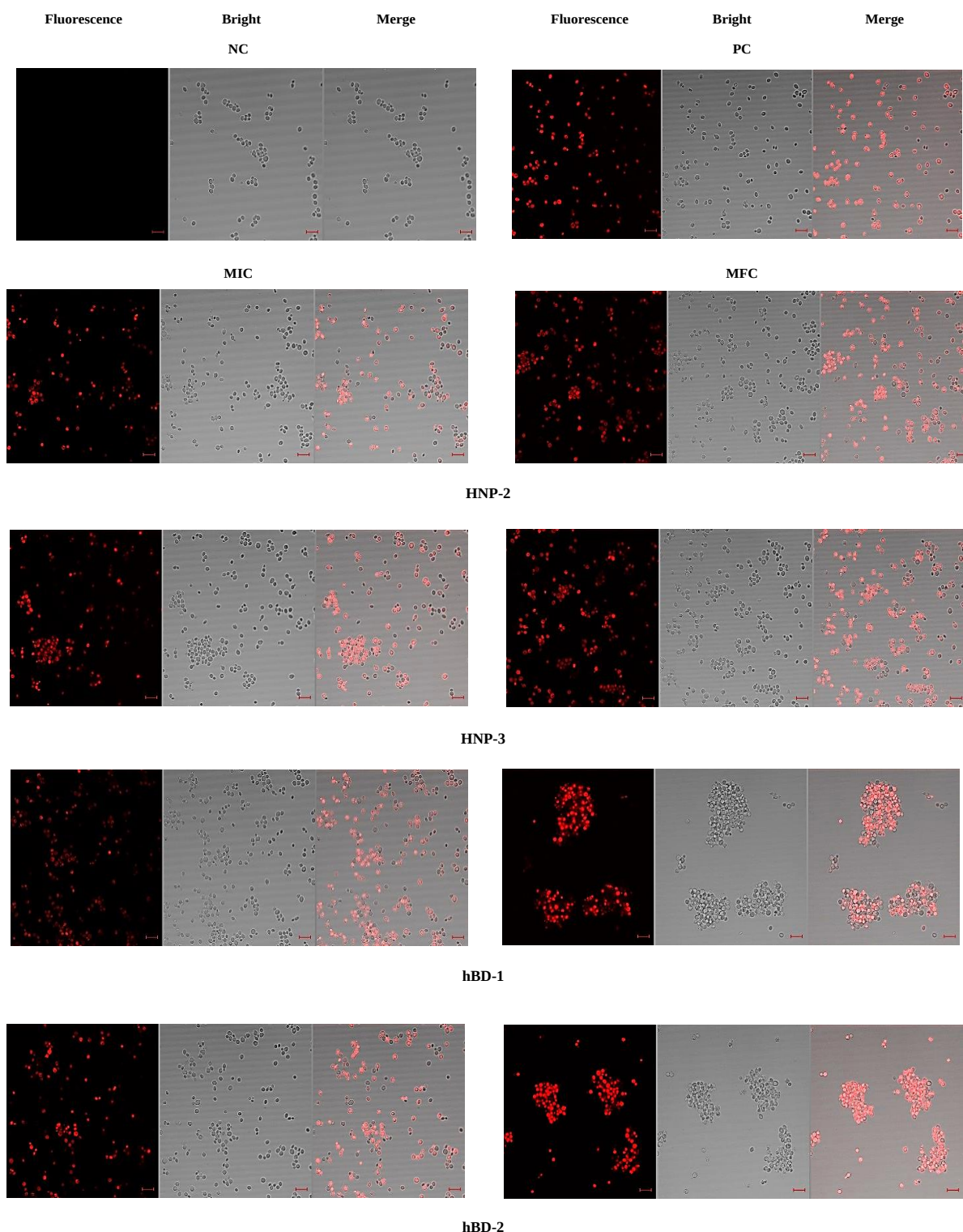


Figure C.3: Confocal microscopy images of the effect of the tested peptides on *Candida auris* cells. Permeabilization of the membrane of *C. auris* strains CAU-09 following exposure to the tested peptides (HNP-2, HNP-3, hBD-1 and hBD-2) at MIC/MFC concentrations for 4 hours. cells with membrane damage were stained with PI red; negative and positive control represent untreated, and cells treated with 10 mM H_2O_2 respectively. *Scale-bar: 10 μm*

Appendix D:

Supplementary data for chapter 4

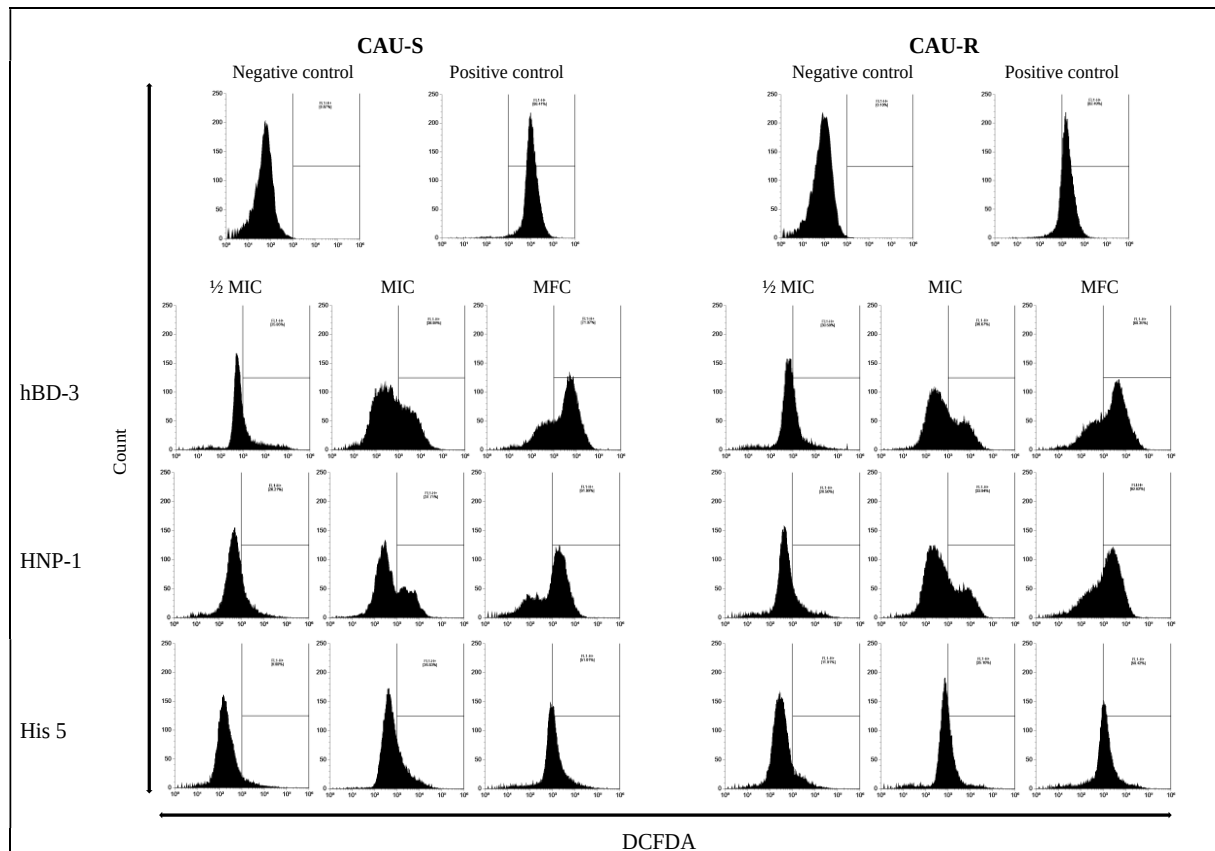


Figure D.1: Flow cytometric analysis of intracellular reactive oxygen species (ROS) accumulation using H₂DCFDA staining assay. *Candida auris* cells were exposed to 0.5 MIC, MIC and MFC of hBD-3, HNP-1 and His 5. Untreated cells and cells exposed to H₂O₂ (10 mM) were used as negative and positive controls respectively. Samples were analysed by flow cytometry in FL1 channel. The figures shown are representative data of three independent experiments.

Appendix E:

Supplementary data for chapter 5

Table E.1: Oligonucleotides sequences for primers (5'—3') used in this study.

Gene	Forward (5'-3')	Reverse (3'-5')	Reference
<i>ACT1</i>	GAAGGAGATCACTGCTTTAGCC	GAGCCACCAATCCACACAG	(1)
<i>CDR1</i>	GAAATCTTGCACTTCCAGCCC	CATCAAGCAAGTAGCCACCG	(1)
<i>CDR2</i>	GTCAACGGTAGCTGTGTG	GTCCCTCCACCGAGTATGG	(1)
<i>MDR1</i>	GAAGTATGATGGCGGGTG	CCCAAGAGAGACGAGCCC	(1)
<i>SAP3</i>	AGGCGAGCCTACTTACGACA	CTCAGAGCCCAAGAACAAGG	(2)
<i>SNQ2</i>	ATCACCGAGGAATTGAGCAC	TCAACCTGTGAGCTTGATGC	(3)
<i>PGA7</i>	GGCAGACTTTTCAGCTTTGG	AATCAATTTCCCGTTTGCAG	(3)
<i>PGA26</i>	CCACGAACCTCCAAACAAGT	TGGTCACTGTGAGGGTGGTA	(3)
<i>PGA52</i>	ACGAACACACCGTTGAATGA	AGTGCCATCTTGAGCGCTAT	(3)

1. Rybak JM, Doorley LA, Nishimoto AT, Barker KS, Palmer GE, Rogers PD. Abrogation of Triazole Resistance upon Deletion of *CDR1* in a Clinical Isolate of *Candida auris*. *Antimicrob Agents Chemother*. 2019 Mar 27;63(4):e00057-19.
2. Kim JS, Lee KT, Bahn YS. Secreted aspartyl protease 3 regulated by the Ras/cAMP/PKA pathway promotes the virulence of *Candida auris*. *Front Cell Infect Microbiol*. 2023 Sep 15;13:1257897.
3. Srivastava V, Ahmad A. Abrogation of pathogenic attributes in drug resistant *Candida auris* strains by farnesol. *PLoS One*. 2020 May 11;15(5):e0233102.