Universidade do Minho
Escola de Ciências

Joana Isabel Carvalho Pereira

Fighting Fungal Infections: Development of a New Diagnosis Method and a Novel Antifungal Treatment

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Fighting Fungal Infections: Development of a New Diagnosis Method and a Novel Antifungal Treatment

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Professora Doutora Paula Sampaio
e da
Professora Doutora Maria João Sousa

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"Se eu vi mais longe, foi por estar sobre ombros de gigantes"

Isaac Newton

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

Combater as Infeções Fúngicas: Desenvolvimento de um Novo Método para Diagnóstico e Novo Tratamento Antifúngico

A incidência das infeções fúngicas invasivas (IFI) tem aumentado significativamente devido ao desenvolvimento de intervenções médicas cada vez mais agressivas, aumentando o número de indivíduos imunocomprometidos. *Candida* spp. e *Aspergillus* spp. são os organismos mais frequentemente presentes, contudo, nos últimos anos a incidência de *Rhizopus* spp. tem aumentado. A eficácia do tratamento das IFI depende da suscetibilidade ao agente antifúngico utilizado e varia com a espécie. Assim, a correta identificação a nível da espécie é fundamental para a seleção de uma estratégia antifúngica. Por outro lado, torna-se também urgente aumentar o leque de alternativas terapêuticas disponíveis.

Nesta tese foram desenvolvidas diferentes abordagens com um objetivo comum: melhorar o combate às infeções fúngicas. Na primeira parte da tese foi desenvolvida uma estratégia de *multiplex PCR* para identificar espécies fúngicas. Foram desenhados dois painéis, o *Painel de Candida* para identificar *C. albicans, C. parapsilosis, C. glabrata, C. krusei* e *C. tropicalis* e o *Painel de Fungos Filamentosos* para identificar *A. fumigatus, A. flavus, A. terreus, A. niger e R. arrhizus*. Esta metodologia foi eficaz na identificação das dez espécies fúngicas, em isolados e amostras clínicas. Apresenta um limite de deteção entre 10 a 1 pg de DNA e é capaz de detetar infeções mistas. Nas amostras clínicas de locais estéreis, a metodologia apresentou uma especificidade de 100% e sensibilidade de 89%.

Os derivados de benzo[a]phenoxazina têm demonstrado boas propriedades antiproliferativas em leveduras. Assim, e face à necessidade de encontrar novos agentes antifúngicos, foi avaliada a atividade antifúngica destes derivados, quer livres quer encapsulados em lipossomas de DODAB:MO. O derivado C34 demonstrou excelente atividade antifúngica contra as espécies de *Candida* e *Aspergillus* testadas, tanto na forma livre como encapsulado. Formulações não toxicas combinadas com fluconazole aumentaram a sua atividade em estirpes fluconazole-resistentes. Este composto também demonstrou aumentar significativamente a morte de leveduras induzida por macrófagos. Estes resultados demonstram que o C34 pode ser considerado numa estratégia antifúngica, melhorando a atividade dos macrófagos na defesa contra infeções fúngicas.

PALAVRAS-CHAVE: *Aspergillus* spp., benzo[a]phenoxazinas, *Candida* spp., diagnóstico *Rhizopus* spp.

ABSTRACT

Fighting Fungal Infections: Development of a New Diagnosis Method and Novel Antifungal Treatment

The incidence of invasive fungal infections (IFI) has increased significantly due to the development of more aggressive medical and therapeutic techniques, which intensify the number of immunocompromised individuals. *Candida* and *Aspergillus* species are the most common etiological agents, however, the incidence of *Rhizopus* spp. has increased in recent years. The susceptibility to antifungal drugs differs according to the species thus, the rapid and correct identification at the species level is crucial for an effective antifungal strategy. Additionally, resistance is often found making it urgent to increase the number of therapeutic options for the treatment of these infections.

In this thesis different approaches were used with a common goal: the combat of fungal infections. In the first part of the thesis a simple and easy to interpret PCR multiplex strategy was developed for the identification of the most common fungal species involved in IFI. Two panels were designed, the *Candida Panel* to identify *C. albicans, C. parapsilosis, C. glabrata, C. krusei* and *C. tropicalis* and the Filamentous Fungi *Panel* to identify *A. fumigatus, A. flavus, A. terreus, A. niger* and *R. arrhizus.* The methodology developed is suitable for the identification of the ten most important fungal species involved in IFI, in isolates and clinical samples. It has a limit of detection between 10 to 1 pg of DNA and is able to detect mixed infections. In clinical samples from sterile places the method presented 100% specificity and 89% sensitivity.

Benzo[a]phenoxazine derivatives, have been shown to present good antiproliferative properties against yeast. Thus, aiming to identify new antifungals, we evaluated the antifungal activity of different benzo(a]phenoxazine derivatives, both free and encapsulated in DODAB:MO liposomes. Benzo[a]phenoxazine derivative C34 presented high activity against all pathogenic *Candida* and *Aspergillus* species tested in both free and encapsulated form. Non-cytotoxic formulations combined with fluconazole improved the fluconazole antifungal activity against fluconazole-resistant strains. Moreover, C34 decreased significantly yeast killing induced by macrophages. These results demonstrate that C34 could be included in an antifungal strategy to improve fluconazole activity and improve the activity of macrophages to combat fungal infections.

KEYWORDS: *Aspergillus* spp., benzo[a]phenoxazines, *Candida* spp., PCR, *Rhizopus* spp.

LIST OF PUBLICATIONS

The work performed during this PhD resulted in the following peer-reviewed scientific publications:

1. Carvalho-Pereira J, Oliveira M, Costa-Barbosa A, Vaz C, Velegraki A, Zancopé-Oliveira RM, Pais C and Sampaio P (2018). High Variability Within *Candida albicans* Transcription Factor *RLM1*: Isolates From Vulvovaginal Infections Show a Clear Bias Toward High Molecular Weight Alleles". *Medical Mycology*, 56(5):649-651. DOI: 10.1093/mmy/myx079

2. Carvalho-Pereira J, Fernandes F, Vaz C, Oliveira M, Araújo R, Springer J, Loffler J, Buítrago MJ, Pais C and Sampaio P. New multiplex PCR based strategy for detection of fungal pathogen DNA in patients with suspected invasive fungal infections. (under review in *Journal of Clinical Microbiology*).

3. Carvalho-Pereira J, Gonçalves MS, Sampaio P and Sousa MJ. Evaluation of free and DODAB:MO liposome-encapsulated benzo[a]phenoxazine derivatives against the most frequent pathogenic fungal species *(manuscript in preparation)*

4. Carvalho-Pereira J, Costa-Barbosa A, Gonçalves MS, Sousa MJ and Sampaio P. Benzo[a]phenoxazine DODAB:MO liposomes enhances the antifungal effect of fluconazole on fluconazole-resistant *Candida* species. *(manuscript in preparation*)

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Peer-reviewed scientific publications not included in this thesis:

1. Miranda C, Bettencourt S, Pozdniakova T, Pereira J, Sampaio P, Franco-Duarte R and Pais C (2020). Modified high-throughput Nile red fluorescence assay for the rapid screening of oleaginous yeasts using acetic acid as carbon source. *BMC Microbiology*, 20:60. DOI: 10.1186/s12866-020-01742-6

2. Mesquita A*, Carvalho-Pereira J*, Franco-Duarte R, Alves A, Gerós H, Pais C and Sampaio P. Oral *Candida albicans* colonization in healthy individuals: prevalence, genotypic diversity, stability along time and transmissibility. *contributed equally. (under review *in Journal of Oral Microbioogy*).

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5-FC	5-Fluorocytosine
ABPA	Allergic Bronchopulmonary Aspergillosis
Als	Agglutinin-like Sequence
BAL	Bronchoalveolar Lavage
BDG	Beta-D-glucan
CaN	Calcineurin
CFU	Colony Forming Unit
CLSI	Clinical Laboratory Standards Institute
CNA	Chronic Necrotizing Aspergillosis
DHODH	Dihydroorotate Dehydrogenase
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DODAB	Dioctadecyldimethylammonium Bromide
EAPCRI	European *Aspergillus* PCR Initiative
ECMM	European Confederation of Medical Mycology
EDTA	Ethylenediamine Tetraacetic acid
ELS	Electrophoretic Light Scattering
EORTC/MSG	Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group
EPA	Epithelial Adhesin
ER	Endoplasmic Reticulum
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAM	6-Carboxyfluorescein
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GM	Galactomannan

HEX	Hexachlorofluorescein
IFI	Invasive Fungal Infections
INPI	Instituto Nacional de Propriedade Industrial
ITS	Internal Transcribed Spacer
LUV	Larger Unilamellar Vesicles
MALDITOF MS	Matrix-Assisted Laser Desorption/Ionization time-of-Flight Mass Spectrometry
MIC	Minimum Inhibitory Concentration
MLV	Multilamellar Vesicles
MO	1-Monooleoyl-Rac-Glycerol
MOPS	3-(N-Morpholino) Propanesulfonic Acid
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide
OPC	Oropharyngeal Candidiasis
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PDI	Polydispersity Index
PEG	Polyethyleneglycol
PL	Phospholipase
PNA-FISH	Peptide Nucleic Acid Fluorescence *in situ* Hybridization
PTX3	Pentraxin 3
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
ROX	6-Carboxy-X-Rhodamine
RT-PCR	Real Time – Polymerase Chain Reaction
Sap	Secreted Aspartyl Proteinase
SUV	Small Unilamellar Vesicles
T2MR	T2 Magnetic Resonance
VVC	Vulvovaginal Candidiasis

CHAPTER 1

Motivation, Thesis Outline and Objectives

1.1 MOTIVATION

The development of medicine has caused an increase in the number immunocompromised individuals, susceptible to the development of severe fungal infections. These infections present high morbidity and mortality rates and involve several hospital resources, associated with high costs. It is known that the early and correct identification of fungal pathogens is crucial to select an appropriate antifungal treatment and enhance survival of infected patients, being also important to contain hospital outbreaks. The main objective of this work is to develop studies to contribute to the combat of fungal infections. Two main problems are described in this combat, the diagnosis and the antifungal treatments, due to the limited number of available antifungal drugs.

The golden standard methodologies for diagnosis of fungal infections are based on culture or histological methods and present several disadvantages since they have low sensitivity and are time-consuming. Thus, to address this problem, the development of new fast and easy to interpret methods are needed. Previous work performed in our research group studied specific regions in several fungal species able to differentiate *Candida* strains and species, using fragment analysis. In the first part of this thesis we exploited the same methodology to identify pathogenic fungal species most frequently isolated from invasive fungal infections (IFI). After the correct fungal identification another main problem arises, which is the limited number of alternatives for antifungal treatment. At the moment, only three groups of antimycotics, classified according to the antifungal mechanism of action, are used in fungal infections. However, current treatments present some issues due to drug toxicity, pathogens resistance, low drug bioavailability and reduced options of oral or intravenous preparations. Our research group studied several benzo[a]phenoxazine chlorides and observed that some compounds presented antifungal activity against *Saccharomyces cerevisiae*. Aiming to identify new antifungals, we decided to exploit the activity of these compounds against the most frequent pathogenic fungal species isolated form IFI.

With this plan of action, we aim to address the two main problems in the combat of fungal infections and contribute to new diagnosis and antifungal treatment strategies.

1.2 THESIS OUTLINE

Considering the work developed during the last four years, the present thesis is divided in seven chapters. Chapters 3, 4, 5 and 6 describe the experimental work and are presented in the form of articles. The manuscript present in Chapter 3 is submitted in *Journal of Clinical Microbiology*, while the manuscript present in Chapter 4 is already published in *Medical Mycology*. Manuscripts presented in Chapter 5 and 6 are being prepared for publication. Chapter 3 also resulted in the patent N° 20181000078137, Instituto Nacional da propriedade Industrial (INPI) "Método para identificar microrganismos patogénicos e marcadores de resistência usando analise de fragmentos de PCR multiplex".

Chapter 1 describes the context, motivation and the global structure, followed by the aims of this work.

In **Chapter 2** an overview of the literature related to the different types of fungal infections is presented. Several subjects are addressed, including the epidemiology and pathogenicity mechanisms associated with the development of the disease. A review on approaches to identify fungal pathogens and methods used for fungal diagnosis in clinical practice is also provided and the antifungal strategies used in fungal infections, including the use of drug delivery systems, are discussed.

Chapter 3 describes the work entitled "New multiplex PCR based strategy for detection of fungal pathogen DNA in patients with suspected invasive fungal infections". In this work a simple and fast methodology to identify DNA from *Candida, Aspergillus* and *Rhizopus* species, was developed. Two Panels were designed, one, the *Candida Panel* to identify *C. albicans, C. parapsilosis, C. glabrata, C. krusei* and *C. tropicalis* and the *Filamentous Fungi Panel* to identify *A. fumigatus, A. flavus, A. terreus, A. niger* and *R. arrhizus.* The methodology developed is suitable for the identification of the ten most important fungal species involved in IFI, can be applied to isolates and clinical samples and presents 100 % specificity and 89 % sensitivity in samples from sterile places.

Chapter 4 presents the work entitled "High variability within *Candida albicans* transcription factor *RLM1:* Isolates from vulvovaginal infections show a clear bias toward high molecular weight alleles". During the

search for specific *C. albicans* DNA regions for Chapter 3, CAI marker was studied as alternative. The search on data about CAI, revealed that specific CAI genotypes in Chinese isolates from vulvovaginal candidiasis (VCC) were correlated with severe recurrent infection. In this context, and since the identification of virulence factors can also aid in the definition of therapeutic strategies, we accessed if the specific genotypes correlated with severity of the infection was also present in Western populations. VVC isolates from other geographic regions presented similar CAI alleles genotypes, which probably confer a higher fitness to the vaginal environment, but not those specific from the Chinese population.

Chapter 5 describes the study of the benzo[a]phenoxazine compounds as potential antifungals, with the work "Evaluation of free and DODAB:MO liposome-encapsulated benzo[a]phenoxazine derivatives against the most frequent pathogenic fungal species". This chapter shows the activity of five benzo[a]phenoxazine compounds against the most frequent pathogenic fungal species isolated from invasive fungal infections and selects the two most effective compounds for the development of an antifungal strategy based on DODAB:MO liposomes. This work also shows the influence of the selected compounds in two important fungal virulence factors, cell adhesion and filamentation.

In **Chapter 6** we continue the study with benzo[a]phenoxazine compounds, now in combination with fluconazole, in the work "Benzo[a]phenoxazine DODAB:MO liposomes enhances the antifungal effect of fluconazole on fluconazole-resistant *Candida* species". This chapter describes the selection of a non-toxic formulation based on benzo[a]phenoxazine compounds and DODAB:MO liposomes and shows its effect with fluconazole, in decreasing the Minimum Inhibitory Concentrations in fluconazole resistant strains and in yeast killing induced by macrophages.

In **Chapter 7**, the overall conclusions and significance of the work are presented. Followed by suggestions for future studies and future perspectives.

1.3 OBJECTIVES

As mentioned above this thesis uses different approaches for a common goal: the aid in the combat against fungal infections, namely, by improving the fungal identification phase and by the development of efficient antifungal alternatives.

In the first part of this thesis we aimed to designed and optimized a multiplex PCR based methodology to identify *Candida*, *Aspergillus* and *Rhizopus* species. The specific objectives were:

- Select specific DNA regions to design specific primers to use in a PCR based methodology
- Design multiplex PCR panels, adjustable to the laboratory needs;
- Evaluate the specificity, sensibility and reproducibility of each panel using fungal isolates;
- Evaluate the methodology effectiveness using clinical samples;

In the development of new therapeutic approaches, the identification highly virulent strains can fine tune selection of compounds. So, considering reports that specific genotypes were correlated with severity of the infection in the Chinese populations we aimed to access if those genotypes were also present in Western populations.

In previous studies, benzo[a]phenoxazines derivatives presented good antifungal activity. Several compounds used in this project were already characterized in what regards physical-chemical properties and antiproliferative activity against *Saccharomyces cerevisiae*. Thereby, in this thesis we aimed to evaluate their potential as antifungal agents against *Candida, Aspergillus* and *Rhizopus* strains, including strains associated with severe infections. The specific objectives were:

- Select fungal strains associated with severity of fungal infection and antifungal resistance.
- Evaluate the antifungal activity of five derivatives of benzo[a]phenoxazines against selected strains from distinct fungal species, namely *Candida albicans, C. glabrata, C. parapsilosis, C. tropicalis*, *C. krusei*, *C. bracarensis, C. auris, Aspergillus fumigatus, A. flavus, A. terreus, A. niger* and *Rhizopus arrhizus;*
- Select the most effective benzo[a]phenoxazine compounds to develop and characterize an antifungal lipidic nanoformulation based on benzo[a]phenoxazines and DODAB:MO liposomes;

- Assess the antifungal activity of selected lipidic nanoformulations against fluconazole-resistant strains;
- Determine the complementary action of the selected lipidic nanoformulations in *in vitro* macrophage yeast killing assays.

CHAPTER 2

General Introduction

2.1 FUNGAL INFECTIONS

In the last three decades, fungal infections evolved as a major worldwide public health problem, affecting especially immunocompromised patients. Several reasons have been proposed to explain this tendency, including the increased lifespan of the populations over the world, the age-related loss of immune competence and the use of more aggressive therapeutic methods [1–3]. Several risk factors for the development of fungal infections have been proposed, such as the use of chemotherapeutic or immunomodulatory agents, bone-marrow or solid-organ transplants, parenteral nutrition, more aggressive surgeries, mechanical ventilation, diabetes, or even previous fungal colonization, among others [4, 5].

Fungal infections present high morbidity and mortality rates, which can range from 2 to 95 %, depending on the etiologic agent involved, and the type of underlying disease [2, 6, 7]. The high hospital costs associated also represent a problem [8–10]. A recent study estimated that in the United States the total medical cost of fungal diseases is more than $7.2 billion, including $4.5 billion for hospitalizations and $2.6 billion for outpatient visits [8]. Moreover, other study determined the cost of neonates hospitalization, in cases of invasive fungal infection, and estimated a median hospital cost of $28,500 per patient, depending on the condition, length of stay and needs [10]. However, these costs underestimate the true economic burden, because many fungal diseases are likely to go unrecognized and their cost analysis did not account for the costs related to unnecessary medical procedures and inappropriate treatment before the fungal diagnosis is established [8].

More than 300 fungal species are considered pathogenic, causing from less-severe superficial lesions in mucosa and skin, to life-threatening disseminated mycosis characterized by the spread of fungi through the tissues and blood circulation [11–13]. The epidemiology of fungal infections varies according to geographic regions, some species are rare or non-existent in certain areas, for example, *Histoplasma* or *Coccidioides* spp. in Europe [14, 15]. However, the predominant fungal pathogens worldwide include *Candida* spp., *Aspergillus* spp., and the emerging species of Mucorales.

2.1.1 *Candida* and candidiasis

Candida species are part of the commensal flora of the genitourinary system, gastrointestinal tract, skin and oral cavity of healthy individuals. Perturbation of the normal flora balance or compromised immune defenses induce opportunistic infections, denominated candidiasis [4, 16]. In the majority of cases,

candidiasis can be considered an endogenous infection, caused by prior colonization of mucosa and gastrointestinal tract, however, it can also be considered exogenous, since many studies isolated *Candida* species from the floor, countertops, doorknobs, food and other inanimate surfaces that were then correlated with infections in hospitalized patients [17–19].

Over the past decades, several epidemiologic studies have been performed in European countries and the USA to evaluate the incidence of the different clinical manifestations of *Candida* spp. Superficial candidiasis is the most common manifestation of *Candida* infections, which affects mucosal epithelial tissues and skin appendages such as nails and air, and, is frequently caused by prior colonization when host physical barriers or immune system integrity are compromised [20–22]. The most common manifestation is Oropharyngeal Candidiasis (OPC), that is a concern in new-born babies, due to the immature immune system, and individuals infected with HIV, in which the prevalence of oral carriage ranges from 43% to 98% [23–26]. Vulvovaginal candidiasis (VVC) is also frequent, being the second most common gynecologic infection. VVC affects more than 75% of women, and about 40–50% of these women experience one recurrence, and up to 5%, more than four episodes during one year [27, 28].

Invasive candidiasis is the fourth leading cause of healthcare-associated bloodstream infections overall and, is considered the third most common cause of bloodstream infections in intensive care units, and *Candida* spp., the second most common pathogen in catheter-associated urinary tract infections [1, 29]. Invasive candidiasis is associated with high morbidity and mortality rates, ranging from 46% to 75% [6, 7]. This clinical manifestation can involve the infection and spread of *Candida* cells via the bloodstream to multiple organs, such as the brain, kidneys, heart, lungs and liver [30, 31]. *Candida* spp. infections are usually a consequence of high colonization combined with a defect in host defenses. However, in critically ill patients, the source of infection may also be intravascular catheters or other devices, colonized by endogenous or exogenous *Candida* species [32, 33].

Not all predisposing factors, such as immune suppression, prior colonization or disruption of normal physiological barriers, equally favor superficial and invasive candidiasis, since host immune protection is site-specific [34, 35]. For example, T-cells immune responses are important in protection against superficial candidiasis, subsequently, individuals infected with HIV suffer frequently from OPC or onychomycosis, due to the decrease of CD4+ cell counts [36]. By contrast, neutropenic patients are particularly susceptible to invasive candidiasis [37].

Data from ECMM (European Confederation of Medical Mycology) suggest that *C. albicans* remains the most common species isolated from patients with invasive fungal infection [38]. However, in the last two

decades, a shift in species distribution has been observed [39]. This change may be explained by the introduction of fluconazole as a therapeutic and prophylactic agent in 1990s. It was demonstrated that patients with infections caused by non-*albicans* species received prophylactic agents before the onset of their infections more frequently than patients with infections caused by *C. albicans* [40].

Epidemiologic studies performed in care units from the USA and Europe demonstrated that non-*albicans* species, specially *C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei* now comprise two-thirds of all *Candida* infections [41–44]. However, the incidence of different species depends on the geographic region. For example, in Denmark or UK, *C. glabrata* is the second most frequent species, with almost 30% of prevalence, however, in Brazil or Japan, the incidence decreases, being *C. parapsilosis,* the second most frequent species (Figure 2.1). The type of hospital and patients studied also influence the incidence distribution. For example, *C. parapsilosis* is most frequent in individuals with parenteral nutrition, intravenous catheters or neonates, probably due to the exogenous contamination by health care workers [45, 46]. Sabino and co-workers studied the incidence of candidemia in a Portuguese oncology hospital for a period of six years and demonstrated that although *C. albicans* was more frequently isolated from patients with solid tumours, non-*albicans* species, particularly *C. tropicalis*, were most frequently found in patients with haematological malignancies [47].

Recently, a new *Candida* specie, *C. auris*, have been described as an emerging problem [48]. This species represents a new problem since is multidrug-resistant, difficult to identify using conventional methods, and has caused several outbreaks in healthcare settings [49, 50]. This trend can also be explained by the introduction of prophylactic antifungal agents, before the onset of the infections, promoting the increasing incidence of non-*albicans* species intrinsically resistant to antifungals [49, 51].

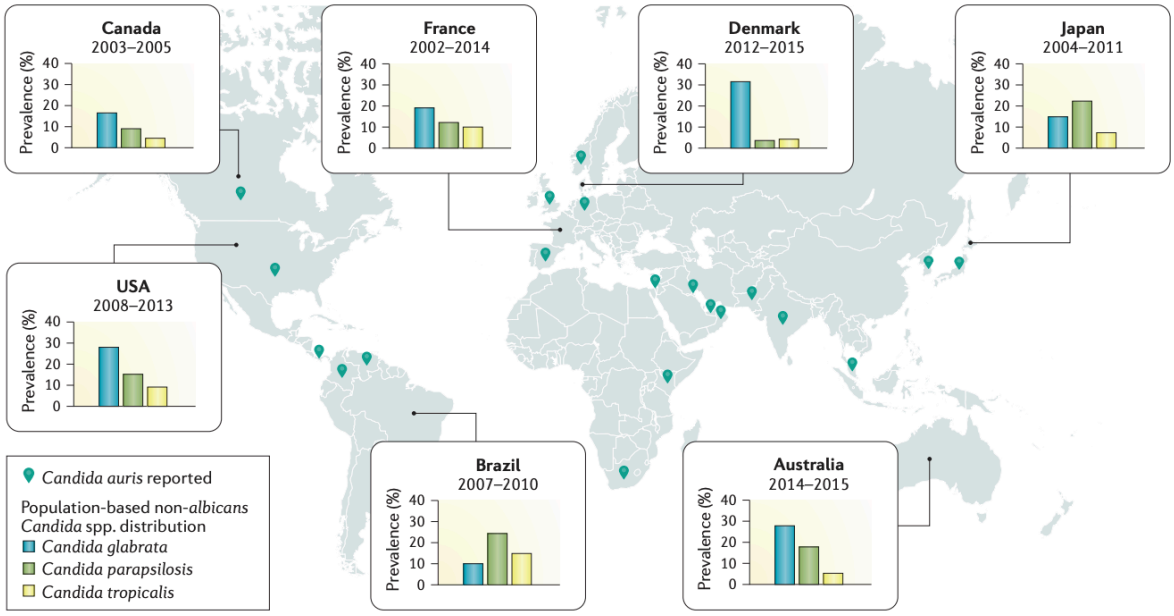


Figure 2.1 Geographical variations in the distribution of Candida species (Adapted from Pappas *et al.* (2018) [31]).

2.1.2 *Aspergillus* and aspergillosis

Aspergillus is a filamentous fungus ubiquitous in nature (soil, water, food, air, among others), which is the second most common genus isolated from invasive infections. Although all individuals inhale *Aspergillus* spores or conidia daily, the development of infection occurs predominantly in severely immunocompromised individuals. Aspergillosis is a major problem in clinical practice due to the high morbidity and mortality rates, which range between 30 – 95% [6, 7]. Moreover, *Aspergillus* outbreaks are frequently identified in hospital environments, mainly related to construction or renovation activities, compromised air quality, improperly function of ventilation systems or poorly maintained air filters [52].

Immunocompromised individuals are more susceptible to aspergillosis, however, some studies also reported the development of invasive aspergillosis in immunocompetent patients. Indeed, Ratermann *et al.* (2014) [53] reported a fulminant case of invasive pulmonary aspergillosis, after a near-drowning accident. Arendrup *et al.* (2006) [54] reported two cases of immunocompetent patients with acute community-acquired invasive pulmonary aspergillosis, developed after spreading bark chippings.

Invasive aspergillosis is strongly corelated with patients with haematological malignancies, such as patients undergoing allogeneic haematopoietic stem cell transplants, acute myeloid leukaemia or diseases of the lymphoid systems, however, the risk to develop invasive aspergillosis depends on other factors such as neutropenia or the type of stem cell transplanted/affected [55, 56]. Chronic obstructive

pulmonary disease (COPD), liver cirrhosis, lung transplantation or use of corticosteroids are also considered risk factors for Invasive aspergillosis [57–59]. *Aspergillus* species are also able to develop other clinical manifestations or syndromes like chronic necrotizing aspergillosis (CNA), allergic bronchopulmonary aspergillosis (ABPA), aspergilloma or superficial disease [60, 61].

Recently, some studies demonstrated that gene polymorphisms affecting the innate immune response are associated with an increased risk of developing aspergillosis. For example, functional polymorphisms in genes encoding interleukin 1β (*IL1B*) and β-defensin 1 (*DEFB1*) increase the susceptibility to infections caused by filamentous fungi in solid organ transplantation recipients [62]. Moreover, genetic deficiency of *PTX3,* which encodes for long pentraxin, decreases the antifungal capacity of neutrophils [63].

Aspergillus genus comprises more than 250 species, being *A. fumigatus* the most frequently isolated species from infections, followed by *A. flavus*. However, other species such as *A. niger* and *A. terreus* are also isolated depending on the clinical group or geographic region [64–66]. *A. flavus* is predominant in Asia, Middle East and Africa due to the great ability to survive in warm and arid climatic conditions [67].

2.1.3 Mucorales and mucormycosis

Mucorales are ubiquitous fungus present in the environment (soil and decaying organic matter) and, are considered emerging pathogens, causing invasive fungal infections in immunocompromised patients. Indeed, outbreaks of mucormycosis have been reported in developed countries, and is now the fifth cause of invasive fungal diseases, with mortality rates between 30–90% [68, 69].

For Mucorales, inhalation of sporangiospores, ingestion of contaminated food, and traumatic inoculation are the most frequent routes of infections [70]. Whereas, diabetes, deferoxamine therapy, injection drugs, solid organ transplant, bone marrow transplantation, and haematological malignancies are the main risk factors associated with the development of mucormycosis [70]. However, the risk factors differ according to geographic regions, for example, diabetes is the most common risk factor in Asia, whereas haematological malignancies, in Europe and the USA [71, 72].

Similarly to invasive aspergillosis, mucormycosis affect essentially paranasal sinuses, lungs and skin. The most common clinical manifestations are rhinocerebral, pulmonary, cutaneous and disseminated mucormycosis [73]. These clinical manifestations seem to be associated with specific risk factors. Rhinocerebral mucormycosis is frequently found in patients with diabetic ketoacidosis, whereas

pulmonary mucormycosis is often seen in patients with haematological malignancies, cutaneous mucormycosis occurs generally after trauma [74, 75].

Different fungal genera associated with the mucormycosis are part of the Mucorales family, namely *Mucor* spp., *Rhizopus* spp., *Apophysomyces* spp., *Lichtheimia* spp. and *Cunninghamella* spp. However, *Rhizopus* spp. remains the most frequent isolated genus, being *Rhizopus oryzae (R. arrhizus*), followed by *Rhizopus microsporus* var. *rhizopodiformis* the most frequent species [69, 76].

2.2 FUNGAL VIRULENCE MECHANISMS

To establish infection, an opportunistic pathogen, has to colonize a host, penetrate the surface, survive, divide in the host environment, and damage the host, manifested as disease symptoms [77]. Although the majority of fungal pathogens are ubiquitous in nature, or part of the human commensal flora, the ability to adapt to the host different environments, including changes in oxygen and carbohydrate levels, pH, osmolality, availability of nutrients and temperature, or the ability to evade from the host immune system, favors the development of infections. These diverse mechanisms, required for the development of infection, are designated as virulence factors [78].

2.2.1 Virulence factors of *Candida* species

Candida species present a set of virulence factors and specific strategies to assist in their ability to colonize host tissues, cause disease and overcome host defenses. The expression of these factors may depend on the specie, the type, local and stage of infection, and the nature of the host response. The virulence factors that mostly contribute to infection are the adhesion capacity, morphogenesis, phenotypic switching, production of hydrolytic enzymes, and biofilms production.

2.2.1.1 Morphogenesis

Some *Candida* species can switch from the unicellular budding yeast to the filamentous form (hyphae or pseudohyphae), including *C. albicans, C. tropicalis, C. parapsilosis* or *C. krusei*. These species, classified as polymorphic, use filamentation in different stages of their pathogenesis process [79, 80]. Although some studies demonstrated that specific *C. glabrata* and *C. auris* strains can filament, these species are described as non-polymorphic, using other virulence mechanisms for infection [81, 82].

Both morphological forms are important in the infection process, playing different roles in the development and progression of the disease. Indeed, studies suggested that yeast cells are important for the dissemination via the bloodstream while hyphae forms are important in the process of invasion of tissues and organs, for adaptation to different niches and to escape from immune system host cells after internalization [83–85]. Moreover, morphogenesis of *Candida* species has also implications for immune evasion due to differential composition and exposure of surface components, affecting fungal sensing by the host immune system [86].

Filamentation is mainly triggered by the presence of unfavorable conditions or hostile environments. These conditions include neutral or high pHs, starvation, presence of human serum, physiological temperature, CO_2 and O_2 levels, among others [87]. Moreover, the yeast-to-hyphae transition may also be induced by quorum sensing, in which low cell densities promote hyphal formation while high cell densities favor yeast form [88]. However, distinct combinations of these conditions are necessary for *Candida* filamentation [89].

Adverse environmental conditions activate a complex network of signal transduction pathways that activate or inhibit Efg1, Cph1, Nrg1, among others. These transcription factors regulate the expression of genes essential for the yeast-to-hyphae transition [90–92]. Filamentation process is also linked to the expression of adhesins, such as Hwp1 or Als3, for anchoring fungal cells to the host tissues, and secreted enzymes, able to degrade proteins, lipids and other cellular constituents, during the infection process [93, 94].

Directional hyphal growth, named thigmotropism, is considered essential for tissues penetration and damage. Indeed, Bran *et al.* (2008) [95] showed that the deletion of specific genes involved in thigmotropism process leads to a reduction of virulence in the mouse model of disseminated infection. This mechanism is regulated by extracellular calcium uptake through the calcium channels Cch1, Mid1 and Fig1, resulting in specific calcium gradients that direct the orientation of growth [96].

2.2.1.2 Phenotypic switching

The white-to-opaque switch of *C. albicans* is the most studied phenotypic transition, in which heterozygous round oval cells from smooth and white colonies can change their morphology to homozygous elongated cells and form opaque colonies. Besides the differences in cell shape, this switching leads to alterations in cell surface structures, which may affect *Candida* survival and virulence in the host. In fact, Solis *et al.* (2018) [97] showed that opaque cells had decreased ability to invade and damage oral epithelial cells due to the reduced surface expression of Als3. Moreover, Sasse *et al.* (2013) [98] showed that induction of filamentation may also be reduced in opaque cells, decreasing the tissue invasion and virulence. In contrast, the loss of some surface proteins may decrease the recognition of opaque cells by the host immune system, and the subsequent elimination [98].

Although the white-to-opaque switch of *C. albicans* is the most studied phenotypic transition, other variants have been identified and described, such as the gastrointestinally induced transition cells or gray cells. These cells differ morphologically and functionally from previously defined cell types, and express

a transcriptome optimized for the digestive tract, for example, are able to adapt their metabolism to the locally available nutrients [99, 100].

Several works also identified different phenotypes in *C. glabrata*, *C. tropicalis, C. parapsilosis* and even in *C. krusei*, suggesting that some of these phenotypes changes the expression of several putative virulence factors involved in biofilm formation, filamentation, antifungals resistance, among others [81, 101–103].

2.2.1.3 Adhesion

The initial step for colonization of *Candida* species is the ability to adhere to host cells, medical devices and even to other fungal cells. The adhesion ability depends on *Candida* surface properties such as hydrophobicity or cell wall composition and is essential for establishing infection [104].

Candida species has a specialized set of adhesion proteins, or adhesins, responsible for adherence. The most studied adhesins are from the agglutinin-like sequence (Als) protein family, encoded by eight ALS genes (*ALS1-7* and *ALS9*) [87]. These proteins present four domains, the N-terminal domain, the T domain, the central tandem repeats and the CT, and differences in the N-terminal end are responsible for the distinct Als adhesive properties. For example, Als1 influence the binding to endothelial and epithelial cells in early stages of infections, whereas Als5 confers adherence to collagen, laminin, bovine serum albumin and fibronectin in late stages of infection. While Als3 is considered essential for adhesion, morphogenesis and biofilm formation, throughout the infection process [105]. Fu *et al.* (2013) [106] showed that this adhesin contributes to the trafficking of *Candida* cells to the brain and renal cortex during the disseminated infection in mice. The expression of adhesins depends on the species or strains, and all eight genes are expressed by the majority of *C. albicans* strains, however, in *C. tropicalis* and *C. parapsilosis* only three and five were identified, respectively [107].

Although Als are the most frequent and studied adhesins, other proteins are considered important for the adhesion and infection process, such as Hwp1 in *C. albicans* or the glycophosphatidylinositol-anchored protein 30 (Pga 30) in *C. parapsilosis* [108]. In *C. glabrata* the major group of adhesins is encoded by the epithelial adhesin gene family (*EPA*), which are similar to *ALS* family [109].

2.2.1.4 Biofilm formation

Biofilm is defined as a three-dimensional structure of a complex and well-organized microbial community, composed by cells embedded in a polysaccharide extracellular matrix, in which planktonic cells can differentiate to gain specialized properties or functions [110]. The biofilm formation involves different stages, including attachment, maturation, the formation of microcolonies, filamentation, production of the extracellular matrix, and detachment, important for the dispersion and formation of novel communities [111]. These structures adhere to both biotic (cell surfaces) and abiotic (medical devices) surfaces, however, the efficiency of biofilm formation depends on several factors, such as the texture of the surface, environmental conditions, the intrinsic ability of the specie to form biofilm and the activity of immune cells.

Candida biofilms present tolerance to several external stresses, such as antimicrobials, immune system attack and even to mechanical stresses. This resistance is conferred by several factors, including the existence of phenotypic variants with specific characteristics, that can resist to different conditions and stresses; the presence of an extracellular matrix, that promotes the retention of water, nutrients and enzymes, and promotes of hyphal growth; or expression of genes encoding antifungal targets or genes involved in cell wall biosynthesis, that allows resistance to antifungal agents [112, 113].

Several *Candida* species, such as *C. albicans, C. glabrata, C. tropicalis*, *C. parapsilosis* or *C. auris*, can produce biofilms being this ability associated with increased pathogenicity and higher mortality rates in patients with invasive fungal infection [114].

2.2.1.5 Secreted hydrolytic enzymes

The secretion of hydrolytic enzymes is considered an essential virulence attribute for *Candida* infections, being associated with the active penetration into host tissues. Hydrolytic enzymes are implicated in different stages of infection. In adhesion, hydrolytic enzymes can degrade host cell surface molecules, and in invasion, degrade host cell membranes. Moreover, hydrolytic enzymes are also considered crucial for the resistance to host immune system and for nutrient acquisition [87].

Extracellular hydrolytic enzymes of *Candida* species include the secreted aspartyl proteinases (Sap), lipases and phospholipases. A family of ten Sap proteins encodes by 10 *SAP* genes (Sap1-10) are able to degrade essential proteins associated with host membranes, such as collagen or keratin, but also important defence proteins, such as immunoglobulin G heavy chains or complement protein [115]. Sap1-

8 are secreted and released to the surrounding environment, while Sap9 and Sap 10 remain attached to the yeast cell surface.

Several studies have suggested that *SAP* genes family is differentially expressed, according to environmental conditions and *Candida* phenotypes. For example, Sap8 is predominantly detected in yeast cells grown at 25°C and Sap9 is usually found in later growth phases. Moreover, Sap1-3 is predominantly observed in the cell wall and cytoplasm of blastopores, Sap4-6 is located at the tips of hyphae and Sap 1 and Sap3 are frequently found in phenotypically switched cells [116]. This variability is considered important for *Candida* virulence since it allows fungal survival and infection on a multiplicity of tissues and conditions [115, 117].

All ten Saps were found in several strains of *C. albicans*, while, only three (Sap1-3) and four (Sap1-4) were found in *C. parapsilosis* and *C. tropicalis,* respectively. The number of Saps in *C. glabrata* and *C. krusei* remains undefined. This aspect shows that expression of *SAP* genes varies according to *Candida* species, and are an essential virulence factor for the majority of species. Singh *et al.* (2019) [118] showed that Sap1 and Sap2 are essential in adhesion to epithelial cells, in host cell damage, and survival within macrophages. Moreover, Wu *et al.* (2013) [119] also suggested that Sap4-6 may induce apoptosis on epithelial cells, demonstrating their involvement in *Candida* pathogenicity.

In addition to Sap, phospholipases (PLs) are also considered essential enzymes for *Candida* pathogenesis. PLs can hydrolyse phospholipids, fatty acid and glycerol. These enzymes are divided into four different classes (A, B, C and D) however, only members of class B (Plb1-5) seems to be extracellular, contributing to the disruption of the host membranes. Studies showed that mutations of *PLB1* and *PLB5* result in attenuated virulence in a mouse model of systemic infection [120, 121]. *C. tropicalis* and *C. parapsilosis* can produce extracellular PLs, however, at much lower levels than *C. albicans*. No clear phospholipase activity was reported for *C. glabrata* and *C. krusei*.

The third class of hydrolytic enzymes are the lipases, which are encoded by 10 *LIP* genes (*LIP1-LIP10*) and are responsible for the hydrolysis and synthesis of triacylglycerols [122]. The virulence of these enzymes was demonstrated by Gácser *et al.* (2007) [123], which showed that a mutation in *LIP8* gene decreases *C. albicans* virulence in a murine model of systemic infection. Expression of all 10 LIP genes was observed in *C. albicans* and *C. tropicalis* however, no studies suggested the existence of these enzymes in the remaining *Candida* species.

Other factors such as ion acquisition, heat shock proteins, specific stress responses, metabolic adaptation and pH-sensing and regulation, also contribute for *Candida* virulence. In fact, all virulence factors described above are correlated with and integrated into different mechanisms of *Candida* pathogenesis.

2.2.2 Virulence factors of *Aspergillus* species

Several factors contribute to the establishment of disease caused by *Aspergillus* species. These organisms present physical characteristics that allow conidia to reach the distal airways and can adapt and survive in a variety of host environments. *Aspergillus* species can survive in a broad range of pH (2.1-8.8) and temperatures (12–65 °C) and presents a versatile metabolism to supply their nutritional necessities according to the host environment. Some studies have been performed to explain the mechanisms of pathogenesis of *A. fumigatus*, however the specific mechanisms of the majority of pathogenic *Aspergillus* species are still unknown.

The ability to survive the host immune system is conferred by a variety of factors. *Aspergillus* species present a hydrophobic rodlet layer, responsible for conidial dispersion and soil fixation, which masks cell wall β–1,3-glucans, being able to prevent the immune recognition and subsequent host immune responses [124]. In *A. fumigatus* seven rodlet proteins RodA to RodG were found, however only RoaA showed to be essential for the immune-inertia of the cell wall of conidia [125]. In *A. nidulans* at least six proteins are involved in hydrophobin rodlet formation, namely RodA and DewB-E, however only the deletion of *rodA* showed the loss of the rodlet structure [126].

The presence of DHL-melanin is important to protect conidia from ultraviolet light, enzymatic lysis and oxidation, by conferring cell wall rigidity and physical resistance to degradation. Melanin also masks immunostimulatory glucans, preventing recognition and killing of inhaled conidia by professional phagocytes and protecting against reactive oxygen species (ROS) [127]. Moreover, Amin *et al*. (2014) [128] also showed that DHN-melanin leads to higher uptake of conidia by lung epithelial cell, specifically induces host cells apoptosis and prevents the acidification of phagolysosomes, improving the survival of *Aspergillus* inside epithelial cells. It was shown that mutations in DHN-melanin genes of *A. fumigatus* alter conidial surface, the amount of ROS release by phagocytes and fungi susceptibility to hydrogen peroxide, resulting in reduced virulence in a murine animal model [129].

Aspergillus also secretes several metabolic products, such as gliotoxin, that are important in virulence. Gliotoxin was identified in several fungal species and is preferentially produced during the infection process. This mycotoxin presents immunosuppressive characteristics since inhibits antigen presentation

and T cell responses, is proapoptotic for mammalian cells, inhibits NADPH oxidase activity responsible for the neutrophil oxidative burst, and also causes epithelial cell damage and inhibition of ciliary beating [130]. Sugui *et al.* (2007) [131] deleted the *gliP* gene in *A. fumigatus*, resulting in the lack of gliotoxin synthesis, and showed a reduced ability to induce apoptosis in mammalian cell and to inhibit the oxidative burst in neutrophils, resulting in reduced virulence in mouse models.

Aspergillus produce extracellular enzymes, such as phospholipases and proteases, which play an important role in virulence. Some examples are serine (Alp), metallo (Mep) and aspartic (Pep) proteases, and phospholipase A, B, C and D, which are responsible for the degradation of host tissues. Expression of extracellular enzymes is important in early fungal invasion and nutrient acquisition [132]. Moreover, elastane production is also considered to be implicated in the pathogenesis of aspergillosis, since elastin is a major structural component of the lung [133].

The ability to adhere to pulmonary epithelial cells is an important feature of *Aspergillus* species, which plays a key role in colonization and infection. Gravelat *et al.* (2013) [134] identified the most important adhesin of *Aspergillus*, namely galactosaminogalactan (GAG), which play an important role in adhesion and biofilm formation. Moreover, this adhesin also masked cell wall β–1,3-glucans, suppressing host inflammatory responses.

2.2.3 Virulence factors of Mucorales

Although Mucorales are considered emerging pathogens, fewer studies to understand the virulence of these organisms were performed, when comparing with *Candida* and *Aspergillus* species.

Mucorales present some characteristics that favor the survival of the fungi in the host, such as the tolerance to high temperatures, to hypoxia, or the ability for dimorphic growth [70]. Although some of these factors are not well defined in Mucorales, two specific mechanisms are considered important for its virulence.

High iron concentrations improve fungal growth by impairing, phagocytosis and decreasing INF-γ secretion [135]. The ability of Mucorales to obtain iron from the host organisms is the most studied virulence factor. For iron uptake, Mucorales uses two mechanisms, the siderophores (iron chelators), such as ferrioxamine, and the high-affinity iron permeases (*FTR1*). Mucorales present specific ferrioxamine receptors (Fob1 and Fob2) in the surface, which are highly expressed in the presence of ferrioxamine, regulating the iron uptake. Moreover, *FTR1* acts in iron uptake and transport, being

expressed in situations of lack of iron in the environment [136]. Ibrahim *et al.* (2010) [137] showed that inhibition of *FTR1* expression compromised the ability of *R. oryzae* to acquire iron and reduced its virulence in mice.

The second important virulence factor described in Mucorales species is the presence of the spore-coat (CotH) protein. This protein is located on the surface of Mucorales spores and acts as invasin, disrupting host cells [138]. Studies suggest that, during Mucorales infections, CotH mediates the attachment to glucose-regulated protein 78 (GRP78), present in endothelial cells, enhancing its virulence [139]. Additionally, the use of anti-CotH antibodies in mice showed to confer protection against infections caused by *R. oryzae.*

Other virulence factors have been described, for specific Mucorales species, such as the alkaline *Rhizopus* protease enzyme (Arp), expressed in invasive rhinocerebral mycosis caused by *R. microsporus* [140], or the calcineurin (CaN) and the ADP-ribosylation factor (Arf), studied in infections caused by *M. circinelloides* [141, 142].

2.3 DIAGNOSIS OF FUNGAL INFECTIONS

The clinical signs or symptoms of fungal infections are not specific and well defined. The presence of fungal infection is suspected, in patients with known risk factors, which shows clinical manifestations that are unresponsive to antibacterial treatment. In the case of invasive fungal infections, an early diagnosis is crucial to ensure a favorable outcome, since a delay in the initiation of an effective antifungal treatment is associated with higher mortality rates [143].

An early and adequate identification of causative species of fungal infections is crucial for three main reasons. (i) To select an appropriate antifungal treatment, since fungal species differ in their susceptibility to antifungal agents. *C. krusei* is intrinsically resistant to azoles, and *A. terreus* to amphotericin B, while *C. glabrata* and *A. fumigatus* easily acquire resistance to fluconazole [144–146]. (ii) To evaluate the risk of develop deep organ involvement, and evaluate the severity of clinical manifestations, since it differs depending on the infecting species. For example, *C. tropicalis* is frequently isolated from patients with hematologic malignancies, and colonization in these patients is predictive of subsequent infection [147]. (iii) To identify hospital outbreaks and act accordingly [148].

In 2019 the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) updated the classification of invasive fungal infections (IFI), dividing the diagnosis as “proven”, “probable” and “possible”. This classification is obtained using some laboratory techniques, previously standardized and validated, for the identification of fungal pathogens in clinical samples. However, the methodologies and thresholds approved vary with the population analyzed (*e.g.* pediatric patients or UCI patients) [149]. Imaging, histopathology, biochemical tests and galactomannans (GM) and Beta-D-glucans (BDG) detection are some methodologies approved and frequently used in IFI diagnosis. However, the golden standard for fungal diagnosis of IFI is a positive hemoculture or the visualization of fungal structures in histopathology.

2.3.1 Conventional methods

The first methodology used in clinical laboratories is the histopathology or microscopic analysis of biopsy specimens and other biological products. These tests evaluate the presence or absence of hyphae, the shape and size of yeast cells or spores, among others, in tissues or other clinical samples. The use of

different stains for fungal cell wall improves the fungal detection. For *Candida* species, this method allows to observe the presence of true hypha, characteristic of *C. albicans*, or the shape of blastopores, that for example in *C. krusei* are elongated. For filamentous fungi, this method allows to differentiate the characteristic morphology of *Aspergillus* from the non-septate Mucorales. However, these methodologies only allow for the presumptive identification of the fungal species [5, 150].

In clinical laboratories, the culture of microorganisms is always essential, or desirable, to confirm microscopic observations. The media used should support the growth of fungal species, inhibit the growth of bacteria, and facilitate the identification of clinical specimens. Culture of *Aspergillus* and Mucorales species is performed in a standard media, but present a low diagnostic yield, since a negative culture does not exclude the presence of disease. Sensitivity of bronchoalveolar lavage (BAL) culture is only around 30% [150].

To differentiate *Candida* species in culture, several chromogenic media have been developed. BBL CHROMagar *Candida*® (BD), CandiSelect4® (BioRad), ChromID*Candida*® (BioMerieux) or CHROMATIC *Candida*® (Liofilchem) are examples of differential media for the identification of *Candida* species. These media contain chromogenic substrates which react with enzymes (hexosaminidade or alkaline phosphatase) secreted by *Candida* species, resulting in colonies with species-specific pigmentations and textures. These differential media allow for the presumptive identification of some species, such as *C. albicans, C. tropicalis* and *C. krusei*. The identification varies with the media used, but *C. albicans, C. tropicalis* and *C. krusei* are frequently identified in all media [151].

For invasive fungal infections, blood culture, using, for example, the BACTEC® Mycosis-IC/F system, remains the reference standard for the microbiological diagnosis [152]. Blood cultures are relatively inexpensive, use widely available/accepted technologies and when positive allow for the evaluation of the anti-microbial pathogen susceptibility. However, the recovery of fungal species from the blood presents several problems. First, most blood cultures that are positive for fungal species become positive within 96 hours, but the time to be positive is species dependent. Factors such as the volume of blood, the specific blood culture technique, previous antifungal drug exposure, fluctuations in pathogen density, also influence the sensitivity of the technique. Moreover, deep-seated infections often yield negative results because conidia can have been cleared from the bloodstream at the time blood is collected. These factors lead to the presence of false-negative results and low sensitivity (21-71%) [153, 154].

After culturing, the identification of yeast species, especially *Candida* species, is frequently performed using commercial biochemical tests based on enzymatic reactions. API *Candida*® (BioMerieux), API 20C

AUX® (BioMerieux), API ID 32C (BioMerieux®), Auxacolor® (BioRad) or Vitek 2 YST ID Card® (BioMerieux) are examples of commercial kits. These tests use carbohydrate and nitrogen assimilation, fermentation tests and enzymes detection to identify the different *Candida* species. However, they present several limitations, such as the variable results interpretation, existence of genetically different species with similar profiles, or even the presence of strains of the same species with different profiles [155]. Pasligh *et al.* (2010) [156], showed that seven different strains of *C. dubliniensis* presented different API ID 32C profiles, and some of them were identified as *C. albicans*.

For *Aspergillus* spp and Mucorales*,* frequently involving pulmonary infections, the diagnosis of invasive infection also depends on radiographic findings, by computed tomography, in patients who are at risk for the disease. However, the results are only suggestive since other invasive infections can originate similar findings [150].

It is known that an early and effective treatment of fungal infections, enhances survival chances. However, the reliance of conventional methods remains an obstacle in clinical practice. Moreover, these techniques require the growth and isolation of fungal species and to make therapeutic decisions in an early stage of infection, the development of new reliable nonculture methods is crucial [157].

2.3.2 Serological tests

Serological tests for diagnosis of fungal infections emerged as a nonculturable alternative to conventional methodologies. In these tests, the identification of fungal infection is based on the detection of specific antigens, antibodies or metabolites in clinical samples, particularly in serum, BAL or urine. Several antigens have been studied as potential targets for the diagnosis of *Candida* and *Aspergillus* infections, such as mannans (MN), galactomannans (GM) and Beta-D-glucans (BDG). Tests based on the identification of these molecules are the most frequently used, and some commercial kits are approved by the Food and Drug Administration (FDA) for the detection in serum specimens. These tests have been optimized for the diagnosis of *Candida* and *Aspergillus* infections. For Mucorales infections, the detection of these antigens have variable success and are not frequently used in clinical practice [158, 159].

Detection of the fungal cell wall component Beta-D-glucan (BDG) is commonly used as a screening and diagnostic tool, in patients with suspected invasive fungal infection since the results are available earlier than with conventional methods [160, 161]. Fungitell® Assay is the only assay approved by FDA for the detection of BDG antigens in serum specimens, however other tests have been developed, such as Fungitec G-MK (Seikagaku Biobusiness) or Glucatell®. It is a pan-fungal diagnostic test since BDG is

present in different fungal species, such as *Candida* spp, *Aspergillus* spp and *Pneumocystis jiroveci* [162–164]. Moreover, in the detection of BDG several sources of false positives have been reported, such as colonization without infection, hemodialysis with cellulose membranes, treatment with antibiotics, serious bacterial infections or surgical devices containing glucan.

The detection of mannan antigen can be useful for the diagnosis of invasive candidiasis. Mannan is an abundant cell wall polysaccharide in *Candida* spp. Pastorex *Candida*® (BioRad) and Platelia *Candida* Ag Plus® (BioRad) are commercial tests that detect the presence of mannan in serum or plasma samples, using agglutination test and EIA technique, respectivel [165]. However, the main disadvantage is the rapid clearance of the antigen from patient sera, leading to false negatives. This methodology also depends on the frequency of sampling, the underlying disease, and the *Candida* species involved in the infection.

Studies suggested that the mannan antigen detection combined with anti-mannan antibodies detection improves, considerably, the diagnosis of *Candida* infections. Platelia *Candida* Ab Plus® (BioRad) was developed to test the presence of anti-mannan antibodies in serum samples, using an EIA technique [166]. The detection of specific antibodies against *C. albicans* germ tube antigen (CAGTA) ((CAGTA) VIRCLIA® IgG) is also used. However antigen (CAGTA) presents low specificity since it detects some non-*albicans* species, although with lower sensitivity [167].

For diagnosis of *Aspergillus* species, the galactomannan (GM) is the antigen most frequently detected. The Platelia *Aspergillus* Ag® (BioRad) is an approved and commonly used test that detects *Aspergillus* GM antigen in serum and BLA fluid samples, using enzyme immunoassay (EIA) technique [148]. Studies suggested that this method present limited sensitivity and specificity when used in serum, however, when BAL samples are used, these parameters increase [168]. False-positive results were reported due to the cross-reactivity with other fungal species, such as *Penicillium* spp, *Paecilomyces* spp or *Histoplasma* spp. Moreover, dietary reactivity, laboratory contamination, prior use of antifungals and antibiotics, presence of *Aspergillus* commensal species, underlying condition or renal clearance may also lead to false-positive or false-negative results [169].

Technologies based on the incorporation of immune-chromatographic assays into lateral-flow devices (LFDs) have been used for rapid and specific detection of *Aspergillus* spp in serum and BAL samples. This test uses a murine monoclonal antibody (JF5) to detect a glycoprotein antigen present in the hyphal cell wall and septa of *Aspergillus* [170]. This method is considered easy to use and evaluate, and presents high specificity. Thornton [171] tested JF5 and a strong reaction with *A. fumigatus* and the closely related

species *A. amstelodami, A. nidulans* and *A. fischeri* was observed. Although a weak cross-reaction with *Penicillium* spp. was observed, the methodology was considered highly specific. This methodology was not extensively validated, since only few trials were performed [172, 173].

2.3.3 Peptide nucleic acid fluorescence in situ hybridization (PNA-FISH)

The Yeast Traffic Light PNA FISH® (PNA FISH® YTL, AdvanDx) is a commercial method approved by the FDA for the identification of the most frequent *Candida* species. This method uses fluorescently labelled peptide nucleic acid (PNA) probes to target species-specific rRNA of different *Candida* species directly from positive blood culture bottles or cultures [174]. After the probe anneals to its target, the diffusion of the fluorescently labelled probe across the cell wall and membrane is detected microscopically. *C. albicans* and *C. parapsilosis* show green fluorescence, *C. tropicalis* present yellow fluorescence and *C. glabrata* and *C. krusei* fluoresce red. However, differentiation between *C. albicans* and *C. parapsilosis* and between *C. glabrata* and *C. krusei* is not possible. In this way, PNA FISH® YTL classify the identification of *Candida* species according to their susceptibility to fluconazole, as susceptible (*C. albicans* and *C. parapsilosis*), intermediate (*C. tropicalis*) and resistant (*C. glabrata* and *C. krusei*) species [175]. This methodology presents good specificity and the time of identification is reduced (30-90 minutes), however, the diagnosis is dependent on positive blood cultures [176].

2.3.4 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI_TOF MS) has recently emerged as a relevant technology for the identification of several fungal pathogens. This methodology uses a laser to ionize biomolecules, which are then detected and measured based on their mass-to-charge ratio. The time-to-flight component generates a peptide mass fingerprint that is used to interrogate a database [177]. VITEK MS® (BioMerieux) and MALDI Biotyper® (Bruker Daltonik GmbH) are the MALDI_TOF MS instruments approved by the FDA for the diagnosis of invasive infections [178, 179].

MALDI-TOF MS showed to be able to distinguish several *Candida* spp, *Aspergillus* spp and Mucorales species. This method presents several advantages since it discriminates at the species level, is reproducible, fast, easy to perform and has low consumable cost [180–182]. However, this methodology also requires a comprehensive reference database, commercial libraries remain incomplete, with a low

number of species and isolates. This problem was reported by Schwarz *et al.* (2019) [183] who used MALDI-TOF MS to discriminate Mucorales species, and by using the National Institutes of Health in-house library were able to identify different isolates of *R. arrhizus*, however, using the Bruker database, the identifications were not possible. Moreover, Imbert *et al.* (2019) [184] also used MALDI-TOF MS to evaluate the online MSI platform for the identification of cryptic *Aspergillus* species, showing that some isolates were not present in the database, being misidentified with closely related species.

Identification of the fungal species by MALDI-TOF MS needs to use pure cultures, since fingerprint variation from mixed cells will induce an erroneous identification, and culture conditions can also induce fingerprint variation. Although MALDO-TOF MS provide species identification within 10-15 minutes, this methodology needs to use cultures and is not able to identify directly from clinical samples.

2.3.5 PCR based methods

PCR based methodologies have been developed and studied as alternatives for the identification of fungal pathogens. These techniques include the conventional polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), Real-Time PCR, among others [185]. PCR based methodologies are characterized by their high specificity, sensitivity and reproducibility. They can be performed using clinical isolates, positive blood cultures or using directly clinical samples, such as whole blood, serum, plasma, urine, BAL, biopsies, among others [186, 187].

At the moment only a few PCR-based methods are approved and used in clinical practice for fungal identification, however, several strategies have been developed and described as alternatives, targeting different DNA regions, namely internal transcribed spacer (ITS) regions, beta-tubulin, among others [188, 189]. T2Candida is a PCR-based assay that uses T2 magnetic resonance (T2MR) detection to identify *C. albicans, C. parapsilosis, C. tropicalis, C. glabrata* and *C. krusei.* However, similarly to PNA FISH® YTL, T2Candida panel cannot identify a specific species, and the results are divided into three groups, namely *C. albicans/ C. tropicalis*, *C. parapsilosis* and *C. glabrata/C. krusei.* The main advantage of this methodology is the rapid diagnosis (3-4 hours), since it uses whole blood samples directly, not requiring previous culture or DNA extraction.

Bomkamp *et al.* (2020) [192] evaluated the T2Candida panel for the diagnosis of candidemia at an Academic Medical Centre and obtained sensitivity and specificity values of 73% and 96%, respectively. Moreover, Lamoth *et al.* (2020) [193] also tested this methodology in blood samples of patients with probable intra-abdominal candidiasis and obtained 33% and 93% of sensitivity and specificity, respectively.

This low sensitivity values, associated with the high cost of the panel, which uses specific equipment (T2Dx Instrument), should be considered as disadvantages of this methodology [194].

In clinical laboratories, Real-Time PCR has been implemented, using different commercial kits, however, they are non-approved methodologies due to the lack of standardization and performance validation. These methodologies are fast, use DNA extracted from several clinical samples, such as serum, plasma, whole blood or BAL and do not require posterior analysis techniques, such as electrophoresis. Sensitivity and specificity values are frequently high, however, they depend on the methodology used [195].

SeptiFast® (Roche Diagnostics) is the most commonly used Real-Time PCR commercial kit and allows the identification of twenty-five clinically important microorganisms, including five *Candida* species (*C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei*) and one *Aspergillus* species (*A. fumigatus*), directly from whole blood samples [193]. This methodology uses the internal transcribed spacer (ITS) region (18S-5.8S) as target, and the species identification is based on the melting curves differences. MycAssay™ *Aspergillus* is also a Real-Time PCR commercial kit for the qualitative detection of *Aspergillus* spp. DNA (18S region) extracted from BAL and serum samples, being able to detect DNA of eighteen different *Aspergillus* species [198].

These methodologies do not require prior fungal culture, and the amplification is performed directly from clinical samples, being alternatives to the conventional and serological methods used. However, they use non-specific targets that can increase the occurence of nonspecific signals from environmental microorganisms or laboratory contamination [199, 200]. Moreover, in mixed microbial infections, the overlapping of the molecular targets and PCR products may interfere with the correct identification [182]. Several studies reported a low overall diagnostic sensitivity for Septifast. However, Dark *et al.* (2015) [201] performed a systematic review and meta-analysis of diagnostic accuracy studies of SeptiFast in the setting of suspected sepsis and showed that negative tests are probably false negatives because pathogens present were not on the PCR test panel.

Real-Time PCR methodologies have also been developed for the identification of emerging and/or multidrug-resistant pathogens [202, 203]. Arastehfar *et al.* (2019) [204] developed a multiplex real-time PCR for the identification of the multi-resistant *C. auris* and related species in serum samples. This methodology is based on the melting curve of the products. No cross-reactivity with other fungal species or human DNA was observed, and the methodology was able to identify mixed infections. However, other studies are needed to determine the sensitivity and specificity in clinical samples.

Scherer *et al.* (2018) [205] [203] also developed three quantitative PCR assays for the detection of DNA of the main genera involved in pulmonary mucormycosis, to be used directly with BAL samples. The performance of this methodology was evaluated with the prospective multicenter protocol ModiMucor (prospective evaluation of a new molecular tool for early diagnosis of mucormycosis – PHRC) and was considered a possible alternative for Mucorales identification in BAL. Moreover, the commercial kit MucorGenious® (Pathonostics) was also described as a suitable assay for Mucorales screening in pulmonary samples [206]. However, these methodologies only allow the identification at the genus level, as the majority of Real-Time PCR methods developed for Mucorales identification [207].

To identify pathogens involved in invasive fungal infections at the species level, several methodologies have been developed, using species-specific targets [208]. Valero *et al.* (2016) [209] developed a new panfungal Real-Time PCR Assay, which combines DNA binding dyes and specific molecular beacon probes, followed by melting curve analysis, for the identification of *Candida, Aspergillus,* Mucorales, and endemic species. This methodology uses DNA extracted from biopsy specimens, BAL, aqueous humor fluid, and others, and showed a sensitivity of 83.3%. However, it also demonstrated some limitations in species identification, particularly with *Candida* species, due to the similarities of the melting curves.

As mentioned above, the main problem of PCR-based methodologies for the diagnosis of fungal infections is the lack of standardization and performance validation. To solve these problems, a multicenter effort was completed by the European *Aspergillus* PCR Initiative (EAPCRI) to provide guidelines for PCR methodology [210]. Several parameters regarding DNA extraction, and amplification from different clinical samples, have been evaluated. For example, Springer *et al.* (2013) [211] used three Real-Time PCR techniques to compare the detection of *Aspergillus* DNA from serum and whole blood and observed no significant differences between the two clinical samples. However, they also concluded that there was a trend for whole blood to be more sensitive and to yield an earlier positive result than serum. On the other hand, serum is easier to process and allows other serological tests to be performed at the same time. In another study, Springer *et al.* (2020) [212] compared the use of serum and BAL for GM detection, and blood for quantitative PCR, in cases of proved/probable invasive aspergillosis. This research showed that the use of combined strategies, independently of the samples used, significantly improves the early diagnosis of aspergillosis. With the parameters evaluated, several recommendations of EAPCRI have been suggested and used by researchers improving the clinical performance of PCR in high-risk patients [195, 213].

2.4 ANTIFUNGAL STRATEGIES

After the diagnosis of fungal infection, and isolation of the fungal species, the characterization of pathogenic organisms regarding antifungal susceptibility is crucial. For that, Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) developed standardized and reproducible methodologies for susceptibility testing and defined interpretative breakpoints for susceptibility, considering the minimum inhibitory concentration (MIC), the pharmacokinetic/pharmacodynamic data and animal model data [214].

Current treatment strategies for fungal infections are limited due to a reduced number of molecular targets. The fact that fungi are eukaryotic organisms, similar to the human host, increases the risk of cross-target toxicity. The cross-interactions with other drugs are also possible, some antifungals are inhibitors of some subtypes of cytochrome P-450 in the liver, leading to increasing concentration of certain drugs in the blood [215]. Moreover, the increasing incidence of resistant species, the low drug bioavailability and the delayed diagnosis contribute to the lack of efficient treatments [216].

At the moment, three main groups of antimycotics, classified according to the mechanism of action, are approved and commonly used in clinical practice, namely polyenes, azoles and echinocandins. However, other antifungals have been used, but with lower regularity [217].

2.4.1 Polyenes

Polyenes are the major class of antifungal agents, including more than 200 compounds, and are produced by *Streptomyces* species [218]. These compounds interact with ergosterol components of the fungal cell membrane, and the complex polyene-sterol created provides an aqueous pore, affecting cell permeability and causing cell leakage and dead [219]. Polyenes can also disturb cell´s homeostasis by the formation of large extramembranous aggregates that extract the essential membrane-lipid ergosterol from the plasma membrane, by the formation of reactive oxygen species (ROS), and by the inhibition of the membrane transport of some amino acids [219, 220].

Nystatin, pimaricin and candicidin are polyenes with a therapeutic application, however amphotericin B is the most frequently used polyene, and was the first antifungal approved for the treatment of fungal infections (in 1958), being the only agent available for the treatment of invasive infections for decades [5].

Amphotericin B is a broad-spectrum antifungal, with known activity against *Candida* species, *Fusarium* species, *Scedosporium* species, among others, and is considered a resistance-evasive antifungal. Moreover, amphotericin B is considered the most active antifungal drug for the treatment of mucormycosis, except when caused by *Cunninghamella* or *Apophysomyces* species [221].

Resistance to amphotericin B is essentially observed in *A. terreus*, however other species, such as *A. flavus* and *A. fumigatus* can acquire resistance. Resistance to amphotericin B remains relatively uncommon among *Candida* species, however, some studies demonstrated the appearance of resistant strains in species of *C. albicans, C. auris* or *C. glabrata* [222–224]. Fernandes *et al.* (2015) [225] demonstrated that amphotericin B is unable to prevent biofilm formation by *C. tropicalis*, and that the increase of the protein and carbohydrate contents in biofilms matrices treated with amphotericin B also increased biofilm resistance.. Several molecular mechanisms are responsible for the polyene resistance, namely the decrease of total ergosterol content, the replacement of some or all polyene-binding sterols, and the reorientation or masking of existing ergosterol [219].

Amphotericin B administration is limited, due to the acute infusion-related reactions (nausea, fever, hypoxia, among others) and nephrotoxicity of its conventional formula [226]. To overcome this limitation, new formulations were developed, which use liposomes or lipid complexes as delivery systems, and are now commercialized. There are several liposomal amphotericin B products available, including AmBisome (AHPL), Ambihope (Abbott Healthcare Pvt. Ltd.), or AmBiL (Taiwan Liposome Co.). The chemical composition, as well as the efficacy and safety, are variable, showing that the manufacturing process can influence liposome properties. Even considering the high prices of liposomal amphotericin B formulations, in 2016 their use was recommended as the first-line therapy for mucormycosis by ESCMID/ECMM guidelines [227].

2.4.2 Azoles

Azoles (2.2) are the second most studies groups of antifungal agents. These molecules bind to cytochrome P450 sterol 14α–demethylase and block demethylation of lanosterol, resulting in ergosterol depletion, and the accumulation of toxic sterol intermediates. This process inhibits cellular growth and division and induces severe membrane stresses [217].

Azoles include the imidazole-based drugs, such as clotrimazole, miconazole, ketoconazole and econazole, which are formulated for superficial infections, due to the high toxicity and low bioavailability, and the triazole-based drugs, such as fluconazole, itraconazole, voriconazole, posaconazole, isavuconazole, which

are typically fungistatic drugs used for both superficial and invasive fungal infections. The differences in the structure of the two azole subgroups are responsible for their variation on antifungal potency, bioavailability, drug interaction and toxicity [228].

In the early 1990s, the introduction of fluconazole, as a prophylactic agent and antifungal of choice, for the treatment of fungal infections, caused the emergence of azoles resistant strains, particularly within *C. glabrata* and *Aspergillus* species. Moreover, this increasing azole resistance may be also explained by the appearance of species intrinsically resistant to fluconazole, such as *C. krusei* or *C. auris*.

Three main mechanisms of resistance to azoles have been reported, and more than one mechanism may operate in any given strain [229]. (i) Induction of multi-drug efflux pumps which decrease the accumulation of the drug at the target enzyme. For example, the class of the ATP binding cassette (ABC) transporters (PDR) includes *CDR1* and *CDR2*, and Cg*SNQ2* (specific for *C. glabrata*) and are the major azole drug transporters, conferring resistance. The expression of these drug transporters is determined by specific regulators *TAC1*, and mutations in these regulators leads to overexpression of the efflux pumps and enhance azole resistance [230, 231]. Moreover, others efflux pumps have been also identified, such as *MDR1* transporter, which belongs to the major facilitator superfamily (MFS) and is regulated by *MRR1* [232]. (ii) Alteration and up-regulation of the target enzyme preventing the binding of azoles to the enzymatic site. For example, in *Candida* azoles inhibit the intracellular target enzyme ERG11p, important in the biosynthesis of ergosterol, and several alterations have been reported in the *ERG11* target gene in both strains resistant and susceptible to azoles. Depending on the location or specific substitution of mutation, these alterations may lead to the development of azole-resistance. The transcription gene *UPC2* regulates *ERG11* and gain of function mutations in these gene leads to an increase of ERG11p, and a consequent azole resistance [233]. (iii) Development of bypass pathways, for example by the replacement of ergosterol with the latter sterol, resulting in functional fungal cell membranes and subsequently preventing the membrane disruptive effects of the azoles [234].

To overcome the problems associated with the use of azoles, novel analogues with improved efficacy and safety have been developed, with special attention to isavuconazole. This triazole exhibits a long elimination half-life and present broad-spectrum activity against *Candida* and *Aspergillus* species. Moreover, it is also active against the majority of Mucorales, similarly with amphotericin B [235]. The FDA has already approved the use of isavuconazole for the treatment of invasive aspergillosis and mucormycosis.

2.4.3 Echinocandins

The echinocandins (2.2) are cyclic hexapeptides that act as inhibitors of the β -1,3- D-glucan synthase Fks1 essential for cell wall synthesis. Alterations in β -1,3- D-glucan destabilizes the integrity of the fungal cell wall and promotes the osmotic instability and cell death [236].

The echinocandins drugs used in antifungal treatment are caspofungin, micafungin and anidulafungin. All these drugs present low toxicity since they are active against a fungal-specific target that is not found in mammals. These agents have broad-spectrum antifungal activity against *Candida* and *Aspergillus* species, however, are not active against *C. neoformans* and non-*Aspergillus* moulds. The echinocandins resistance is unusual, however, some case reports have illustrated the potential for resistance development. Some reports showed that *C. albicans*, *C. glabrata* and A*. fumigatus* strains can acquire resistance by promoting changes in amino acids in the hot spot region of the β-glucan synthase Fks1 [237, 238]. Moreover, *C. parapsilosis* has a naturally occurring polymorphism in Fks1, resulting in an acquired susceptibility to echinocandins [239, 240]. Resistance in *A. fumigatus* strains was also reported by an Fks1-independent mechanism, that uses CAS-induced ROS-mediated changes in the lipid composition of the microenvironment of glucan synthase, altering the drug target interaction [241].

The mains disadvantage of echinocandins is their low oral bioavailability, only intravenous infusion are available. Enfumafungin has been developed and tested to overcome oral bioavailability limitation and presented potent *in vitro* activity against *Candida* and *Aspergillus* species. Jiménez-Ortigosa *et al.* (2014) [242] also showed that this compound presented dose-dependent activity, against echinocandins-resistant *Candida* and *Aspergillus* strains.

2.4.4 Other antifungal agents

Although polyenes (2.2), azoles and echinocandins represent the major classes of antifungal agents used in clinical practice, other compounds were developed and approved for clinical application, such as allylamines, pyrimidines, sordarins or ciclopiroxalamines.

Pyrimidines are one of the oldest classes of antifungal agents and include flucytosine (5-fluorocytosine or 5-FC). This drug is a synthetic analogue of cytosine, that when in the cytosol becomes deaminated to generate 5-fluorouracil. 5-fluorouracil acts as a potent antimetabolite that causes RNA miscoding and inhibits DNA synthesis [217].

Flucytosine is selectively toxic to fungal cells because mammalian cells lack cytosine permease and do not convert flucytosine into 5-fluorouracil, being the "gold standard" for the treatment of *Cryptococcus* spp infections [243]. This drug is also active against *Candida* species, but the development of resistance is frequent. The resistance mechanisms include (i) mutations in genes which encodes for essential enzymes in the cellular transport and uptake of 5-FC, and (ii) increasing synthesis of pyrimidines, which compete with the fluorinated antimetabolites of 5-FC and reduce its antimycotic activity [244].

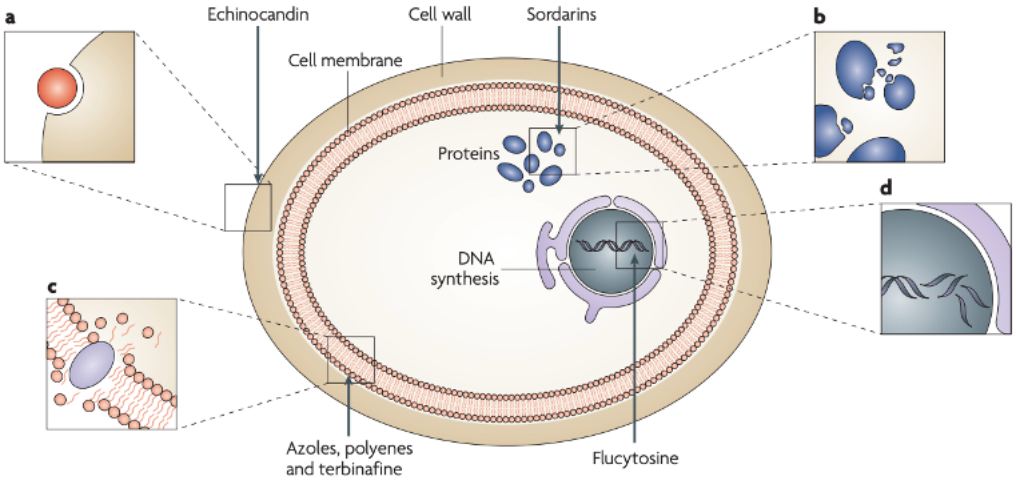


Figure 2.2 Mechanisms of action of clinically used antifungal drugs. **a** echinocandins, **b** sordarins, **c** azoles, polyenes and terbinafine and **d** flucytosine. (Adapted from Ostrosky-Zeichner *et al.* (2010) [228].

2.4.5 Combined antifungal drugs

The combination of two antifungal drugs is a promising strategy for preventing drug-resistant fungal species. This strategy responds to the biological compensation, reduces doses of each compound overcoming the problem of toxicity, and access different multi-target mechanisms, inhibiting the development resistance [245].

A total of twenty-four patents of combinations for antifungal use were released between 2000 and 2015. These patents included the combinations of several compounds, including two commercial antifungal drugs, a commercial antifungal drug and new compounds, an antifungal drug and an immunomodulatory agent, an antifungal drug and an antibiotic, an antifungal drug and antibodies, an antifungal drug and an anti-inflammatory agent, among others [246]. However, at the moment only one combination was well studied and validated in clinical trials for fungal infections, namely the combination of amphotericin B and flucytosine to treat cryptococcosis. This combination was initially tested by Bennett *et al.* [247] in

patients with cryptococcal meningitis, showing an improvement in the number of patients treated and a decrease of nephrotoxicity. Same results were observed by Day *et al.* (2013) [248] in patients with cryptococcal meningitis, in which amphotericin B and flucytosine combination was associated with improved survival of the patients.

2.4.6 New antifungal strategies

At the moment, ergosterol synthesis, ergosterol itself or cell wall synthesis are the only targets of the most frequently used antifungal drugs, except the echinocandins. These molecular targets and mechanisms are well known and for that reason new classes of antifungal have been developed against the same molecular targets.

Recently, new molecular targets have been identified and studied as alternatives, and at least three compounds developed are now in clinical trials. E1210 (Amplyx Pharmaceutical) targets inositol acyltransferase (Gwp1), important in the biosynthesis of the GPI anchoring proteins, which are vital to cell wall regeneration and adhesion to host cell [249]. This compound showed a potent broad-spectrum antifungal activity against clinically important fungal species, including several *Candida* and *Aspergillus* species, and also *Rhizopus* and *Scedosporium* species [250], and is now in Phase 1 clinical trials.

VL-2397 (Vical) is an antifungal drug isolated from the fungus *Acremonium persicinum* and present a new mechanism of action, with a ferrichrometype structure with unusual amino acids in the siderophore backbone, however, the specific mechanism remains unknown. This compound is already in Phase 2 clinical trial for the treatment of invasive aspergillosis [251].

The third compound in clinical trials is the orotomide compound F901318 (F2G), that targets the fungal dihydroorotate dehydrogenase (DHODH), essential for DNA/RNA synthesis, the cell cycle, protein synthesis, cell wall synthesis and phospholipid synthesis. Although DHODH is also found in mammals, F901318 is not active against human DHODH. This compound present good activity against *Aspergillus* species and is now in Phase 2 clinical trial [252].

Besides the described antifungal targets, other mechanisms have been studied and tested, targeting glycosphingolipids biosynthesis, amino acids biosynthesis, glyoxylate cycle, signaling pathways, transcriptional factors or histone deacetylases [253].

For a successful fungal infection, pathogens have to adapt to host stressors and evade hosts' immunity. In this way, approaches to modulate the host immune system in fighting fungal pathogens have been suggested as antifungal strategies. These strategies include the application of effector and regulatory cells, such as granulocytes, the administration of recombinant cytokines and growth factors, such as interferon-γ, TNF-α and IL-15, and vaccination. For example, Pentraxin 3 (PTX3) is receiving particular attention as a potential antifungal agent with anti-inflammatory properties in aspergillosis. This opsonin form complexes on the surface of *Aspergillus* conidia, increasing the innate immune response [254, 255]. However, immunomodulatory treatments are associated with high toxicity, excessive immune response and inflammation, or pathogen replacement [256].

2.4.7 New compounds with potential antifungal activity – Benzo[*a*]phenoxazines

The use of new compounds with different targets, associated with different therapeutic strategies, could be an excellent alternative against fungal pathogens. In the last years several compounds with a variety of applications have been synthetized.

The benzo[*a*]phenoxazine dyes are organic fluorophores presenting predominantly lipophilic nature. They derive from a polycyclic phenoxazine, being characterized by the presence of a benzene ring fused with the *a* face of the phenoxazine structure (Figure 2.4). Attached to the central benzophenoxazine structure, these compounds may present functional groups, such as an amine function at the position 9 that may be primary, secondary or tertiary, with a positive or neutral charge. Moreover, they may also present a functional group (amine, carboxyl or hydroxyl group) at the position 5, essential for covalent labelling molecules or to confer them the possibility of other chemical adaptations [257, 258]. The synthetic applications of phenoxazines appeared to be problematic due to the insoluble nature of molecule, however, changes in the molecule, such as the introduction of a nitro group, may increase compounds solubility [259].

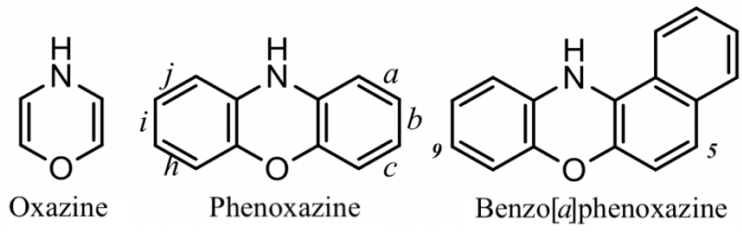


Figure 2.3 Molecular structure of oxazine, phenoxazine and benzo[a]phenoxazine.

The most studied and used benzo[*a*]phenoxazines are Meldola's Blue, Nile Blue and Nile Red, and the majority of compounds used derive from them. These compounds or derivatives absorb and emit florescence in 600-900 region of spectrum being frequently used for labelling of nucleic acids in blotting experiments, gel electrophoresis, living cells assays, among others [260, 261]. However, several studies have demonstrated their potential in other fields. For example, recently these compounds showed potential to be used as photosensitizers in antimicrobial photodynamic therapy against *Candida albicans* biofilms or for the photodynamic therapy in cancer treatment [262, 263].

Benzo[*a*]phenoxazines have been also studied due to their antiproliferative properties, being tested as immunosuppressors, anti-tumour, anti-inflammatory and antimicrobial agents [264, 265]. Suzuki *et al.* (2007) [266] tested the activity of several phenoxazine derivatives in normal and tumor cell lines, and observed that depending on the chemical structure of compounds, some presented specific activity against tumor cells, inducing different types of cell dead. Moreover, Schleiferböck *et al.* (2013) [267] also showed that the benzo[*a*]phenoxazine derivative SSJ-183 present antimalarial properties, being active even against parasite strains resistant to the conventional drugs.

Recent studies showed the antifungal activity of several benzo[*a*]phenoxazines against *Saccharomyces cerevisiae* [264, 265, 268]. The antiproliferative activity of benzo[*a*]phenoxazines is traditionally explained by their ability to form stable complexes with DNA, in which the phenoxazine ring intercalates into DNA, inhibiting DNA-dependent RNA polymerase, leading to cell death. However, these interactions and the biologic effect in yeast cells are highly dependent on benzo[*a*]phenoxazine structure in which for example a hydroxyl group at the position 5 decrease antifungal activity [269]. Moreover, some studies suggested that phenoxazine derivatives can suffer a metabolic conversions intro free radical intermediates leading to oxidative stress and DNA damage [270]. Recently, studies also suggested that compounds with higher antifungal activity accumulate in perinuclear membrane of endoplasmic reticulum and the enzyme oxidosqualene cyclase (important for sterol biosynthesis) has been considered as a potential target [268]. However, the exact mechanism of benzo[a]phenoxazines antifungal activity, as well as the possibility of fungal species to acquire resistance remains unknown and more studies are needed.

2.4.8 Liposomes as drug delivery systems

The use of nanoparticles as drug delivery systems is a recently studied alternative to solve problems such as the high toxicity, bioavailability, solubility, poor distribution or lack of selectivity of drugs. The small sizes of these particles allow to target specific body sites, minimizing undesirable side effects [266]. However, the therapeutic potential of formulations depends on several factors, such as hydrodynamic size, shape, quantity, surface chemistry, rout of administration, length of stay in circulation and reaction with the immune system.

Different nanosystems have been used to deliver bioactive substances structures, including liposomes, micro- and nanoemulsions, cyclodextrins, solid lipid nanoparticles, polymers, dendrimers, silica, carbon or magnetic nanoparticles [262, 263 264].

Liposomes are the most studied vesicular systems for drug delivery and are characterized as small (from 50 to 500 nm) and spherical vesicles composed by polar lipids, which are amphiphilic molecules that have a hydrophilic head and two apolar hydrophobic chains. Due to their amphipathic nature, in aqueous environments lipids can self-assemble into colloidal particles encapsulating both hydrophobic and hydrophilic molecules [275, 276]. In aqueous systems liposomes can form one or more phospholipid bilayers, in which hydrophilic region is composed by a polar headgroup and the hydrophobic regions presents nonpolar hydrocarbon tails (Figure 2.3) [277]. However, this final organization depends on lipids nature, concentration or temperature [278].

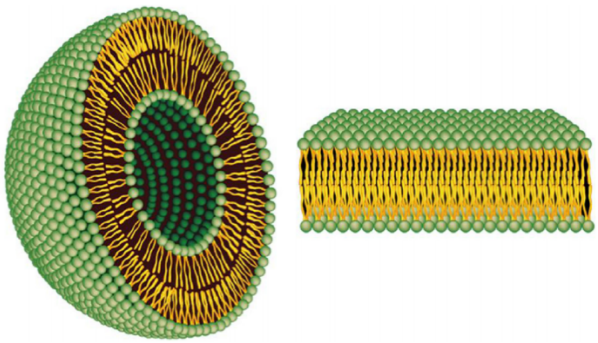


Figure 2.4 Representation of a liposome and a lipid bilayer (Adapted from Bozzuto and Molinari (2015) [279]).

Considering the production method, and consequently size and number of bilayers, liposomes are classified in two categories. (i) Multilamellar vesicles (MLVs), which present sizes between 500 and 1000 nm and are composed by two or more concentric lipid bilayers and (ii) unilamellar vesicles (ULVs), which

are divided into larger unilamellar vesicles (LUVs) with sizes > 50 nm and small unilamellar vesicles (SUVs) with sizes < 50 nm and (iii multivesicular vesicles (MVVs) [280].

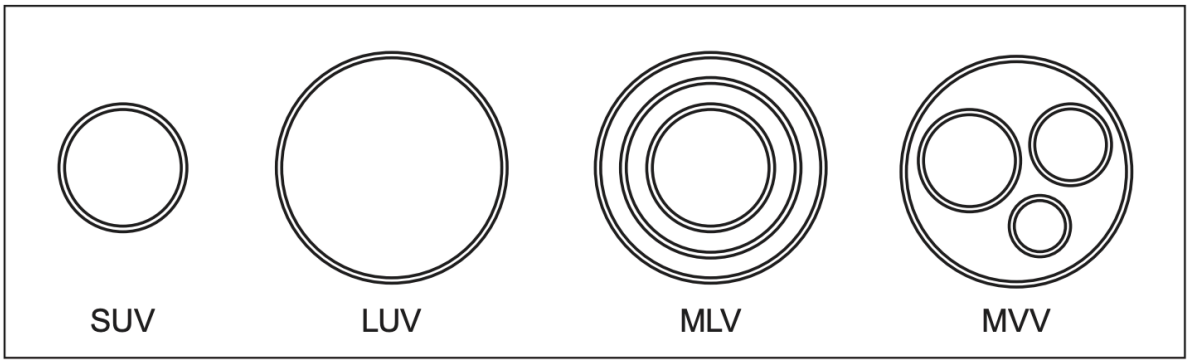


Figure 2.5 Types of liposomes depending on size and lamellarity, namely small unilamellar vesicle (SUV), large unilamellar vesicle (LUV), multilamellar vesicle (MLV) and multivesicular vesicle (MVV) (Adapted from Elizondo *et al.* (2011) [281].

Liposomes present a high entrapment efficiency and are able to encapsulate a variety of amphiphilic, lipophilic and hydrophilic compounds, such as drug molecules, proteins, nucleotides and even plasmids [282]. As bilayer structures, depending on compounds characteristics these may be absorbed on the liposome surface (hydrophilic compounds), be incorporated between hydrocarbon chains (hydrophobic compounds), or be encapsulated in the hydrophilic region (hydrophilic compounds) [277].

In the last years liposomes have been studied as nanocarriers for drug delivery due to several advantages: i) increased efficacy and therapeutic index of drugs; ii) increase stability of drugs; iii) they are non-toxic, flexible, biocompatible, biodegradable and non-immunogenic; iv) reduced toxicity of the encapsulated agent; v) they help to reduce the exposure of sensitive tissues to toxic drugs and; vi) it is possible to couple them with site-specific ligands to achieve active targeting [283, 284].

Liposomes could interact with cells using several methods: i) liposomes adsorb to the cell surface, their lipids are degraded by cellular enzymes releasing the liposome content into the extracellular fluid, followed by diffusion of the content into the cell, ii) liposomes (<150 nm) are totally incorporated by the cell and release the content directly into the cytoplasm, or iii) liposome (>150 nm) are phagocytised and release their content [285, 286].

The development of an effective liposome-based drug is influenced by the liposome physiochemical properties [287]. The size of liposomes influences their circulation half time, since large liposomes (>150

nm) activate the immunological complement and rapidly leads to clearance from bloodstream by phagocytes, whereas small liposomes <100 nm interact less with plasma proteins and evade from phagocytes. However, it is known that the latter ones present less drug-storage capacity [279].

The liposomes superficial charge also affects the effectiveness of liposome-based drugs, for example, in positively charged liposomes repulsion among liposomes due to ζ-potential prevents their aggregation and flocculation but promotes interaction with cells, whereas in neutral liposomes aggregation occurs and they do not interact with cells [288]. Moreover, negatively charged liposomes are less stable and interact with biological system, increasing the uptake by phagocytes. Extended circulation times of these delivery systems can be achieved by modification of the liposomal surface with hydrophilic polymers such as polyethylene glycol (PEG), that forms a hydrated steric barrier at the surface of the liposomes, endowing them with stealth properties [289].

The number of bilayers is also important since it affects the type and amount of drug encapsulated and respective release. ULVs preferentially entrap hydrophilic compounds due to the large aqueous core, while MLVs presents several concentric lipid bilayers, encapsulating preferentially hydrophobic drugs. Moreover, MLVs and MVVs due to the large diameter are able to encapsulate higher compound amount. However, the number of phospholipid bilayers also hamper the drug release [277].

There are numerous studies using liposomes as nanodelivery systems, but few are approved to use in the clinical practice. As described previously, there are three liposomal formulations with antifungals used in clinical practice for the treatment of fungal infections, namely Abelcet®, Ambisome® and Amphotec®. All these formulations are approved for intravenous administration of amphotericin B.

Ambisome®, approved in 1997, is the most frequently used. This liposomal formulation of amphotericin B is composed of hydrogenated soy phosphatidylcholine (HSPC), cholesterol and distearoyl phosphatidylglycerol (DSPG). In aqueous media spontaneously arrange into unilamellar vesicles (<100 nm), encapsulating amphotericin B [290].

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CHAPTER 3

New multiplex PCR based strategy for detection of fungal pathogen DNA in patients with suspected invasive fungal infections

Adapted from:

New multiplex PCR based strategy for detection of fungal pathogen DNA in patients with suspected invasive fungal infections

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3.1 ABSTRACT

The incidence of invasive fungal infections (IFI) has increased due to the development of more aggressive medical and therapeutic techniques, which increase the number of immunocompromised individuals. *Candida* and *Aspergillus* species are the most common etiological agents isolated from IFI, however, the incidence of *Rhizopus* species has increased in recent years. The susceptibility to antifungal drugs differs according to the species thus, the rapid and correct identification at the species level is crucial.

The main objective of this work was to develop a simple and easy to interpret PCR multiplex strategy for the identification of the most common fungal species involved in IFI, in which the PCR products are fluorescently labelled according to the species and the fragment sizes. Two panels were designed, the *Candida Panel* for the identification of five *Candida* species, and the *Filamentous Panel* for the identification of four *Aspergillus* species and *Rhizopus arrhizus.* The panels were design with species-specific markers and tested in 121 strains of 26 different species and in 29 clinical samples from hospitalized patients.

The multiplex PCR showed the correct identification of all 10 targeted pathogens and exhibited no cross-reactivity with nontargeted pathogens, being 100% specific. It also performed well in colony-PCR. The sensitivity reached 10 to 1 pg of DNA and, when DNA concentration was equal to or higher than 10 pg of DNA, the method gave reproducible results in all replicates (10 to 20) for all 10 targeted pathogens. This methodology was also able to identify the different species in mixed samples, and human serum did not inhibit the detection of the specific markers. The analysis of the performance of the methodology with DNA from clinical samples revealed that this method was not suitable for bronchoalveolar samples, but for samples from sterile places the sensitivity was 89% and the specificity 100%.

This proof-of-concept showed the potential of this multiplex PCR methodology to be used for the identification of pathogenic species, even in mixed infection. The possibility of the user to see the peak in the specific region of the panel with the correct fluorescence dye is a unique characteristic of this assay that aids rolling-out unspecific amplifications. The methodology developed is suitable for the identification of the ten most important fungal species involved in IFI, not only from positive blood cultures strains but also from clinical sample from sterile places. It is easy to perform and interpret and can be implemented at relatively low costs for routine identification in microbiological laboratories. In addition, the panels can be customized, selecting markers for different species and/or resistance gene.

Keywords: *Aspergillus* spp, *Candida* spp, Multiplex, PCR, *Rhizopus* spp.

3.2 INTRODUCTION

The development of medicine has caused an increase in the number of immunocompromised individuals. Cancer chemotherapy, organ transplantation, antimicrobial therapy and abdominal surgery are among the medical procedures that constitute the main risk factors predisposing to invasive fungal infections (IFI) [1, 2]. IFI are associated with mortality rates, ranging from 2 to 60% depending on the etiology of invasive infection and the type of underlying disease of the patient [3] and to with high hospital costs [4, 5]. A recent analysis estimated, in the United States, the total direct medical cost of fungal-disease related hospitalizations to be $4.6 billion [5]. A total cost of $1.4 billion with *Candida* infections (26,735 hospitalizations,) and $1.2 billion with *Aspergillus* infections (14,820 hospitalizations) [5]. Notably, the authors also reported that the presented costs underestimate the true economic burden because many fungal diseases probable go unrecognized and also their cost analysis did not account for the costs related to unnecessary medical procedures and inappropriate treatment before the fungal diagnosis is established.

Several etiological agents are associated with IFI but species of genus *Candida* followed by *Aspergillus* spp. are the most frequently isolated [6]. Although *Candida albicans* is most often isolated, species other *Candida* species such as *C. tropicalis, C. glabrata, C. parapsilosis* and *C. krusei* are also increasingly recognized as widespread pathogens [7]. Concerning *Aspergillus* sp., *A. fumigatus* is the dominant species, but *A. flavus, A. niger* and *A. terreus* are also largely associated with invasive aspergillosis in severely immunocompromised individuals [8]. More recently, outbreaks of Mucorales infections (mucormycosis) associated with IFI were reported in developed countries [6, 9]. Mucorales are considered emerging pathogenic species, particularly *Rhizopus, Lichtheimia* and *Mucor* genus, being *Rhizopus arrhizus (Rhizopus oryzae)* the most frequently found [10].

To enhance survival of infected patients, and early and adequate identification of the infecting species is crucial, since it allows the selection of appropriate antifungal treatment, as different species differ in their susceptibility to antifungal agents [11, 12]. Moreover, the fact that the risk of developing deep organ involvement, and the severity of clinical manifestations, differs depending on the infecting species the significance of correct diagnosis is high [2]. Species identification is also essential for the rapid identification of hospital outbreaks [13].

The European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG)

defines that for proven diagnosis of IFI a microbiological and/or histopathological diagnostic method is requires [14]. Thus, blood cultures are still the reference standard for the microbiological diagnosis of IFI [15]. However, this culture-based method has several limitations, such as false-negative results because of on-going antimicrobial therapy, and long time to positivity (from 12 hours to 72 hours). These drawbacks reflect the low numbers of circulating microorganisms (from 1 to 10 CFU/mL), the possible presence of fastidious pathogens, and insufficient blood sample volumes (especially in children). Therefore, their sensitivity is low, only 50–75% even when sampling recommendations are correctly followed [16]. Bacteremia coexists in approximately 20% of cases, hampering even more fungi detection [16]. However, blood cultures are relatively inexpensive, use widely available/accepted technologies and allow the evaluation of anti-microbial pathogen susceptibility.

To overcome limitations of blood cultures and achieve a faster diagnosis, laboratory techniques that do not require growth of the organism in culture or identification in tissue samples play an important role in clinical decision-making related to IFI [17]. Detection of the fungal cell wall component (1, 3)-β-D-glucan (BDG) is commonly performed as a screening and diagnostic tool for patients with suspected IFI [18, 19]. BDG is a pan-fungal antigen identified by several assays, being Fungitell Assay (Associates of Cape Code Inc; Falmouth, MA) approved by the Food and Drug Administration (FDA) for the detection in serum specimens. Fungitell is approved for detection of BDG in serum of suspected patients infected with *Candida* species, *Pneumocystis jiroveci, Aspergillus* spp, *Fusarium* spp, and *Acremonium* spp. [20–22]. Another FDA-approved assay is the Platelia *Aspergillus* EIA (Bio-Rad; Marnes-la-Coquette, France), designed to detect galactomannan (GM) in the serum and bronchoalveolar lavages of suspected patients. GM is produced by a variety of fungi, including *Aspergillus* spp., *Penicillium* spp., *Paecilomyces* spp., *Purpureocillium licacinum*, and *Histoplasma* spp..

The combined use of multiple diagnostic assays generally increases the accuracy of IFI diagnosis, by interpreting the results of two individual tests used in conjunction. Several molecular methods have been developed to help diagnosis of IFI, improving the clinical utility and performance of current fungal diagnostics. These molecular approaches range from Laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) to Real-time PCR and, can be performed in various samples, such as positive blood culture, whole blood, plasma, or sera [23].

Molecular methodologies, based on the analysis of DNA, are characterized by their high specificity, sensibility and reproducibility. The majority of DNA based techniques require DNA detection by single or multiplex specific PCRs (using species-specific primers) amplification. The PCR fragments can then be

identified by several methods, including sequencing, melting curve analysis, electrophoretic separation, fluorescence in situ hybridization (FISH), electrospray ionization mass spectrometry (ESI-MS), microarray, or surface-enhanced Raman scattering (SERS) [23]. SeptiFast (Roche Diagnostics) and MycAssay *Aspergillus* kits are some examples of commercial kits used in clinical laboratories to identify fungal pathogens by real-time PCR. These kits do not require prior fungal culture and the amplification is performed directly from clinical samples [24, 25]. These methodologies usually use nonspecific targets (ex. mitochondrial DNA, 28S rRNA, 18S rRNA, and internal transcribed spacer (ITS) regions of ribosome DNA) with a high probability of nonspecific signals from environmental microorganisms present in the laboratory [26, 27]. Also, in mixed microbial infections, the overlapping of the molecular targets may interfere with the correct identification [28].

In this work, we designed a sensitive and specific multiplex PCR strategy for the identification of the most clinically important pathogenic species of *Candida* sp., *Aspergillus* sp. and *Rhizopus*. Two multiplex panels were tested and optimized, namely, the *Candida Panel*, using primer specific for *C. albicans, C. parapsilosis, C. glabrata, C. krusei, C. tropicalis* and the *Filamentous Fungi Panel*, using primers pairs designed for identification of *A. fumigatus, A. flavus, A. terreus, A. niger* and *R. arrhizus*. The novelty of this method is that is uses species specific primers outside the mitochondrial or ribosome DNA, reducing cross-amplification with closely related species and that the visualization of the results is straightforward, based on the presence of fragments with the correct size and fluorescent color. This methodology was tested in DNA isolated from strains from clinical samples and in DNA isolated directly from clinical samples from biopsies, bronchoalveolar lavages, cerebrospinal fluid, and vitreous humor.

3.3 MATHERIAL AND METHODS

3.3.1 Primers Selection and Panel Design

A search for specific DNA regions was performed and specific primers designed by using Primer 3Plus (https://primer3plus.com) software. The selected reverse primers were fluorescently labelled with HEX (hexachlorofluorescein), FAM (6-carboxyfluorescein) or NED (Proprietary to Applied Biosystems) for DNA size determination in 3130 Genetic Analyzer (Applied Biosystems®). According to the expected fragment sizes, two panels were designed combining the sizes with the fluorescent label.

3.3.2 Strains Tested

DNA from 121 previously identified clinical and environmental isolates was used to optimize the methodology. To assess specificity of the methodology strains of the following species were used: *C. albicans, C. parapsilosis* sensu stricto, *C. glabrata* sensu stricto, *C. tropicalis, C. krusei, C. metapsilosis, C. orthopsilosis, C. bracarensis, C. guilliermondii, C. lusitaniae, L. elongisporus, A. fumigatus, A. flavus, A. terreus, A. niger, A. versicolor, A. unguis, A. westerdijkiae, A. tamarii, A. wentii, A. nidulans, A. tubingensis, R. arrhizus, R. microsporus, Mucor* spp. and *L. corymbifera* recovered from different sources, such as saliva, vagina, respiratory tract, feces, skin, blood culture, urine, catheter, wine and hospital environment. All these isolates are conserved at the collection of Biology Department of Minho University. The type strains, *C. albicans* (PYCC 3436), *C. tropicalis* (PYCC 3097), *C. krusei* (PYCC 3341), *C. glabrata* (PYCC 2418) and *C. bracarensis* (153 MT), obtained from the Portuguese Yeast Culture Collection (PYCC), New University of Lisbon, Lisbon, Portugal, were also included in this study. Human Genomic DNA (Promega®) was also tested.

3.3.3 Genomic DNA Extraction

Before DNA extraction, yeasts and filamentous strains were grown at 30 °C for 48 hours and five days, respectively, on YPD agar medium (Yeast extract 1%, Bactopeptone 1%, Glucose 2% and Agar 2%).

DNA extraction was performed using different methodologies, always in a category 2 laminar flow cabinet. Yeast DNA was extracted using the commercial kit JetQuickTM DNA Purification Kit, according to manufacturer's instructions. Yeast strains were also tested without previous DNA extraction, using colony-

PCR, which is a simple and fast methodology that uses directly the colony in the PCR tube to obtain the DNA template by thermal shock [29].

Genomic DNA from filamentous fungi was extracted using an in-house protocol. Briefly, fungal biomass was frozen in liquid nitrogen and macerated using a pestle. Maceration step was repeated three times with the addition of TES buffer (0.05 M EDTA, 20% sucrose, 1 M Tris pH 8.0). After maceration, samples were heated at 100 °C, centrifuged 10 minutes at 3000 rpm, 4 °C, and centrifuged again at 13000 rpm, 3 minutes, 4 °C. Chloroform: Isoamyl Alcohol (24:1) added and samples centrifuged at 14000 rpm for 10 minutes, at 4 °C. The top layer transferred to a new 2 mL tube and 1/10 of the total volume, of sodium acetate 3 M and 2.5X of the total volume of absolute ethanol, was added. Samples centrifuged over 30 minutes at 14000 rpm, the supernatant discarded, and the pellet washed with 70% (v/v) ethanol. Samples washed again with 70% (v/v) ethanol and pellets dried at room temperature. Finally, the pellets were resuspended in ultrapure H_2O and heated at 65 °C for 45 minutes.

3.3.4 DNA Extraction from Serum Spiked with DNA

All manipulations were performed in a category 2 laminar flow cabinet to prevent contamination. Clotted-blood samples were collected from consenting healthy donors, and serum fractionated by centrifugation was divided into 500 µl aliquots. Then, serum aliquots were spiked with 5 ng, 500 pg and 50 pg of DNA from *A. fumigatus, A. terreus, C. albicans, C. krusei* or *R. arrhizus* and total DNA extracted using the QIAmp Ultrasens Virus Kit (QIAGEN), according to European *Aspergillus* PCR Initiative (EAPCRI) for testing serum [30]. The DNA obtained was stored for amplification with the corresponding panel. The yield of extraction was determined, comparing results from RT-PCR using DNA before *vs* DNA after QIAmp Ultrasens Virus Kit extraction, according to Springer *et al.* (2017) [31].

3.3.5 PCR Amplification Conditions

3.3.5.1 Singleplex amplification

For all primer pairs, singleplex PCRs were performed using several strains, to confirm locus-specific amplification and PCR fragment sizes. All locus were amplified with 25 ng of genomic DNA, in a 10 µl reaction volume, containing 1X PCR buffer (20 mM Tris HCl [pH 8.4], 50 mM KCl), 0.2 mM of each of the four deoxynucleoside triphosphates (dNTPs), 1.5 mM $MgCl_2$, 1 U of PhusionTM High-Fidelity DNA Polymerase, 0.2 µM of each primer, and 2 µl of fungal DNA. The PCR program consisted of a pre-

incubation step for 5 min at 95 °C, followed by 30 cycles of 30 sec at 94 °C, 30 sec at 64 °C and 1 min at 72 °C, with a final extension step of 10 min at 72 °C.

3.3.5.2 Multiplex amplification

Multiplex PCRs were performed by combining 1X PCR buffer, 0.2 mM of each of the four deoxynucleoside triphosphates (dNTPs), 2 mM $MgCl_2$, 2 U of PlatinumTM II Taq Hot-Start DNA polymerase in a 20 µl total volume. The PCR program consisted in a pre-incubation for 5 min at 95 °C, followed by 45 cycles of denaturation 30 sec at 95 °C, annealing 30 sec at 60 °C and extension 1 min at 72 °C, with a final extension 10 min at 72 °C. The primer concentration used was adjusted according to the PCR panel used.

3.3.6 PCR Fragment Size Determination

Following amplification, PCR products were added to a mixture (9:1) of Hi-Di formamide and internal size standard (GeneScan 500 6-carboxy-X-rhodamine [ROX]; Applied Biosystems Inc.®), and PCR fragments separated in 3130/3130xl Genetic Analyzers (Applied Biosystems®). Fragment sizes of the PCR products were determined automatically using the Peak Scanner software (www.thermofisher.com).

3.3.7 Clinical Samples

A total of 29 clinical samples, obtained from DNA collection of clinical samples from the Instituto de Salud Carlos III, Centro Nacional de Microbiologia (Madrid, Spain) and Medizinische Klinik II, Universitätklinikum Würzburg (Würzburg, Germany), were tested using the multiplex PCR Panels. These samples included 19 samples of patients with probable/proven fungal infection and 10 negative controls, and were obtained from biopsies, bronchoalveolar lavages (BAL), cerebrospinal fluid, and vitreous humor.

3.4 RESULTS

3.4.1 Design of the Multiplex Strategy

The search for specific regions of *Candida, Aspergillus* and *Rhizopus* species allowed the design of ten specific primer with annealing temperatures around 60 °C, to ensure specificity and reproducibility. The differences in the molecular weight of the expected PCR fragments amplified and the possibility of combining different fluorescent dyes made possible the design of two panels to identify different sets of species. Namely, the *Candida Panel*, for identification of *C. albicans, C. parapsilosis, C. glabrata, C. krusei* and *C. tropicalis* (Figure 3.1a), and the *Filamentous Fungi Panel*, for identification of *A. fumigatus, A. flavus, A. terreus, A. niger* and *R. arrhizus* (Figure 3.1b).

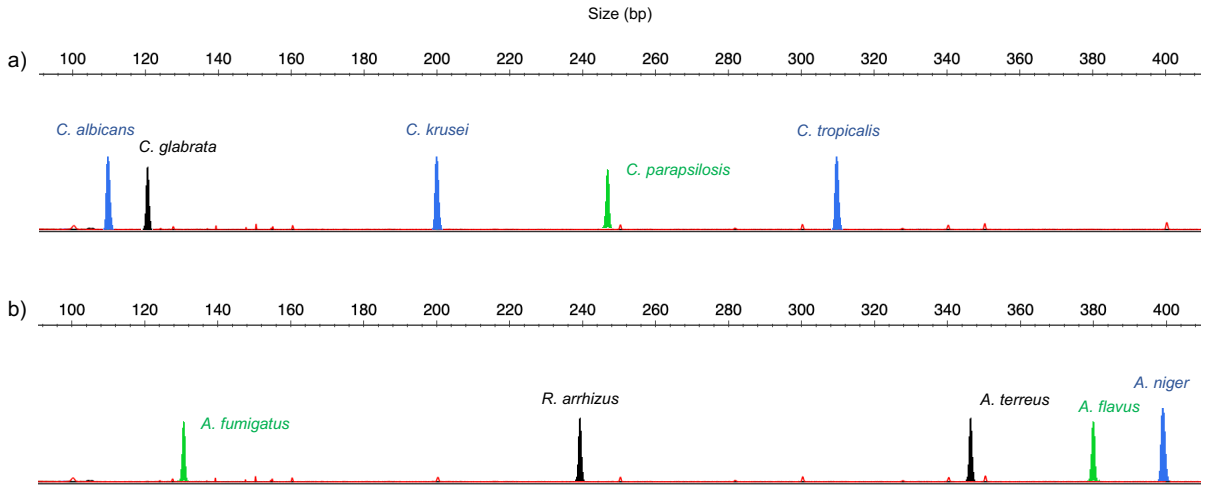


Figure 3.1 Schematic representation of (a) *Candida Panel* and (b) *Filamentous Fungi Panel*, combining the molecular weight of the PCR products with different dyes (FAM=blue, HEX=green, NED=black).

All primers were first tested in singleplex PCR, using different strains to confirm their specificity. The amplification was successful for all markers, and the fragment sizes were within the expected size (Figure 3.2).

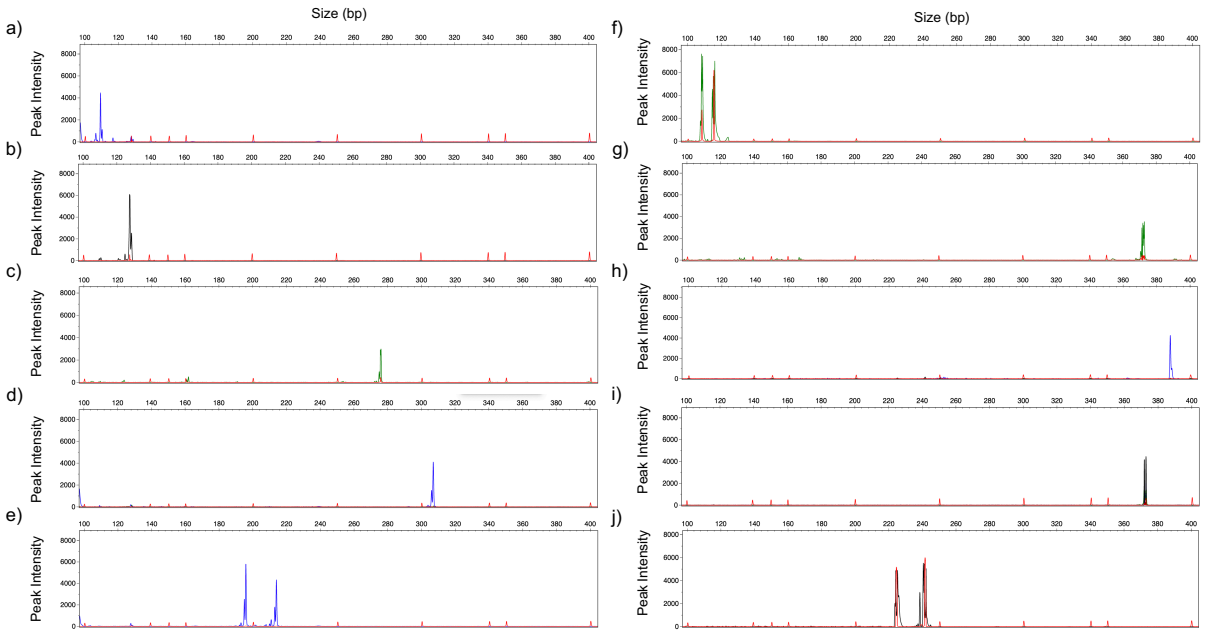


Figure 3.2 Representative GeneScan profiles obtained by singleplex analysis with 2 ng of DNA from (a) *C. albicans* (b) *C. glabrata*, (c) *C. parapsilosis*, (d) *C. tropicalis*, (e) *C. krusei*, (f) *A. fumigatus*, (g) *A. flavus*, (h) *A. niger*, (i) *A. terreus* and (j) *R. arrhizus.*

These markers presented 100% of specificity since no cross-amplification products were obtained when primers and PCR conditions, were used to amplify other species (Table 3.1). The specificity regarding phylogenetical related or frequently miss-identified species was noteworthy (Figure 3.3).

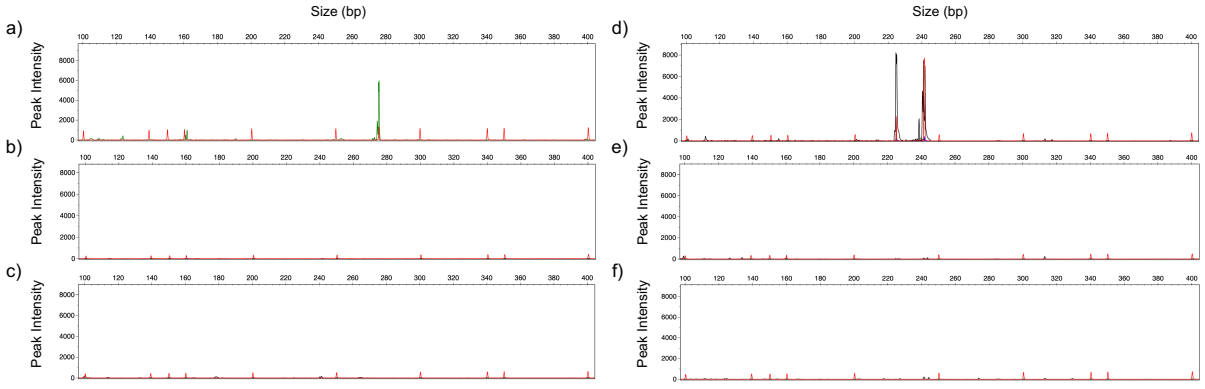


Figure 3.3 Representative GeneScan profiles obtained by singleplex analysis with 2 ng of DNA of (a) *C. parapsilosis*, and the phylogenetically related species (b) *C. orthopsilosis* and (c) *C. metapsilosis*; and (d) *R. arrhizus*, and the second most frequently isolated mucorales (e) *R. microsporus* and (f) *L. corymbifera.*

Table 3.1 List of species tested and results obtained by singleplex analysis. All strains were tested with all primer pairs, and numbers represent the strains with positive peak with the respective primer pair. Ref. and GeneBank Access indicates the bibliographic reference or GeneBank accession number were the strain was identified, – means absence of amplification.

Species	Total of isolates tested	No. of isolates amplified with each primer pair:											Ref. and GenBank Access. Nº
		CA	CP	CG	CT	CK	AF	AFL	AN	AT	RA	Absence of amplification	
C. albicans	21	21	-	-	-	-	-	-	-	-	-	-	(23)
C. parapsilosis	14	-	14	-	-	-	-	-	-	-	-	-	(25)
C. glabrata	20	-	-	20	-	-	-	-	-	-	-	-	(29)
C. tropicalis	7	-	-	-	7	-	-	-	-	-	-	-	(28)
C. krusei	5	-	-	-	-	5	-	-	-	-	-	-	(28)
C. bracarensis	4	-	-	-	-	-	-	-	-	-	-	4	(28,30)
C. metapsilosis	5	-	-	-	-	-	-	-	-	-	-	5	(28,31)
C. orthopsilosis	5	-	-	-	-	-	-	-	-	-	-	5	(28)
C. guiliermondii	1	-	-	-	-	-	-	-	-	-	-	1	(32)
C. lusitaniae	1	-	-	-	-	-	-	-	-	-	-	1	(28)
L. elongisporus	1	-	-	-	-	-	-	-	-	-	-	1	(28)
A. fumigatus	2	-	-	-	-	-	2	-	-	-	-	-	(26)
A.flavus	8	-	-	-	-	-	-	8	-	-	-	-	(33,34) KX897149 KX897152 KX897155
A. terreus	4	-	-	-	-	-	-	-	-	4	-	-	(33,34)
A. niger	6	-	-	-	-	-	-	-	6	-	-	-	(33,34) KX897144 KX897146
A. versicolor	3	-	-	-	-	-	-	-	-	-	-	3	(33) KX897142 KX897154
A.unguis	1	-	-	-	-	-	-	-	-	-	-	1	KX897143
A. westerdijkiae	1	-	-	-	-	-	-	-	-	-	-	1	KX897151
A. tamarii	1	-	-	-	-	-	-	-	-	-	-	1	KX897153
A. wentii	1	-	-	-	-	-	-	-	-	-	-	1	KX897147
A. nidulans	3	-	-	-	-	-	-	-	-	-	-	3	KX897148 KX897156 KX897150
A. tubingensis	1	-	-	-	-	-	-	-	-	-	-	1	KX897145
R. arrhizus	3	-	-	-	-	-	-	-	-	-	3	-	-
R. microsporus	1	-	-	-	-	-	-	-	-	-	-	1	-
L. corymbifera	1	-	-	-	-	-	-	-	-	-	-	1	-
Mucor sp.	1	-	-	-	-	-	-	-	-	-	-	1	-
TOTAL	**121**	21	14	20	7	5	2	8	6	4		28	-

3.4.2 Optimization of Multiplex Amplification Conditions

Amplification conditions for multiplex PCR were then optimized and results compared with the ones obtained in the singleplex analyses. Different concentrations of $MgCl_2$ (from 1.5 to 2.5 mM), primers concentration (from 0.11 to 0.25 µM of each primer) and annealing temperatures (from 55 °C to 64 °C) were tested. Results showed that in multiplex, $MgCl_2$ 2.0 mM promote the best amplification profile, but no significant differences were observed in primers concentrations (data not shown). All annealing temperatures ensured the presence of the expected PCR products, however, at 60 °C a balance between the peak intensity of the expected fragment and reduced unspecific peaks was observed (Figure 3.4).

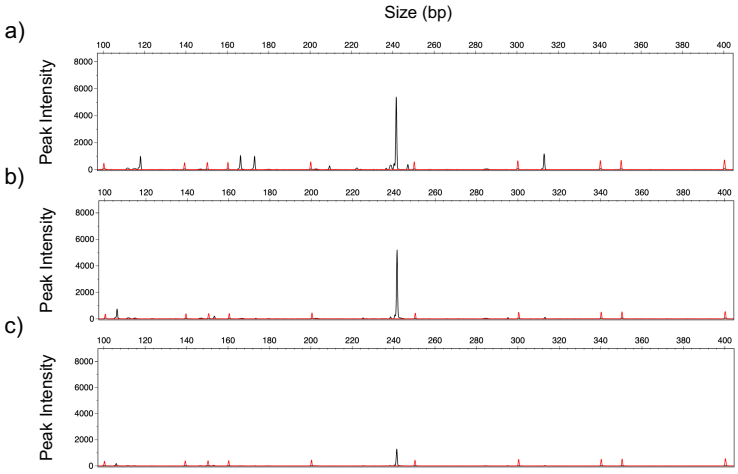


Figure 3.4 GeneScan profiles obtained by multiplex analysis using *Filamentous Fungi Panel*, of 200 pg DNA of *R. arrhizus* using annealing temperatures of (a) 55 °C, (b) 60 °C and (c) 64 °C.

PCR profile of the same strains analyzed in Figure 3.2, were obtained using 2 mM $MgCl_2$, 0.2 µM of each primer, and annealing temperature of 60 °C (Figure 3.5). Comparing both panels, some differences were observed regarding the intensity of the peaks and the presence/absence of a second PCR fragment (allele). These differences do not compromise the correct identification of the different species.

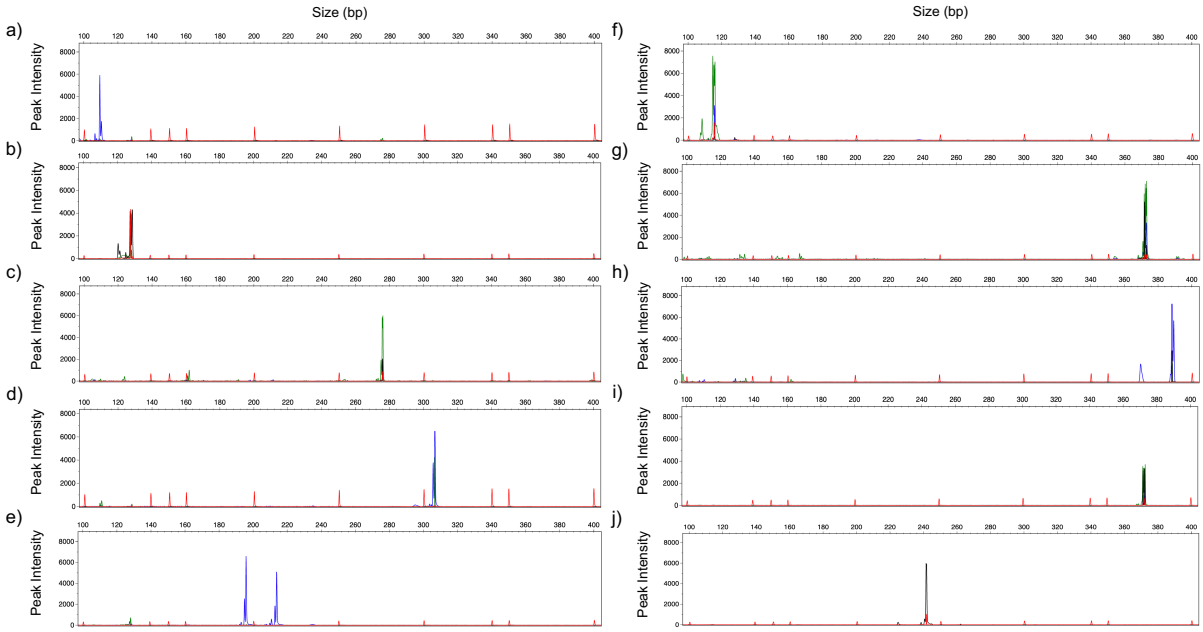


Figure 3.5 Representative GeneScan profiles obtained by multiplex analysis with 2 ng of DNA using *Candida Panel* of (a) *C. albicans* (b) *C. glabrata*, (c) *C. parapsilosis*, (d) *C. tropicalis* and (e) *C. krusei*, and *Filamentous Fungi Panel* of (f) *A. fumigatus*, (g) *A. flavus*, (h) *A. niger*, (i) *A. terreus* and (j) *R. arrhizus*.

These same multiplex PCR conditions were also tested using a protocol for colony PCR [29], showing similar results (data not shown).

These results indicate that the multiplex PCR panels could be used for rapid identification of positive blood-cultures, either by using DNA extracted from the positive fungal cultures or directly using the fungal cells in the PCR.

3.4.3 Limit of Detection of Methodology

In clinical practice, low amounts of fungal DNA are present in biological samples. So, the number of PCR amplification cycles, namely 35, 40, 45 and 50, was tested to enhance the sensitivity of the methodology, using serial dilutions of fungal DNA (from 10 ng to 1 pg). Table 3.2 present the results observed for all species tested. All species amplified very well with high concentrations of DNA, 1 ng, at all PCR cycles.

Table 3.2 Results obtained with 35, 40, 45 and 50 amplifications cycles using different concentrations of DNA of all ten species, with the respective panels. – means absence of amplification, +++ high, ++ medium and + low intensity.

***Candida* Panel**						**Filamentous Fungi Panel**					
Species	**DNA conc.**	**Amplification cycles**				**Species**	**DNA conc.**	**Amplification cycles**			
		35	**40**	**45**	**50**			**35**	**40**	**45**	**50**
C. albicans	1 ng	+++	+++	+++	+++	*A. fumigatus*	1 ng	+++	+++	+++	+++
	100 pg	++	++	++	++		100 pg	++	++	+++	+++
	10 pg	-	+	++	++		10 pg	-	+	++	++
	1 pg	-	-	+	+		1 pg	-	+	+	+
C. glabrata	1 ng	+++	+++	+++	+++	*A. flavus*	1 ng	+++	+++	+++	+++
	100 pg	+++	+++	+++	+++		100 pg	+++	+++	+++	+++
	10 pg	-	++	+++	+++		10 pg	-	+	+	+
	1 pg	-	+	++	++		1 pg	-	-	-	-
C. parapsilosis	1 ng	++	++	++	++	*A. niger*	1 ng	+++	+++	+++	+++
	100 pg	++	++	++	++		100 pg	+++	+++	+++	+++
	10 pg	-	+	+	+		10 pg	-	+	++	++
	1 pg	-	-	-	-		1 pg	-	-	+	+
C. tropicalis	1 ng	++	++	++	++	*A. terreus*	1 ng	+++	+++	+++	+++
	100 pg	++	++	++	++		100 pg	++	+++	+++	+++
	10 pg	-	+	+	+		10 pg	-	-	+	+
	1 pg	-	-	-	-		1 pg	-	-	-	-
C. krusei	1 ng	+++	+++	+++	+++	*R. arrhizus*	1 ng	+++	+++	+++	+++
	100 pg	+++	+++	+++	+++		100 pg	+++	+++	+++	+++
	10 pg	-	+	++	++		10 pg	-	+	++	++
	1 pg	-	+	++	++		1 pg	-	-	-	-

However, with the lowest DNA concentration tested, 1 pg, not all species amplified and those that amplified needed 40 or more PCR cycles. With 10 pg of fungal DNA amplification was observed with 45 and 50 amplification cycles, and since no significant differences were observed, amplifications with 45 cycles were selected.

Figure 3.6 presents amplification profiles using 100 pg to 1 pg of DNA from *C. glabrata* and *A. flavus*, using the corresponding panels. No amplification peaks were observed in electropherogram analysis with the *Candida Panel* or with the *Filamentous Fungi Panel* when both Panes were tested with human DNA (Figure 3.6).

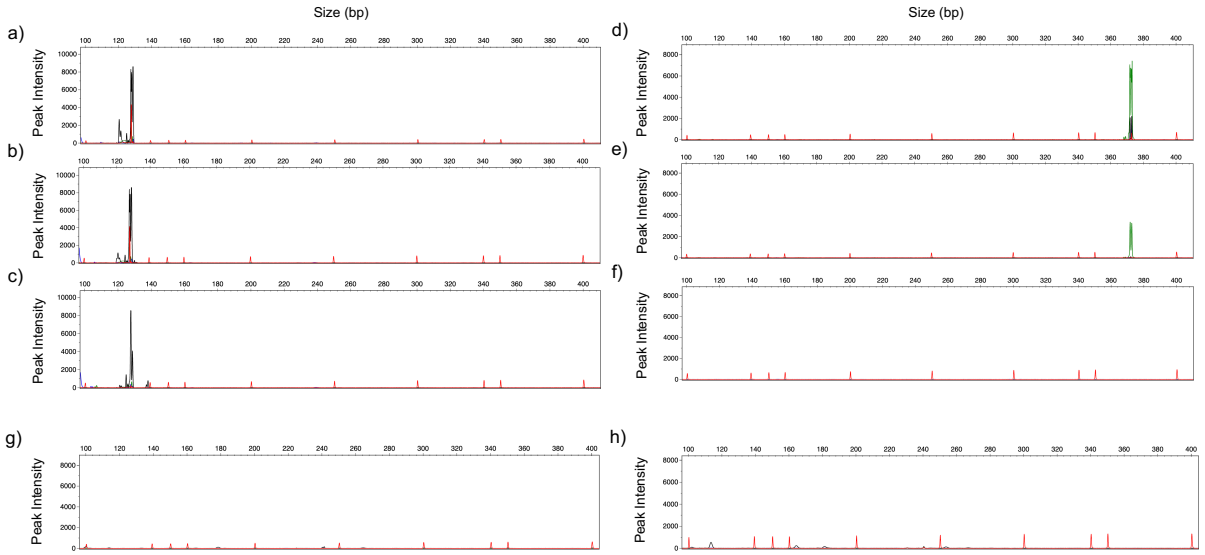


Figure 3.6 GeneScan profiles obtained by multiplex analysis testing (a) 100 pg, (b) 10 pg and (c) 1 pg of DNA of *C. glabrata* with *Candida Panel,* and (d) 100 pg, (e) 10 pg and (f) 1p g of DNA of *A. flavus* with *Filamentous Fungi Panel*. (g) and (h) represent the multiplex profile with 486 ng of commercial Human DNA, with *Candida* and *Filamentous Fungi Panels*, respectively.

3.4.4 Identification of Mixed Fungal DNA

The ability of the multiplex strategy to identify the different species in mixed infections was also evaluated. Two combinations, DNA of *C. albicans* and *C. tropicalis,* and DNA of *A. fumigatus* and *C. niger,* were deliverable mixed in the same tube and multiplex PCR using the respective fungal Panels was performed as previously. In both combinations, results identified two amplification profiles, consistent with the two different species mixed (Figure 3.7).

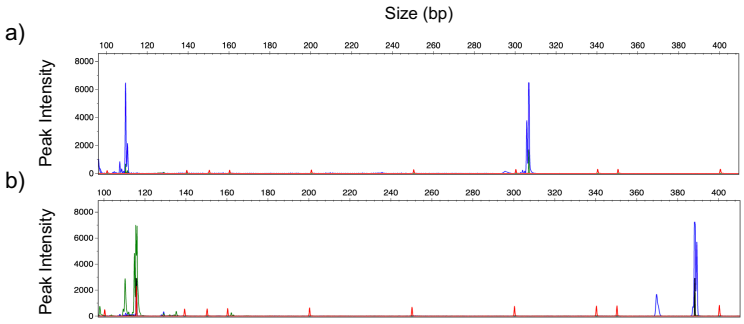


Figure 3.7 GeneScan profiles obtained by multiplex analysis of 100 pg of mixed gDNA of (a) *C. albicans* and *C. tropicalis* using *Candida* Panel and (b) *A. fumigatus* and *A. niger* using *Filamentous Fungi Panel*.

3.4.5 Human Serum Spiked with Fungal DNA

To mimic clinical samples, serum from healthy donors was spiked with 5 ng, 500 pg and 50 pg of fungal DNA (*A. fumigatus*, *A. terreus*, *C. albicans*, *C. krusei* or *R. arrhizus)*, and analysed with the corresponding multiplex Panels. The yield of extraction was approximately 76.5%, so the DNA final concentrations tested ranged between 1 ng and 10 pg of fungal DNA. Results obtained showed the presence of the expected peak in electropherogram, indicating the correct species identification. Figure 3.8 shows the amplification profile of serum spiked with 500 pg of fungal DNA (final DNA concentration of 100 pg).

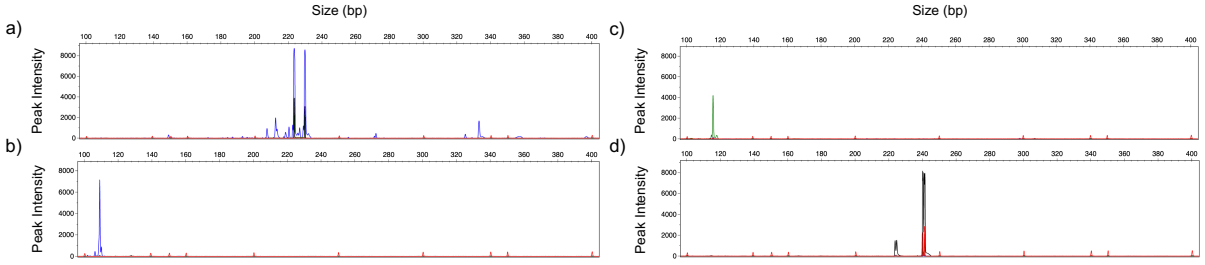


Figure 3.8 GeneScan profiles obtained by multiplex analysis of DNA extracted from serum spiked with 500 pg (final DNA concentration of 100 pg) for (a) *C. krusei* and (b) *C.* albicans using *Candida Panel*, and for (d) A. *fumigatus* and (e) *R. arrhizus* using *Filamentous Fungi Panel*.

3.4.6 Clinical Samples

To determine if the methodology can detect fungal DNA from clinical samples of IFI patients, total DNA was extracted from 29 clinical samples, 19 positive samples and ten negative controls. Samples were obtained from biopsies, bronchoalveolar lavages, cerebrospinal fluid, and vitreous humor. A total of, were tested using the methodology (Table 3.3).

Table 3.3 Clinical samples tested in this study with the correspondent multiplex Panels. Their origin, previous identification by quantitative PCR (qPCR) and results obtained using both panels are indicated. – means absence of amplification.

Samples		EORTC/MSGERC Classification	Previous identification (qPCR)	Multiplex PCR results	
Code	Origin			***Candida* Panel**	***Filamentous Fungi* Panel**
P16-345	Vitreous humour	Probable	*C. albicans*	*C. albicans*	-
P16-126	Cerebrospinal fluid	Probable	*C. albicans*	*C. albicans*	-
P15-31	Bronchoaspirate	Probable	*A. fumigatus*	-	-
P15-239	Liver biopsy	Probable	*C. parapsilosis*	*C. parapsilosis*	-
P15-29	Liver biopsy	Probable	*C. albicans*	*C. albicans*	-
P14-293	Bronchoalveolar lavage	Probable	*C. albicans*	*C. albicans*	-
P14-92	Cerebrospinal fluid	Proven	*C. albicans*	*C. albicans*	-
P13-177	Biopsy	Proven	*C. albicans*	*C. albicans*	-
P13-166	Brain biopsy	Probable	*A. fumigatus*	-	*A. fumigatus*
P13-16	Bronchoalveolar lavage	Probable	*A. fumigatus*	-	-
P16-42	Bronchoalveolar lavage	Probable	*C. krusei*	*C. krusei*	-
P15-8	Biopsy	Probable	*C. tropicalis*	*C. tropicalis*	-
P15-43	Biopsy	Probable	*C. glabrata*	-	-
P15-228	Bronchoalveolar lavage	Probable	*C. parapsilosis*	-	-
KOE 007-1	Bronchoalveolar lavage	NA	*A. fumigatus*	-	Inconclusive
M107.1	Bronchoalveolar lavage	NA	*A. fumigatus*	-	Inconclusive
M119.6	Bronchoalveolar lavage	NA	*A. fumigatus*	-	Inconclusive
M254.9	Bronchoalveolar lavage	NA	*A. fumigatus*	-	Inconclusive
M259.5	Bronchoalveolar lavage	NA	*A. fumigatus*	-	Inconclusive
Controls					
P18-68	Pulmonary biopsy	NA	*C. guilliermondii*	-	-
P15-318	LCR	NA	*T. asahii*	-	-
P18-115	Biopsy	NA	*L. corymbifera*	-	-
P16-316	Intestinal biopsy	NA	*Rhizopus* spp.	-	-
P18/143	Bronchoalveolar lavage	NA	*P. jiroveccii*	-	-
P18-137	Bronchoalveolar lavage	Not classified	Neg. controls	-	-
P18-130	Bronchoaspirate	Not classified	Neg. controls	-	-
P18-55	Pulmonary biopsy	Not classified	Neg. controls	-	-
P18-20	Whole blood	Not classified	Neg. controls	-	-
P18-29	Hepatic biopsy	Not classified	Neg. controls	-	-

NA – information not available

Results obtained indicated no amplification profile with all ten samples from patients without fungal infections while from the 19 clinical samples from patients with *Candida* or *Aspergillus* infections ten

samples confirmed the respective infection. Figure 3.9 shows the amplification profile of DNA extracted from samples of patient n° 5 and n° 9, with infections due to *C. albicans* and *A. fumigatus,* respectively.

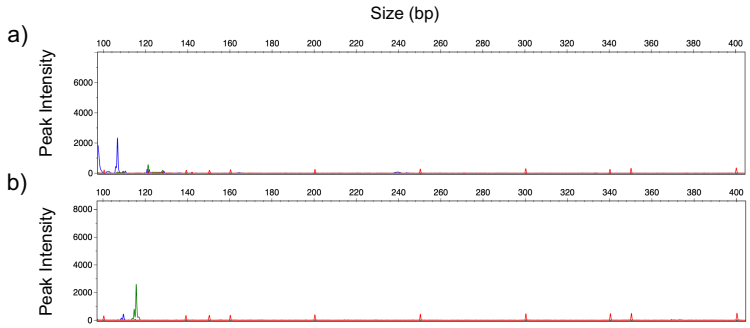


Figure 3.9 GeneScan profiles obtained by multiplex analysis of clinical samples identified as (a) *C. albicans* and (b) *A. fumigatus*, using *Candida* and *Filamentous Fungi Panels* respectively.

However, some problems with samples from bronchoalveolar lavages and bronchoaspirates were observed. From those 10 clinical samples, 5 did not amplified at all, 3 were classified as inconclusive and 2 amplifies as expected. In the inconclusive samples the expected PCR peak was present however, the high background could hinder interpretation (Figure 3.10).

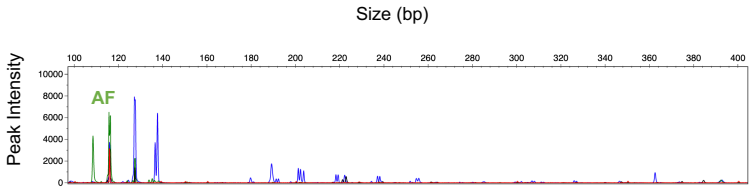


Figure 3.10 GeneScan profiles obtained by multiplex analysis of BAL sample classified as inconclusive. **AF** represents the expected peak, according to qPCR results, for the identification of *A. fumigatus.*

Although the number of clinical samples is reduced, we calculated the sensitivity and specificity of the methodology. Considering all clinical samples, the methodology presented 52% of sensitivity and 100% specificity. However, if samples we consider only samples from sterile places, the sensitivity was 89%. Considering the different Panels, the overall sensitivity for *Candida Panel* was 82% and specificity 100% but considering samples from sterile places the sensitivity was 88% and specificity 100%. These values were not calculated for the *Filamentous Fungi Panel* because the majority of the samples were from bronchoalveolar lavages and bronchoaspirates, which were the problematic samples.

3.5 DISCUSSION

The rapid and accurate identification of pathogens responsible for invasive fungal infections is of extreme relevance for a prompt diagnosis and selection of a correct therapeutic strategy. The identification of these species remains challenging since the conventional methodologies, such as biopsy and culture methods are time-consuming and present low sensitivity and specificity. A way of improving the clinical utility and performance of current fungal diagnostics is by interpreting the results of two individual tests used in conjunction, such as combining molecular and antigen testing results. The approach applied most frequently is the combination of GM testing and Aspergillus polymerase chain reaction (PCR) diagnostics [32].

In this study, we design a new PCR-based methodology that can also be used in combination with other diagnostic tests. The main advantages of our methodology, apart from all of the PCR based methods, are the ability to identify the ten most frequently isolated fungal species, to identify species in mixed infections, and the easy of interpreting results. This last characteristic is due to the possibility of the user to effectively see the peak in the corresponding panel position (molecular weight) with the corresponding color (fluorescent dye). Another important characteristic of this methodology is the possibility of customization. New markers can be included in the multiplex panels, according to the epidemiology of the specific geographic region.

This work presents a proof-of-concept, demonstrating how this methodology could be designed and applied. The DNA fragment sizes are crucial in planning the multiplex strategy since differences in the size of the alleles amplified at each of the selected loci must be combined with different fluorescent dyes to make possible the simultaneous amplification. Also, all the primer pairs in the multiplex PCR should enable similar amplification efficiencies for their respective target. Thus, in this work, primers were carefully selected and designed to compose the desired panels. This design implicated the knowledge of the expected fragment sizes and the combination of different fluorescent dyes when the expected sizes were similar (Figure 3.1). The windows of the designed panels are not saturated, and other markers (new species or resistant genes) can be included.

The methodology was tested in singleplex and in multiplex PCR conditions, and the results compared. We observed that in multiplex, the intensity of some peaks was lower and the presence a second allele was in some samples was absent. These differences could be due to a lower PCR efficiency in multiplex, but the identifications were still clear.

The specificity, reproducibility and sensitivity of this multiplex assay were evaluated first in analytical tests with DNA from different fungal species. Results showed that the proposed method detected correctly all ten targeted pathogens and exhibited no cross-reactivity with nontargeted species. The sensitivity reached 10 to 1 pg of DNA, and results were reproducible in all replicates (between 10-20) when DNA concentration was equal to or higher than 10 pg of DNA. The fact that this methodology can distinguish the different pathogens in mixed infections is a clear advantage over other assays. Also, the possibility of the user to see the peak in the specific region of the panel, with the correct fluorescence dye, is a unique characteristic of this assay that aids in rolling-out unspecific amplifications.

The ability of the methodology to correctly identify the fungal species in human serum spiked with fungal DNA, without interference with human DNA, was a promising indication that it could be tested with clinical samples. Results from the analysis with clinical samples showed that the method is not suitable for bronchoalveolar samples, but is suitable for clinical samples from sterile places, such as from biopsies, cerebrospinal fluid, and vitreous humor. The sensibility observed with this samples was 89% and the specificity was 100%. Although this analysis allowed us to determine the type of suitable samples, we must test a higher number of clinical samples, to include all ten species of the Panels the number and other types of samples.

This proof-of-concept showed the potential of this multiplex PCR methodology to be used for the identification of pathogenic species. We developed panels suitable for the identification of the ten most important fungal species involved in IFI. But similar panels can be developed, not only for fungal species, but also for other clinically important microbial pathogens and genes of interest. If markers are well designed, the methodology shows high stability and capacity to discriminate the different species, even if more than one species is simultaneously present in the sample. Moreover, the methodology developed is easy to perform, the visual discrimination of the amplified peaks helps roll-out unspecific amplifications and can be implemented at relatively low cost for routine identification in microbiological laboratories. If performed correctly, this analysis could be performed directly from the cell biomass of positive blood cultures, without the need for DNA extraction. The methodology can be used for the identification of the ten of fungal species of the panels, either from positive blood cultures or DNA extracted from sterile places of patient with fungal infections.

ACKNOWLEDGMENTS

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CHAPTER 4

High variability within *Candida albicans* transcription factor *RLM1*: Isolates from vulvovaginal infections show a clear bias toward high molecular weight alleles

Adapted from:

High variability within *Candida albicans* transcription factor *RLM1:* Isolates from vulvovaginal infections show a clear bias toward high molecular weight alleles.

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4.1 ABSTRACT

Previous studies have correlated the severity of recurrent vulvovaginal *Candida* infections (VVC) and balanitis in patients from China with the presence of some dominant genotypes at the ORF *RLM1*. Here we tested VVC vs non-VVC isolates from Portugal, Brazil and Greece and, although the same genotypes were identified in VVC isolates, they were present in only five out of 150 strains. However, this analysis showed that VVC isolates presented a higher percentage of genotypes with similar high molecular weight alleles, in comparison with strains isolated from other biological sources.

Keywords: *Candida albicans*, *RLM1*, vulvovaginal candidiasis, alleles, stress resistance.

4.2 INTRODUCTION

Vulvovaginal candidiasis (VVC) is the second most common gynecologic infection and affects over 75% of women. It is known that about 40–50% of these women experience a recurrence, and up to 5% suffer more than four episodes for 1 year [1, 2]. VVC decreases women's life quality, and the high cost of constant medical visits stimulates the use of un-prescribed therapies, which may increase antifungal resistance.

The commensal yeast *Candida albicans* is the most common etiological agent of VVC, being responsible for 70-80% of all cases [3]. The success of this pathogenic yeast depends on their dynamic interactions with the competitive microbiota and with the host that enable it to adjust to a changing environment. Microorganisms developed molecular mechanisms for increasing genetic variations, such as the addition or deletion of repeat units through slipped-strand mispairing or gene conversion, in loci that are involved in critical interaction with the host [4].

The genome of the human pathogen *C. albicans* contains approximately 2600 repeat-containing open reading frames (ORFs), three and 10 times more, respectively, than those of the ascomycete yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [5, 6]. To date, only a few of these genes have been characterized, including *RLM1* that encodes for a transcription factor important in the cell wall integrity (CWI) pathway. Rlm1p presents great variability at its C-terminus, conferred by the CAI microsatellite with around 35 different alleles identified [7, 8]. More than 70% of the CAI alleles identified previously had between 17 and 28 (CAA/G) repetitions, being the most frequent the alleles with 21 and 25 repetitions [7, 8]. Curiously, several studies identified specific CAI genotypes in Chinese isolates from vulvovaginal infections and balanitis that were correlated with severe recurrent infection [9–12]. These genotypes were composed of alleles with 30, 32, 36, 45, 46, and 47 (CAA/G) repetitions, high molecular weight alleles. Phenotypic analysis of strains harboring alleles with higher number of (CAA/G) repetitions, higher than 30, showed that they displayed higher tolerance to combined cell wall and oxidative stress [8] and a high ability to undergo phenotypic switching [13]. This information suggested that these alleles, with higher number of repetitions, may be correlate with the strain capacity to resist and rapidly adjust to this particular niche.

In this context, we addressed the question if these genotypes were also present in *C. albicans* strains from VVC infection from other geographic regions, in comparison with isolated from other biological sources.

4.3 MATERIAL AND METHODS

A total of 329 *C. albicans* strains from Portugal, Greece, and Brazil of different body locations, including 150 strains from vulvovaginal infections and 179 strains from other different sources (79 from the oral cavity, 27 form the urine, 43 from the respiratory tract, and 30 from blood cultures) were genotyped in order to identify the alleles from ORF *RLM1* by amplifying CAI microsatellite. Strain SC5314 (ATCC MYA-2876) was used to correlate genotypes.

Yeast cells were grown at 30 °C for 48 hours on YPD-agar medium and CAI amplification was performed by colony PCR as previously described [14], using the primers reported by Sampaio *et al.* (2003) [7].

Automatic allele size determination was carried out in an ABI310 Genetic Analyzer (Applied Biosystems Inc., Foster City, California), using the GeneScan 3.5 Analysis Software.

Allelic and genotypic frequencies, as well as genetic and genotypic population differentiation tests were performed using GENEPOP (version 4.0.7) [15].

4.4 RESULTS AND DISCUSSION

Genotyping of all 329 *C. albicans* isolates within ORF *RLM1* identified a total of 39 alleles with fragments varying from 183 bp (11 CAA/G repeat units) to 306 bp (52 repeat units), four new alleles than those reported previously. The most frequent CAI genotype was 21–25 (35 strains, 10.6%), followed by 21–22, 21–26, and 25-25 (17 strains, 4.0%). Once more, the most frequent alleles were the ones that presented between 17 and 28 repetitions.

In order to search for the VVCs specific CAI genotypes described by Li *et al.* (2008) [9] in our analysis, we first correlated and adjusted the molecular weight of both alleles from strain SC5314 presented in Li *et al.* (2008) [9] with the GeneScan values obtained in our study for the same strain. This correlation showed that the molecular weights obtained were identical so, all other alleles could also be directly correlated.

Li *et al.* (2008) [9] described that *C. albicans* strains isolated from vulvovaginal candidiasis (VVC) and balanitis showed mainly four CAI genotypes (30-45, 32–46, 30–36, and 30–47), when compared with strains isolated from asymptomatic women. Our analysis showed that only five out of 150 strains, all isolated from VVC, presented the VVCs specific Chinese genotypes, namely two strains with the genotype 30–45, two strains with the genotype 30–47 and one strain with the genotype 32–46. Since this was a very low frequency in comparison with the Chinese studies, we decided to compare the VVC CAI genotypes with the ones obtained in the non-VVC strains. The differentiation tests, concerning allelic/genotypic distribution between the group of VVC isolates and the group of non-VVC isolates showed highly significant differences ($P < 0.0001$), both in the genetic and genotypic distribution. In order to identify these differences, CAI genotype frequencies of VVC versus non-VVC strains were visually compared (Figure 4.1). This analysis showed a clear bias toward high molecular weight alleles in strains from VVCs in comparison with strains from other biological sources. If we consider the frequency of strains presenting genotypes with one allele with 30 or more repetitions (molecular weight of 240 bp or higher) the VVCs group has 46.7%, while the other group has only 20.1% ($\chi^2 = 16.3618$, $P = 0.00052$). Furthermore, if we consider both alleles with 30 or more repetitions a higher difference is observed, the VVCs group having 21.3%, while to the other group belonging only 4.5% of the strains ($\chi^2 = 1.1272$, $P = 0.00851$).

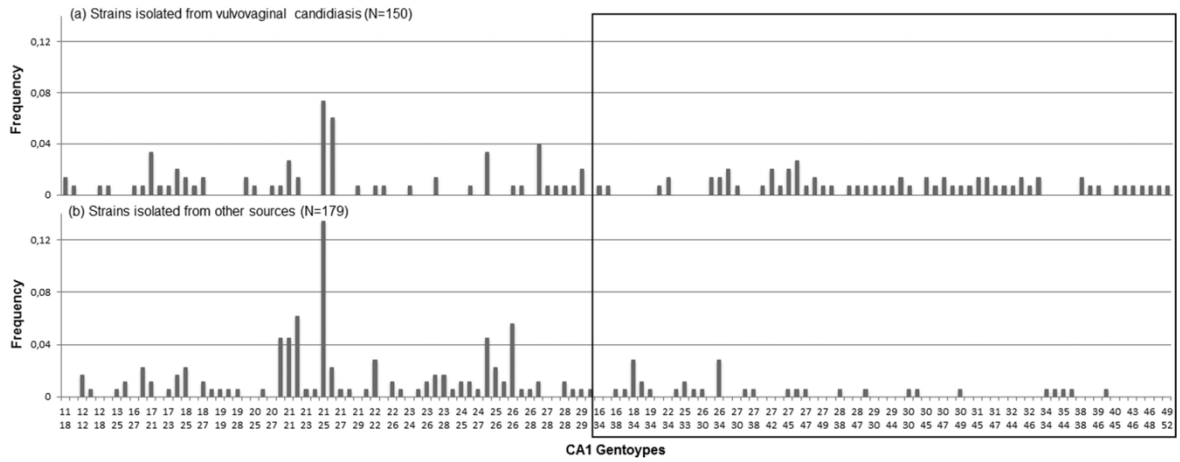


Figure 4.1 Genotypic frequencies based on CAI microsatellite analysis of *C. albicans* strains from (a) vulvovaginal infections and (b) from nonvaginal sources. Highlighted with the square are the genotypes with higher molecular weight.

In the present study we gathered VVC and non-VVC isolates from Brazil, Portugal, and Greece to test for the CAI genotypes that were previously correlated with the severity of vulvovaginal candidiasis and balanitis in the Chinese population. However, those specific genotypes were found only in 3.33% of strains from VVCs. What we observed was that the VVC isolates presented a bias in CAI genotypes with higher molecular weight alleles, such as 46–48, 47-47 or 49–52, similar to the Chinese genotypes [9, 10].

4.5 CONCLUSION

Our previous studies demonstrated a relationship between genotype and phenotype at ORF *RLM1*, in which strains with higher molecular weight alleles presented a higher resistance to combined stress conditions including lower pH and ROS inducing agents [8]. Recently, a correlation between higher white–opaque switching in strains with longer CAA/G repeats in both CAI alleles was also identified [13]. Additionally, we have also demonstrated that *RLM1* is involved in *C. albicans* cell wall biogenesis and its deletion greatly reduce the yeast virulence in the murine model disseminated infection [16]. In this view, and considering that the vagina has a characteristic environment, with a low pH, which is known to induce phenotypic switching, and specific competing microbiota that secretes cell wall damaging factors, the higher molecular weight CAI alleles in ORF *RLM1*, may confer a higher fitness to this environment however, further studies are needed.

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CHAPTER 5

Evaluation of free and DODAB:MO liposome-encapsulated benzo[a]phenoxazine derivatives against the most frequent pathogenic fungal species

Adapted from:

Evaluation of free and DODAB:MO liposome-encapsulated benzo[*a*]phenoxazine derivatives against the most frequent pathogenic fungal species

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5.1 ABSTRACT

Fungal infections represent a major health problem due to their prevalence, increasing number of cases and high complexity of the etiological agents involved. *Candida, Aspergillus* and *Rhizopus* species are the most common and clinically important fungal pathogens being able to cause from less-severe superficial lesions to life-threatening disseminated mycosis.

Effective treatments for fungal infections have some problems due to drug toxicity, pathogen resistance, low drug bioavailability and late diagnosis. At the moment, few groups of antimycotics are available to deal with these infections and the loss of their clinical efficacy creates a potential risk of spreading of resistant strains. This illustrates that the development of new antimicrobials is crucial.

Phenoxazine derivatives, namely benzo[*a*]phenoxazine chlorides, have been shown to present good antifungal properties. Thus, in this work we evaluated the antifungal activity of five benzo(*a*)phenoxazine derivatives, against distinct *Candida* and *Aspergillus* species, and the most promising compound was selected to develop an effective antifungal nanoformulation.

Results demonstrated that all five benzo[*a*]phenoxazines tested presented antifungal activity, with Minimum Inhibitory Concentrations (MICs) between 3.75 to 60 μM. Two of these derivatives, C13 and C34, presented MICs between 3.75-15 μM against all species tested, being selected to develop DODAB:MO formulations.

The liposomal formulations were positively charged, presenting mean sizes between 120-184 nm and polydispersity indexes of 0.3-0.4. The encapsulation efficiency was between 48-70% for both benzo[*a*]phenoxazine derivatives tested. After encapsulation the antifungal activity of C13 and C34 remained similar. Finally, adhesion and filamentation studies were performed, showing the potential of free and encapsulated C34 to reduce these virulence factors.

In conclusion, the results obtained demonstrated that the benzo[*a*]phenoxazine derivative C34 could be a potential antifungal drug due to the great activity against all fungal species tested, including the multidrug resistant *C. auris*.

Key-words: *Aspergillus* spp, benzo[*a*]phenoxazines, *Candida* spp, DODAB:MO liposomes, *Rhizopus* spp.

5.2 INTRODUCTION

The incidence of fungal infections in intensive care units has been increasing over time. Cancer chemotherapy, transplantation or antimicrobial therapies are among the main risk factors for bloodstream infections [1, 2]. *Candida* spp. is considered the third most common cause of bloodstream infections in intensive care units and the fourth leading cause of healthcare-associated bloodstream infections overall, being associated with high morbidity and mortality rates as well as high hospital costs [3, 4].

Although *C. albicans* remains the most common fungal species isolated, a shift in species distribution has been observed in the last two decades [5]. An epidemiologic study performed in Atlanta and Baltimore care units demonstrated that non-*C. albicans* species, specially *C. glabrata, C. parapsilosis* and *C. tropicalis*, now comprise two-thirds of all candidemia's [6, 7]. In Europe, several surveys have been performed, the majority focused on selected clinical groups or European regions and some differences in species prevalence where observed, however, all studies reported the increasing incidence of non-*albicans* species, specially *C. parapsilosis, C. glabrata, C. krusei* and *C. tropicalis* [8, 9]. *Candida auris* has been recently described [10] as a new problem since is multidrug resistant, it is difficult to identify using conventional methods, and has caused several outbreaks in healthcare settings [11, 12]. These trends may be explained by the introduction of prophylactic antifungal agents, before the onset of the infections, promoting the increasing incidence of non-albicans species intrinsically resistant to antifungals [11, 13].

Invasive aspergillosis also represents an important problem in clinical practice, being associated with high fatality rates in immunocompromised patients. Usually, these species are considered susceptible to the newer generation of triazole antifungals, however, resistance has become an emerging clinical challenge [14]. *Aspergillus* species are the most common moulds isolated from human invasive infections, being *A. fumigatus* the most frequently isolated. However, other species such as *A. flavus, A. niger* and *A. terreus* are also frequently isolated depending on clinical groups or geographic region [15–17]. Recently, outbreaks of mucormycosis have been reported in developed countries, being now the fifth cause of invasive fungal diseases [18]. Mucorales have been considered emerging pathogens, with special attention to *Rhizopus arrhizus* [19, 20].

Changes in species incidence influence the treatment recommendations because of differences in susceptibility among these species. Current treatments for fungal infections are limited by the drug toxicity, pathogen resistance and delayed diagnosis. At the moment, only three groups of antimycotics

are commonly used in systemic infections, namely the polyenes (amphotericin B), azoles (fluconazole, itraconazole, isavuconazole and voriconazole) and echinocandins (caspofungin, anidulafungin, and micafungin) [21]. Although fluconazole and echinocandins are the recommended drugs for empirical treatment of fungal infections [22], *C. krusei* or *Aspergillus* species are intrinsically resistant to fluconazole [23, 24], *C. auris* is multi-resistant to several antifungal drugs [25], and *C. glabrata* may acquire resistance to azoles or echinocandins [26]. Several reports demonstrated the loss of clinical efficacy of these drugs, creating a potential risk of spreading of resistant strains, through the emergence of clones with high pathogenic potential [27]. This fact illustrates that the development of new antimicrobials is crucial.

The use of new compounds, alone or associated with different established therapeutic strategies, could be an excellent alternative against fungal pathogens. The benzo[a]phenoxazine dyes are organic fluorophores used for labelling of nucleic acids in blotting experiments, gel electrophoresis, living cells assays, among others. These compounds show antiproliferative properties, being tested as anti-tumour, anti-inflammatory and as antimicrobial agents [28, 29]. Their antiproliferative activity is probably due to the DNA intercalative binding of their polycycles. However, a recent study suggests the perinuclear membrane of endoplasmic reticulum and the enzyme oxidosqualene cyclase (important for sterol biosynthesis) as potential targets [30].

In this work, we evaluated the antifungal activity of five benzo[a]phenoxazine derivatives, against distinct fungal species responsible for invasive and superficial infections and selected the most promising to develop an effective antifungal nanoformulation.

5.3 MATERIAL AND METHODS

5.3.1 Antifungal Activity of Benzo[a]Phenoxazine Derivatives

5.3.1.1 Fungal strains

A total of nineteen isolates of *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. bracarensis, C. auris, A. fumigatus, A. flavus, A. niger, A. terreus* and *R. arrhizus*, were used in this work, including reference strains *C. albicans* SC5314, *C. glabrata* IGC2418, *C. parapsilosis* ATCC22019, *C. krusei* ATCC6258 and *C. bracarensis* NCYC5133. Strains were obtained from different sources and identified using internal transcribed spacer (ITS) sequencing [31]. All these isolates are conserved at the collection of Biology Department of Minho University in 30% glycerol at −80 °C.

Yeast strains were grown at 30 °C for 48 hours on YPD agar medium (Yeast extract 1%, Bactopeptone 1%, Glucose 2% and Agar 2%) and filamentous species on Sabouraud dextrose agar (BDH Prolabo®) for 5 days at 30 °C.

5.3.1.2 Antifungal drugs

Five benzo[a]phenoxazinium chlorides, synthetized in Centre of Chemistry of University of Minho with purity higher than 95%, were tested [30, 32]. Stock solutions were prepared in dimethylsulphoxide (DMSO) (Sigma-Aldrich) to a final concentration of 12 M.

Commercial fluconazole, caspofungin and micafungin solutions were also prepared in DMSO to final concentrations of 25600 µg/mL for fluconazole and 1600 µg/mL for caspofungin and micafungin.

5.3.1.3 Susceptibility tests

In vitro susceptibility of all isolates to benzo[a]phenoxazine derivatives and commercial antifungals was evaluated according to the European Committee on Antimicrobial Susceptibility testing (EUCAST) protocols for yeasts and filamentous fungi, previously described (EUCAST definitive document E.DEF 7.3.1 and EUCAST definitive document E.DEF.9.3.1). Susceptibility to benzo[a]phenoxazine derivatives was assessed testing concentrations ranging from 0.234 to 60 µM. In the case of commercial antifungals, the range tested for fluconazole was 0.125 to 128 µg/mL, and 0.008 to 8 µg/mL for caspofungin and micafungin. All strains were tested in triplicate in three independent assays.

Both EUCAST protocols use RPMI 1640 (with L-glutamine and a pH indicator but without bicarbonate) (Sigma-Aldrich) supplemented with 2% glucose (RPMI 2% G) and buffered to pH 7.0 with 0.165 M 3-(N-morpholino) propanesulfonic acid (MOPS) (Sigma-Aldrich). Serial dilutions of all benzo[a]phenoxazine derivatives and commercial antifungals were performed in supplemented RPMI 1640 medium and added to 96-well plates, to obtain the intended final concentrations. Compounds activity, against fungal cells, was also evaluated at pH 4, 5 and 6.

- *Susceptibility tests in Candida species*

After cultured on YPD agar medium for 24 hours at 30 °C, isolates were suspended on sterile water and cellular density adjusted to 1-3 x 10^6 cell/mL. Concentrations from 0.5-1.5 x 10^5 cell/mL were used. Sterility control and growth control were included in each microplate. A growth control with 2% of DMSO was also included.

After 24h and 48h of incubation at 30 °C the absorbance was measured at 530 nm using a microplate absorbance reader. The Minimum Inhibitory Concentration (MIC) of each compound was considered as the lower drug concentration with growth inhibition of ≥50% after 24 h in comparison with the growth control. EUCAST clinical breakpoints values were used to classify Candida strains as susceptible or resistant to fluconazole and echinocandins.

- *Susceptibility tests in filamentous fungi*

Comparing with the yeast protocol, the filamentous fungi EUCAST protocol present two main variations. First, prior cultures were performed in Sabouraud agar for 2-5 days (or until proper sporulation) at 30 °C and isolates were recovered in 3-5mL of sterile water supplemented with 0.1% Tween 20. Then, conidia were rubbed with a sterile cotton swab and transferred with a pipette to a sterile tube. All inoculum preparations were examined for the presence of hyphae or clumps (<5%). After conidia counting, the suspension was adjusted to 1-3 x 10^6 CFU/mL in sterile water. A final concentration of 0.5-1.5 x 10^5 CFU/mL was used.

MIC lectures were determined visually after 24 h of growth for *R. arrhizus* and after 48 h for *Aspergillus* species. For all benzo[a]phenoxazine derivatives and fluconazole the concentration yielding no visible visual growth was considered the MIC value. For echinocandins, the Minimum Effective Concentration

(MED) was considered the lowest concentration in which abnormal hyphal clusters are observed comparing with growth control.

5.3.2 Preparation and Characterization of Nanoformulations

5.3.2.1 Materials

Dioctadecyldimethylammonium bromide (DODAB) (Tokyo Kasei) and 1-monooleoyl-rac-glycerol (MO) (Sigma-Aldrich) were used for liposome production. Ethanol (high spectral purity) was purchased from Usavol.

5.3.2.2 Preparation of liposomes by lipid-film hydration method

The most active benzo[a]phenoxazine derivatives were selected and encapsulated in DODAB: MO based liposomes prepared by film hydration method. In this protocol, the MO molar fractions tested (χMO) were 0.66 (DODAB: MO molar ratio 1:2) and 0.8 (DODAB: MO molar ratio 1:4). Briefly, DODAB (20 mM) and MO (20 mM) were dissolved in ethanol, vortexed in a round-bottom tube, and the organic solvent removed by rotary evaporation at 60 °C (safely above the lipids phase transition temperature). The liposomal film was prepared at 4 mM total lipid concentration. Then, the lipid film was hydrated with solutions of benzo[a]phenoxazine derivatives at 60 °C, to obtain final concentrations of 23.4 μM, 46.9 μM and 93.8 μM of derivatives and final lipid concentration of 2 mM (888 μg/mL). Finally, the dispersions were sonicated in a bath sonicator for 6 minutes. Empty liposomes were produced and used at 2 mM with ultrapure water with 2% DMSO.

5.3.2.3 Size, polydispersity index and ζ -potential

The ζ-potential and the mean size were determined for all formulations by dynamic light scattering (DLS) at 25 °C with a Malvern ZetaSizer Nano ZS particle analyzer. The Malvern Dispersion Technology Software (DTS) was used with multiple narrow mode (high resolution) data processing, and mean size (nm), polydispersity index (PDI) and error values determined. The surface charge of liposomes was measured indirectly, by ζ-potential analysis, using electrophoretic light scattering (ELS) at 25 °C. DTS with monomodal mode data processing was used to determine average ζ-potential (mV) and error values. All characterizations were performed in quintupled.

5.3.2.4 Quantification of encapsulation efficiency

- *Electronic absorption spectra and standard curve*

To determine the maximum absorption wavelength (λmax) of the benzo[a]phenoxazine derivatives tested (in ultrapure water) an electronic absorption spectrum was measured from 250-2500 nm. Then, the wavelength of maximum absorption was selected to prepare a calibration curve using concentrations between 0.4 to 100 μM.

- *Quantification of encapsulation percentage*

All formulations prepared were pelleted by ultracentrifugation (100 000 g during 1 hour at 4 °C) and the supernatants removed. The concentration of non-encapsulate derivative, in the supernatant, was determined by spectrophotometry using the standard curve previously determined.

5.3.2.5 Antifungal activity of nanoformulations

The activity of all nanoformulations against *Candida, Aspergillus* and *Rhizopus* species was evaluated, by using the same strains described above. The protocols used were also based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocols for yeasts and filamentous fungi, previously described (EUCAST definitive document E.DEF 7.3.1 and EUCAST definitive document E.DEF.9.3.1).

The final concentrations of the encapsulated compounds in the test microplates were 1.88, 3.75 and 7.5 μM, and 71 μg/mL of lipids. Empty liposomes and free compounds were also tested, as controls. These formulations were tested against fungal strains, in triplicate in three independent assays. The activity of liposomes against fungal cell was also evaluated, at pH 4, 5 and 6.

5.3.2.5 Evaluation of nanoformulations effect on *Candida* species filamentation

Candida strains were incubated for 24h with the encapsulated and free C13 and C34, as described above, and morphologic characteristics of yeast cells analysed in the optic microscope. The size of filaments was measured to assess the influence of compounds in filamentation.

5.3.2.6 Evaluation of nanoformulations effect on yeast cells adhesion

To evaluate the effect of nanoformulations in yeast cells adhesion, culture plates were prepared as described above. After 48 hours, the absorbance was measured at 530 nm using a microplate absorbance reader. Then, medium was carefully removed, and wells washed twice with PBS 1X. Finally, 200 µL of RPMI 1640 was added, and absorbance was measured again. The percentage of adhesion was calculated by comparing absorbance before and after wash.

5.3.3 Statistical Analysis

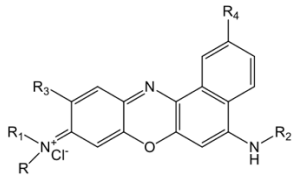
All data were statistically analysed, by GraphPad Prism (5.0 version, GraphPad Software, USA). Comparative analysis of different groups was performed, by using analysis of variance (ANOVA) followed by the Bonferroni test. Unless otherwise stated, results shown are from at least three independent experiments with three replicates. In all cases, <0.05 values were considered statistically significant.

5.4 RESULTS AND DISCUSSION

5.4.1 Antifungal Activity of five Benzo[a]Phenoxazine Compounds

Previous work performed in our group tested the activity of more than forty benzophenoxazinium[a]chlorides against *Saccharomyces cerevisiae* PYCC 4072 [29, 30, 32]. From the compounds with the highest antifungal activity, five were selected for the present study (Table 5.1).

Table 5.1 Benzophenoxazinium[a]chlorides used in this study, molecular structure and MICs against *S. cerevisiae* PYCC 4072.



	R	R_1	R_2	R_3	R_4	MIC (μM)	Ref.
BaP1 (1a)	H	CH_2CH_3	$(CH_2)_3CO_2CH_2CH_3$	CH_3	H	25	[33, 34]
A11	H	CH_2CH_3	$(CH_2)_{19}CH_3$	CH_3	H	6,25	-
C11	H	CH_2CH_3	$(CH_2)_2CH_3$	CH_3	H	6.25	[30]
C13	H	CH_2CH_3	$(CH_2)_3Cl$	CH_3	H	3.75	[30]
C34	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	H	H	1.56	[30, 32]

The compounds were tested against nineteen strains from *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. bracarensis, C. auris, A. fumigatus, A. flavus, A. niger, A. terreus* and *R. arrhizus*. The minimum inhibitory concentration (MIC) values for the different compounds against all species/strains tested ranged between 3.75 and 60 μM at pH 7.0 (Table 5.2).

When testing *Candida* species, BaP1 presented the highest MICs (15-30 μM) and MIC geometric mean (24 μM). C11 also showed high MIC geometric mean (21 μM) and high MIC variability among species, varying between 7.5 μM for *C. tropicalis* and C. *krusei* to 60 μM for *C. parapsilosis*. These results demonstrate that these two compounds are not the best alternative for the development of an antifungal strategy. In contrast, A11 presented high antifungal activity against fungal cells, with MIC geometric mean of 12.75 μM however, the high hydrophobicity of this compound presented a challenge in the assays.

Table 5.2 Minimum Inhibitory Concentrations (MICs) of benzo[*a*]phenoxazinium chlorides and commercial antifungals against fungal species after 24 hours of growth.

	MIC (μM)					MIC (μg/mL)		
Isolates	**BaP1**	**A11**	**C11**	**C13**	**C34**	**Flu**	**Casp**	**Mic**
***C. albicans* SC 5314**	30	15	30	15	15	0.125	0.125	0.016
***C. albicans* SER 7**	30	15	30	15	15	>128	0.03	0.008
***C. glabrata* IGC 2418**	15	15	15	15	7.5	1	0.125	0.016
***C. glabrata* 182**	15	15	15	7.5	15	4	0.06	0.008
***C. parapsilosis* ATCC 22019**	30	15	30	7.5	7.5	1	0.5	1
***C. parapsilosis* 160a**	30	15	60	15	7.5	0.5	0.125	0.5
***C. tropicalis* 2D**	30	15	7.5	7.5	7.5	0.125	0.06	0.03
***C. tropicalis* 45**	30	15	7.5	7.5	7.5	0.5	0.25	0.016
***C. krusei* ATCC 6258**	15	3.75	7.5	3.75	3.75	16	0.25	0.06
***C. krusei* H11**	15	3.75	7.5	3.75	3.75	32	0.5	0.125
***C. bracarensis* 153 MT**	-	-	-	15	15	-	-	-
***C. bracarensis* NCYC 5133**	-	-	-	15	7.5	4	0.5	0.26
***C. auris* 17-274**	-	-	-	7.5	7.5	>128	0.125	0.25
***C. auris* 17-270**	-	-	-	7.5	7.5	>128	0.125	0.25
***A. fumigatus* VA125**	-	-	-	15*	7.5*	-	-	-
***A. flavus* VA26**	-	-	-	15*	15*	-	-	-
***A. niger* VA91**	-	-	-	7.5*	3.75*	-	-	-
***A. terreus* VA39**	-	-	-	7.5*	7.5*	-	-	-
***R. arrhizus* MII**	-	-	-	15	7.5	-	-	-
Mode	30	15	7.5	15	7.5			
Range MIC	15-30	3.75-15	7.5-60	3.75-15	3.75-15			
MIC GM	24	12.75	21	10.7	8.9			

- Isolates not tested; GM geometric mean; * MIC determination after 48 hours;

As observed in *S. cerevisiae* PYCC 4072, the benzo[*a*]phenoxazine derivatives C13 and C34 presented the lowest MIC ranges (3.75-15 μM) and the lowest MIC GM (10.7 and 8.9 respectively). *C. albicans* was the specie with the highest MICs, 15 μM, for both compounds, whereas fluconazole-resistant *C. krusei*,

presented the lowest MIC value 3.75 μM. Moreover, these two compounds also demonstrated activity against other strains resistant to fluconazole, such as *C. albicans* SER7 and *C. auris* 17-274.

The treatment of vaginal infections may be challenging due to the normal acidic vaginal pH (4 to 4.5), which remains acidic during infection. *In vitro* susceptibility studies showed that pH can alter the MICs of azoles and amphotericin B for *Candida* species, whereas echinocandins and caspofungin presented the same activity at low pHs [35, 36]. To determine the effect of acidic pH on these compounds, their activity at pH 4, 5 and 6 was tested. The MICs obtained for all compounds using all acidic mediums were ≥30, showing that acidic pH affects antifungal activity of these compounds, increasing their MICs (data not shown).

The effect of pH in the antifungal activity of benzo[*a*]phenoxazine derivatives at low pHs remains unknown. However, this result indicates that the uses of these compounds as antifungals in acidic environments, such as in vaginal infections, is limited.

Due to the better antifungal performance of C13 and C34, both were selected, to following studies.

5.4.2 Preparation and Characterization of Liposomes with Different DODAB:MO Molar Ratios

5.4.2.1 Preparation of DODAB:MO liposomes

Liposomes have been studied as drug delivery systems to overcome limitations of several pharmaceutical compounds. Formulations confer protection to active compounds, increase the permeation rate of drugs, direct to the desired target site, and may reduce drugs toxicity [35, 36]. AmBisome or Ampholip are examples of liposome formulations with Amphotericin B approved for the treatment of invasive fungal infections, which reduces the nephrotoxicity of this compound [37]. In cases of low efficiency of non-encapsulated compounds, liposomes can concentrate effective dose of drug locally, enhancing its efficiency. The functionalization of liposomes, by directing the drug to a specific local, can further aid the antifungal local therapeutic action [36].

Our research group has previously used N,N-dioleyl-N,Ndimethylammonium bromide (DODAB) liposomes toiled with Monoolein (MO) for encapsulation of *C. albicans* proteins as an antigen delivery system [38]. Previous studies also demonstrated that the presence of MO in DODAB: MO formulations was advantageous for drug delivery purposes, since MO can enhance solubilization of the encapsulated

molecules [39, 40]. Moreover, MO also acts as stabilizer, conferring fluidity to liposomes by favoring lipid chain mobility, important to improve the fusion of liposomes with cell membranes [41].

In this study, the same lipidic vehicle was used to encapsulate benzo[a]phenoxazinium chloride derivatives. Due to the importance of the content of MO in liposomes stability and internalization efficiency, different DODAB: MO lipid ratios (1:4 and 1:2) were tested. The physicochemical properties of empty DODAB:MO liposomes at molar ratios 1:2 and 1:4, and formulations with 93.8 μM of C13 and C34 are presented in Figure 5.1.

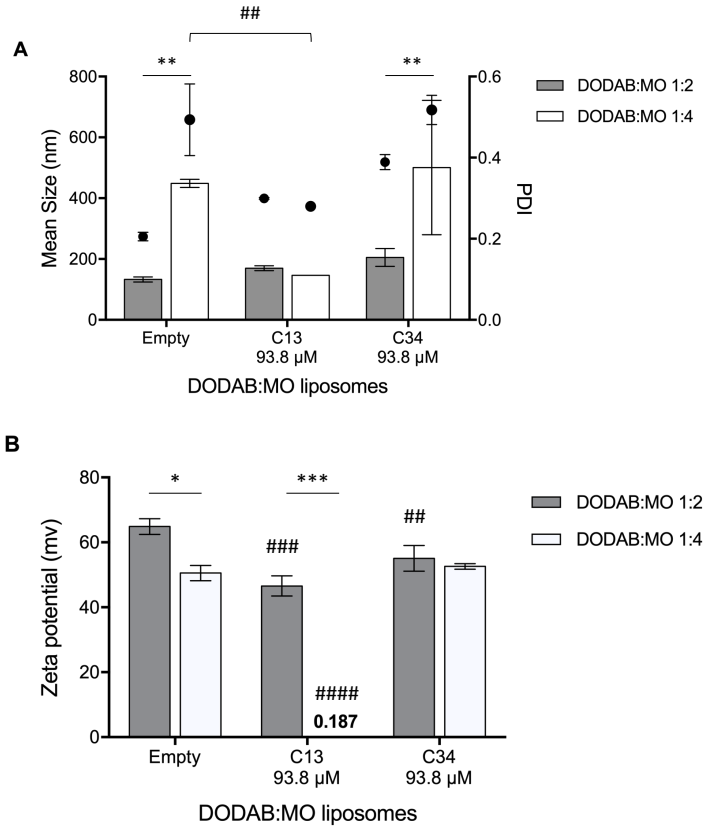


Figure 5.1 Size (A bars), PDI (A spherical symbols) and ζ-potential (B bars) of nanoparticles prepared with 93.8 μM of C13 and C34 and with two different χMO. Results indicate the mean ± SD of independent assays. Significant differences between (1:2) and (1:4) liposomes are represented by: *P<0.05 and ***P<0.001. Significant differences between Empty liposomes and liposomes loaded with C13 or C34 are represented by: ##P<0.01, ###P<0.001 and ####P<0.0001.

The empty DODAB:MO (1:2) liposomes presented mean size (132.8 ± 8 nm) and polydispersity index (PDI) (0.21 ± 0.01) significantly smaller than DODAB:MO (1:4) (448.7 ± 13 nm and PDI 0.49 ± 0.09). The high surface area of DODAB:MO (1:4) liposomes was explained by the fact that MO, due to its natural

negative curvature, is preferentially located in the interior of the lipidic structures [42]. As expected, both formulations presented a high net positive charge (> +50 mV) however, DODAB:MO (1:4) superficial charge was significantly low (Figure 5.1). The lower surface charge of DODAB:MO (1:4) liposomes is directly related to its higher mean size.

Analyzing characteristics of the liposomes loaded with C13 and C34, the mean size and PDI values of both liposomes were similar for DODAB:MO (1:2), but for DODAB:MO (1:4), liposomes with C34 presented significantly higher mean size (500.89 ± 220 nm) and PDI values (0.52 ± 0.02). The zeta potential was positively charged, for all liposomes, except for DODAB:MO (1:4) with C13 (+0.2 mV). This formulation aggregated hours after preparation.

These results indicated that liposomes with DODAB:MO lipid ratio of 1:2 were more homogeneous and stable.

5.4.2.2 Determination of encapsulation efficiency

Electronic absorption spectra of 1 x 10^{-6} M solutions of C13 and C34 compounds in deionized water were measured. The wavelengths of maximum absorption (λmax) of C13 and C34 were 620 nm and 645 nm, respectively (Figure 5.2). After determination of λmax a calibration curve for each compound was constructed.

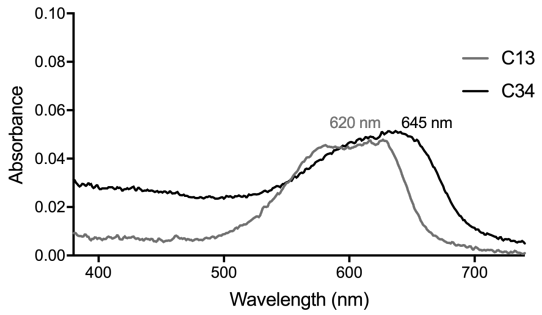


Figure 5.2 Normalized absorbance spectra of compounds C13 and C34, in deionized water.

The encapsulation efficiency of the four formulations was determined by measuring the free compound in the supernatants after ultracentrifugation, by spectrophotometry (Table 5.3). The encapsulation efficiency of C34 was higher than C13 in both DODAB:MO ratios 1:2 and 1:4 liposomal formulations, but

differences were not significant (P >0.05). Although higher content of the neutral MO could help encapsulation, since benzo[a]phenoxazinium chloride derivatives are positively charged, this was not observed.

Table 5.3 Encapsulation efficiency of C13 and C34 93.8 μM in formulations with different DODAB:MO ratios.

Encapsulation (%)				
Compound	**DODAB:MO (1:2) 2mM**		**DODAB:MO (1:4) 2mM**	
	Mean	**SD**	**Mean**	**SD**
C13	63.3	3	64.4	0.1
C34	71.8	2	70.4	0.9

Charged liposomes present several advantages compared with neutral liposomes, since the surface charge induces electrostatic repulsion among liposomes, inhibiting liposomes aggregation or flocculation, and also promote interaction with the fungal cell. Moreover, homogeneous (PDI <0.4) small liposomes shows prolonged blood circulation, allowing better distribution into different organs, whereas bigger liposomes are usually rapidly eliminated from circulation [43]. In this study, liposomes with DODAB:MO molar ratio (1:2) were positively charged and presented smaller mean sizes and PDI with no significant differences in encapsulation efficiency thus, they were selected for antifungal activity tests.

5.4.3 Influence of Benzo[a]Phenoxazine derivatives C13 and C34 Concentration in Liposomes Properties

Three concentrations of C13 and C34 were used, to produce nanoformulations with DODAB:MO (1:2), namely 23.4, 46.9 and 93.8 μM. These liposomes were prepared as such, so that the final concentrations with fungal cells were within the MICs values, previously determined in the susceptibility tests, 1.88, 3.75 and 7.5 μM. The main size, PDI and zeta potential of the new formulations are represented in Figure 5.3.

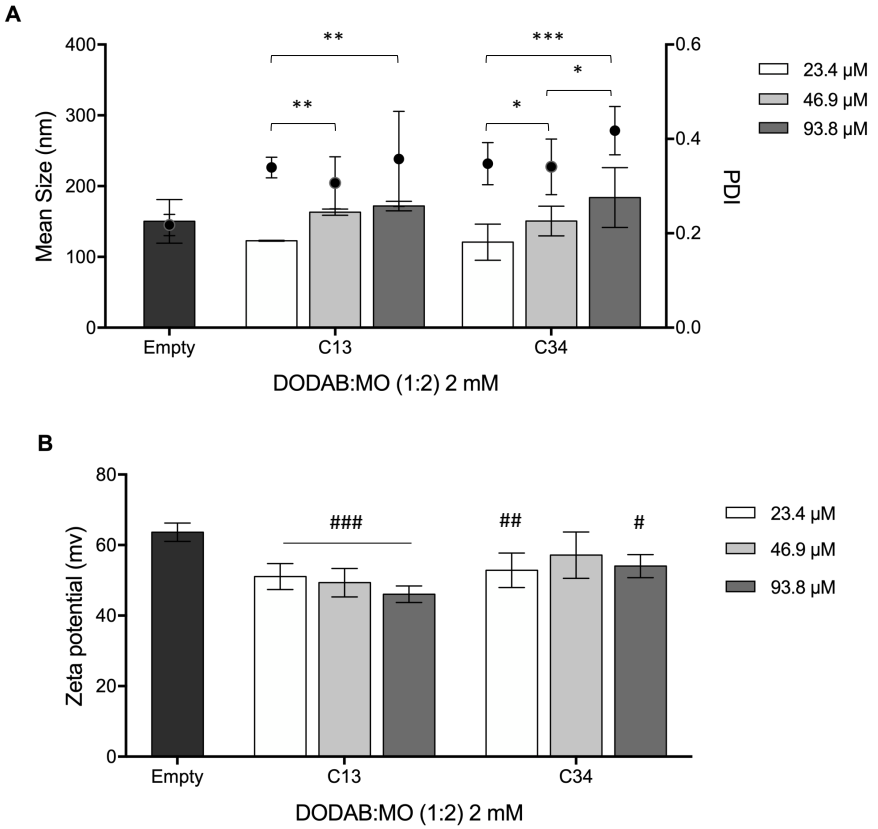


Figure 5.3 Size (A bars), PDI (A spherical symbols) and ζ-potential (B bars) of DODAB:MO (1:2) nanoparticles prepared with 23.4, 46.9 and 93.8 µM of C13 and C34. Results indicate the mean ± SD of independent assays. Significant differences between mean sizes of liposomes with different C13 or C34 concentrations are represented by: *P<0.05, **P<0.01 and ***P<0.001. Significant differences between empty liposomes and liposomes loaded with C13 or C34 are represented by: #P<0.05, ##P<0.01 and ###P<0.001.

As expected, DODAB:MO (1:2) empty liposomes presented a small mean size (150.8 ± 30.5 nm) and PDI (0.22 ± 0.02), and are positively charged (+64 ± 3 mV). Regarding nanoformulations with C13 and C34, all liposomes also presented small mean size and PDI values, similar to empty liposomes. Increasing concentration of the compound slightly increased the mean size of liposomes, but displayed similar PDI values (≤0.4), showing some homogeneity in liposomes sizes. As expected, formulations were positively charged (between +46 and +57 mV), but the superficial charge was slightly lower than empty liposomes, except for liposomes loaded with 46.9 µM of C34. The differences in superficial charge between liposomes loaded with compound and empty liposomes were probably due to the liposome's rearrangement after encapsulation since benzo[*a*]phenoxazine compounds are slightly lipophilic and were probably located in the lipidic bilayer. This can also be evident in the slight increase of liposome mean size with increasing concentrations of the compounds.

The stability of these liposomes was evaluated, by measuring the mean size, PDI and superficial charge of all liposomes after 1, 2 and 3 weeks after production. No significant differences were observed (P >0.05) in these characteristics, showing that these liposomes are stable after 3 weeks of production (data not shown).

Table 5.4 presents the results of the encapsulation percentage of the six formulations. Results indicate that the encapsulation percentage increased with increasing concentrations of C13 and C34. C34 93.8 μM presented the highest encapsulation percentage (70.4%); while C34 23.4 μM the lowest, 48.1%.

Table 5.4 Encapsulation efficiency of different concentrations of C13 and C34 in DODAB:MO (1:2) 2 mM liposomes.

Liposome DODAB:MO (1:2) 2mM	**Encapsulation (%)**	**SD**
C13 23.4 μM	-	-
C13 46.9 μM	58.0	4.2
C13 93.8 μM	62.1	3.1
C34 23.4 μM	48.1	0.9
C34 46.9 μM	50.6	3.5
C34 93.8 μM	70.4	1.7

- Not tested

5.4.4 Antifungal Activity of Liposome Formulations of Benzo[*a*]Phenoxazine derivatives

As described above, liposomes have been studied as drug delivery systems to overcome some limitations, including to increase the therapeutic index of drugs [36]. To evaluate the of antifungal activity of C13 and C34 after liposome encapsulation all formulations were tested by modification of EUCAST methodology. The activity of empty liposomes (71 μg/mL) and free compounds was also tested.

According to EUCAST methodology, if a compound induces more than 50% of growth inhibition, comparing with growth control, the strain is considered susceptible. Figures 5.4A and 5.4B represent the percentage of inhibition of all formulations tested against seven *Candida* species.

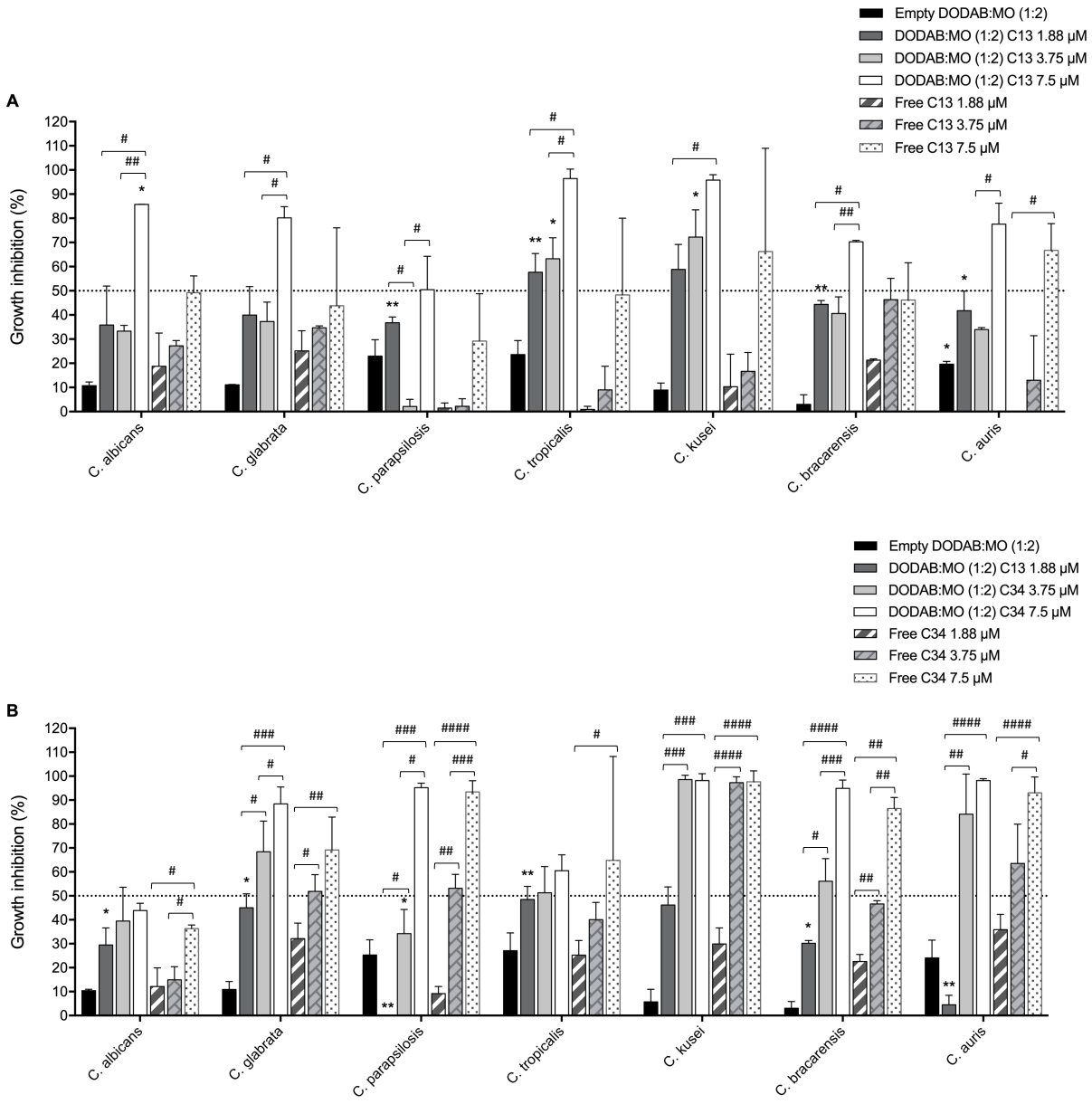


Figure 5.4 Percentage of inhibition of three concentrations of (A) C13 and (B) C34 formulations and free compounds against seven *Candida* species. All values shown are the average values from three independent experiments ± SD. Significant differences between free and encapsulated compound, at the same concentration, are represented by: *P <0.05, **P <0.01. Significant differences between the different concentrations of the compounds are represented by: #P <0.05, ##P <0.01, ###P <0.001 and ####P <0.0001.

Growth inhibition of empty liposomes DODAB:MO (1:2) (71 µg/mL) varied between 3% (*C. bracarensis*) and 23% (*C. tropicalis*), showing that lipids did not present activity against any of the strains tested (inhibition <50%). Overall, increasing concentrations of the compound, either free or encapsulated increased growth inhibition in all strains tested, except for *C. albicans* and *C. tropicalis* (P <0.05).

In previous studies, *S. cerevisiae* PYCC 4072 presented lower MIC values with C34 free compound in comparison with C13 [30, 32]. Similarly, in this study, C34 formulations showed to be more effective than C13 formulations against the majority of species. Growth inhibition of more than 50% was observed for all species, except for *C. albicans,* and in 50% (21 out of 42) of the condition tested with C34. On the other hand, C13 induced growth inhibition of more than 50% in only 29% (12 out of 42) of the conditions tested. Curiously, for C13 92% (10 out of 12) of the conditions that induced more than 50% of growth inhibition were with the encapsulated compound, while for C34 only 52% (11 out of 21) accounted for liposomal formulations. These results suggest that the use of DODAB:MO (1:2) as a drug delivery system could increase the antifungal activity C34 and C13, but C13 benefits the most from encapsulation.

Comparing free and encapsulated compound, in the same condition, globally, encapsulation of C13 enhances the growth inhibition in all species. However, due to the high standard deviation significant differences were observed only in a few conditions. The same conclusion can be made with C34, encapsulation also enhances its activity, but in a lower extent. In two cases encapsulation of C34 even decreased its action, in *C. auris* with C34 at 1.88 μM and *C. parapsilosis* with C34 at 3.75 μM. These results are in agreement with the fact that DODAB:MO (1:2) delivery system is more critical for C13 than for C34 activity.

For filamentous fungi the growth inhibition is determined by visual observation, so the percentage of inhibition with C13 and C34 formulations was not possible. The activity of formulations was characterized, according to the presence or absence of fungal growth. C13 and C34 formulations at 1.88 and 3.75 μM were not active against all five species tested, however at 7.5 μM growth inhibition of *A. terreus, A. niger* and *R. arrhizus* was observed. These results are in agreement with the previously obtained MICs for these species and DODAB:MO (1:2) delivery system did not affect C13 or C34 activity against filamentous fungi.

Previously in this work, the antifungal activity of free benzo[a]phenoxazinium chloride derivatives at low pHs was determined and it was observed that acidic pHs reduces their activity. Since liposomal formulations confer protection to active compounds [37], it would be important to evaluate if encapsulation in DODAB:MO liposomes protects antifungal activity of C13 and C34 at low pHs (Tables 5.5 and 5.6).

Table 5.5 Percentage of growth inhibition of three concentrations of C13, encapsulated and free, against seven *Candida* species, at pH 4, 5, 6 and 7. Results significant different (P<0.05) in comparison with growth inhibition (%) at pH 7are presented at **Bold**.

		Growth Inhibition (%)													
	pH	***C. albicans***		***C. glabrata***		***C. parapsilosis***		***C. tropicalis***		***C. krusei***		***C. bracarensis***		***C. auris***	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Empty DODAB:MO liposomes	**4**	**0**	0	**0**	0	**2.9**	3.5	**0**	0	0	0	0	0	**0**	0
	5	**4.8**	4.5	7.0	4.2	**6.3**	3.9	**0**	0	0.8	0.7	8.9	1.9	**0**	0
	6	**0**	0	7.8	5.2	25.7	3.2	**15.9**	3.5	3.6	4.3	0.2	0.8	**7.1**	12.5
	7	10.8	0.6	10.9	3.3	25.3	6.3	27.1	7.4	5.7	5.2	3.1	2.8	24.1	7.4
DODAB:MO C34 1.88 μM	**4**	**0**	0	**0**	0	**0**	0	**0**	0	**10.4**	0	**0**	0	**0**	0
	5	**0**	0	**0**	0	**0**	0	**26.6**	5.8	**27.9**	2.9	**0**	0	**3.3**	12.3
	6	**0**	0	**4.4**	2.0	**16.4**	0	**18.4**	7.8	**29.7**	1.2	**0**	0	**0**	0
	7	35.8	16.1	39.9	11.8	36.8	2.3	57.7	7.8	58.9	10.3	44.4	1.6	41.8	8.1
DODAB:MO C34 3.75 μM	**4**	**0**	0	**0**	0	0	0	**0**	0	**0**	0	**0**	0	**0**	0
	5	**0**	0	**0**	0	0	0	**14.7**	9.4	**0**	0	**0**	0	**1.2**	5.5
	6	**0**	0	**0**	0	0	0	**7.8**	11.0	**0**	0	**0**	0	**3.95**	2.6
	7	33.3	2.4	37.3	8.0	2.1	3.0	63.3	8.7	72.2	11.3	40.6	6.9	34.0	0.8
DODAB:MO C34 7.5 μM	**4**	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**10.8**	2.4
	5	**7.1**	1.4	**9.7**	2.7	**0**	0	**17.8**	12.2	**17.4**	2.4	**11.2**	1.8	**23.3**	14.3
	6	**4.16**	1.9	**8.57**	3.9	**10.4**	1.4	**31.5**	2.4	**30.7**	15.3	**13.0**	2.2	**32.5**	4.9
	7	85.7	0.1	80.2	4.6	50.4	13.9	96.5	3.9	95.8	2.3	70.3	0.6	77.6	8.7
Free C34 1.88 μM	**4**	**0**	0	**0**	0	0	0	0.8	10.5	4.5	0.5	**0**	0	0	0
	5	**2.4**	3.4	**0**	0	0	0	3.5	1.1	10.3	6.2	**2.7**	3.8	0	0
	6	**0.5**	3.9	**11.7**	0.6	0	0	**10.0**	0.8	10.4	3.7	**0**	0	0	0
	7	18.8	13.7	25.2	8.3	1.5	2.1	0.9	1.3	10.3	13.5	21.3	0.5	0	0
Free C34 3.75 μM	**4**	**0**	0	**0**	0	0	0	**0**	0	**2.4**	5.1	**0**	0	**1.3**	0.9
	5	**7.4**	4.7	**0**	0	0	0	3.5	0.7	8.1	3.1	**0**	0	**0**	0
	6	**3.2**	2.8	**0.6**	3.1	0	0	10.5	0.6	6.2	4.7	**0**	0	**0**	0
	7	27.3	2.1	34.7	0.8	2.2	3.1	9.0	9.7	16.7	7.9	46.3	8.8	13.0	18.4
Free C34 7.5 μM	**4**	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**2.48**	4.9
	5	**0**	0	**0**	0	**0**	0	**0.5**	0.5	**9.2**	1.7	**1.9**	2.9	**0**	0
	6	**1.1**	1.8	**7.4**	0.8	**0**	0	**14.5**	0.4	**16.1**	10.6	**1.9**	4.7	**1.9**	3.6
	7	49.2	7.0	43.8	32.3	29.2	19.6	48.3	31.8	66.3	42.7	46.2	15.4	66.7	11.1

Table 5.6 Percentage of growth inhibition of three concentrations of C34, encapsulated and free, against seven *Candida* species, at pH 4, 5, 6 and 7. Results significant different (P<0.05) in comparison with growth inhibition (%) at pH 7are presented at **Bold**.

		Growth Inhibition (%)													
	pH	***C. albicans***		***C. glabrata***		***C. parapsilosis***		***C. tropicalis***		***C. krusei***		***C. bracarensis***		***C. auris***	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Empty DODAB:MO liposomes	**4**	**0**	0	**0**	0	**2.9**	3.5	**0**	0	0	0	0	0	**0**	0
	5	**4.8**	4.5	7.0	4.2	**6.3**	3.9	**0**	0	0.8	0.7	8.9	1.9	**0**	0
	6	**0**	0	7.8	5.2	25.7	3.2	**15.9**	3.5	3.6	4.3	0.2	0.8	**7.1**	12.5
	7	10.8	0.6	10.9	3.3	25.3	6.3	27.1	7.4	5.7	5.2	3.1	2.8	24.1	7.4
DODAB:MO C34 1.88 μM	**4**	**0**	0	**0**	0	0	0	**3.2**	4.5	**0**	0	**0**	0	0	0
	5	**0**	0	**6.9**	1.2	0	0	**28.7**	3.0	**22.8**	3.8	**0**	0	8.4	7.5
	6	**0**	0	**10.2**	0.6	7.4	6.9	**14.3**	0.7	29.0	5.1	**0**	0	2.12	3.4
	7	29.5	7.1	45.1	5.8	0	0	48.4	5.5	46.2	7.6	30.2	1.1	4.5	3.9
DODAB:MO C34 3.75 μM	**4**	**0**	0	**0**	0	**1.0**	1.9	**0**	0	**3.5**	2.4	**0**	0	**5.1**	1.2
	5	**6.2**	6.3	**3.9**	0.4	**0**	0	**28.2**	7.3	**26.4**	5.1	**0.5**	1.8	**3.8**	9.2
	6	**0**	0	**12.8**	1.1	29.2	9.6	**17.4**	3.9	**30.4**	1.8	**0**	0.7	**0**	0
	7	39.5	14.1	68.4	12.8	34.2	10.1	51.3	10.9	98.6	1.8	56.1	9.4	84.1	16.7
DODAB:MO C34 7.5 μM	**4**	**0**	0	**0**	0	**0**	0	**0**	0	**0.9**	3.1	**0**	0	**0**	0
	5	**0**	0	**2.7**	5.4	**20.0**	12.7	**22.7**	9.5	**29.3**	4.1	**7.2**	0.5	**0.8**	11.3
	6	**0**	0	**16.7**	7.5	81.1	5.9	**21.1**	2.1	**54.4**	0.2	**0**	0	**15.1**	8.1
	7	43.9	3.0	88.4	7.1	95.2	1.9	60.5	6.6	98.2	2.9	94.9	3.5	98.1	0.8
Free C34 1.88 μM	**4**	**3.4**	1.2	**0**	0	**0**	0	0	**0**	2.3	3.8	**0**	0	**1.2**	2.2
	5	**4.8**	4.4	**0**	0	**0**	0	4.8	0.7	9.7	**1.4**	**4.3**	0.1	**0**	0
	6	**0.4**	1.5	**10.3**	3.6	**0**	0	10.1	3.1	9.2	2.0	**0**	1.7	**0**	0
	7	12.2	7.7	32.1	6.5	9.2	2.9	25.3	6.1	29.9	6.7	22.5	3.0	35.9	6.4
Free C34 3.75 μM	**4**	**0**	0	**0**	0	**0**	0	**0.9**	0.3	**3.6**	0.4	**0**	0	**0**	0
	5	**1.0**	1.2	**4.1**	0.1	**0**	0	**4.1**	0.4	**7.6**	7.7	**0.3**	3.1	**0**	0
	6	**0**	0	**8.3**	1.4	**0**	0	**10.1**	3.1	**8.2**	8.6	**0**	0	**0**	0
	7	14.9	5.5	51.8	7.0	53.2	5.8	40.1	7.2	97.3	2.5	46.6	1.4	63.6	16.4
Free C34 7.5 μM	**4**	**0**	0	**0**	0	**0**	0	**0**	0	**5.9**	2.3	**0**	0	**0.1**	3.4
	5	**0**	0	**0**	0	**0**	0	**3.3**	1.2	**7.8**	3.9	**0**	0	**0**	0
	6	**3.3**	1.5	**10.3**	3.6	**0**	0	**10.1**	3.1	**9.2**	2.0	**0**	1.7	**0**	0
	7	36.4	1.5	69.1	13.7	93.4	4.7	64.8	43.4	97.7	4.6	86.5	4.6	93.1	6.6

Results obtained indicated that DODAB:MO (1:2) liposomes did not confer protection to C13 or C34 at pH 4, 5 and 6, since growth inhibition reduced drastically to levels of 0% of inhibition. These results confirm the previous observations that a low pHs benzophenoxazinium[a]chlorides derivatives loss their antifungal activity. The fact that decreasing pH leads contributes to DODAB:MO liposomal degradation [39], can explain why not even encapsulation in this liposomal system protected C13 or C34 antifungal activity.

5.4.5 Effect of Benzo[α]Phenoxazine derivative C14 and C34 in *Candida* Filamentation

Some *Candida* species are able to switch from the yeast-form growth to the hyphal growth [44]. The phenotypic switching is one of the most important virulence factors and contributes to disease, playing different functions during infection. Whereas yeast morphology is usually observed in early stages of infection or for dissemination in the bloodstream, the filamentous form is responsible for invasion and damage of tissues and organs [45, 46].

The effect of free and encapsulated C13 and C34 in *Candida* morphology switching was observed by optic microscopy after cell growth in the presence of the two compounds.

C. glabrata, C. bracarensis and *C. auris* are do not present phenotypic switching [47, 48], so they were not analyzed. No differences in phenotypic switching were observed with *C. parapsilosis*, all cells remained in the yeast form, even in the control. However, *C. parapsilosis* ability for hyphal transition is strain-dependent [47]. So, in this study, no conclusion on the effect of C13 and C34 on *C. parapsilosis* hyphal transition can be made. Regarding *C. krusei*, pseudo-hyphae was observed in cells from growth control and incubated with empty liposomes. However, due to high growth inhibition of the formulations and free C13 and C34, no cells were observed at the microscope so, no comparison could be done.

After 24h of incubation, filamentation was observed in the analysis of *C. albicans* and *C. tropicalis* cells, in control growth and incubated with empty liposomes (Figure 5.5 and 5.6).

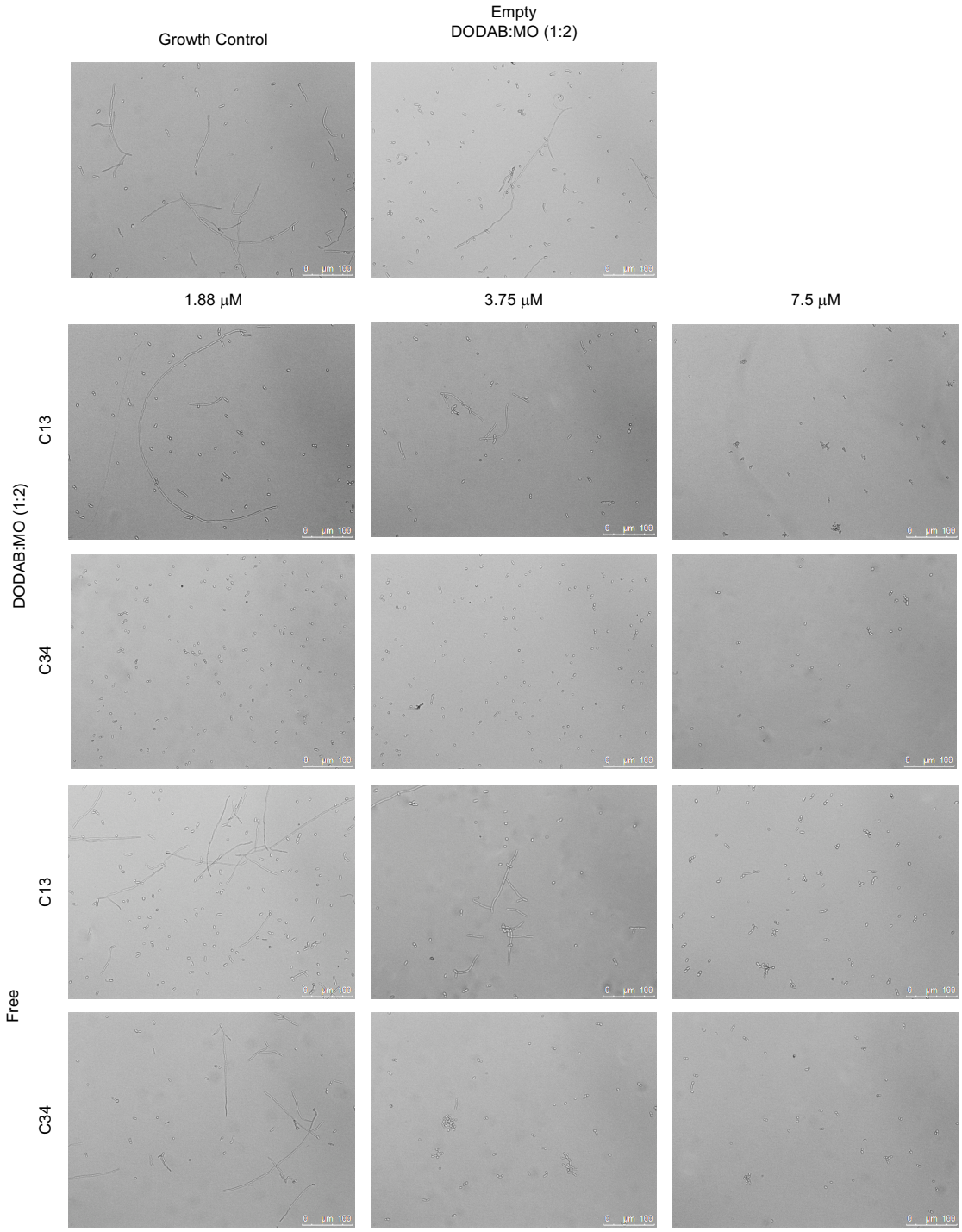


Figure 5.5 Representative microscopy images of *Candida albicans* cells after 24 h incubation in RMPI with empty liposomes (71 μg/mL), C13 and C34 nanoformulations and free C13 and C34, at 1.88, 3.75 and 7.5 μM.

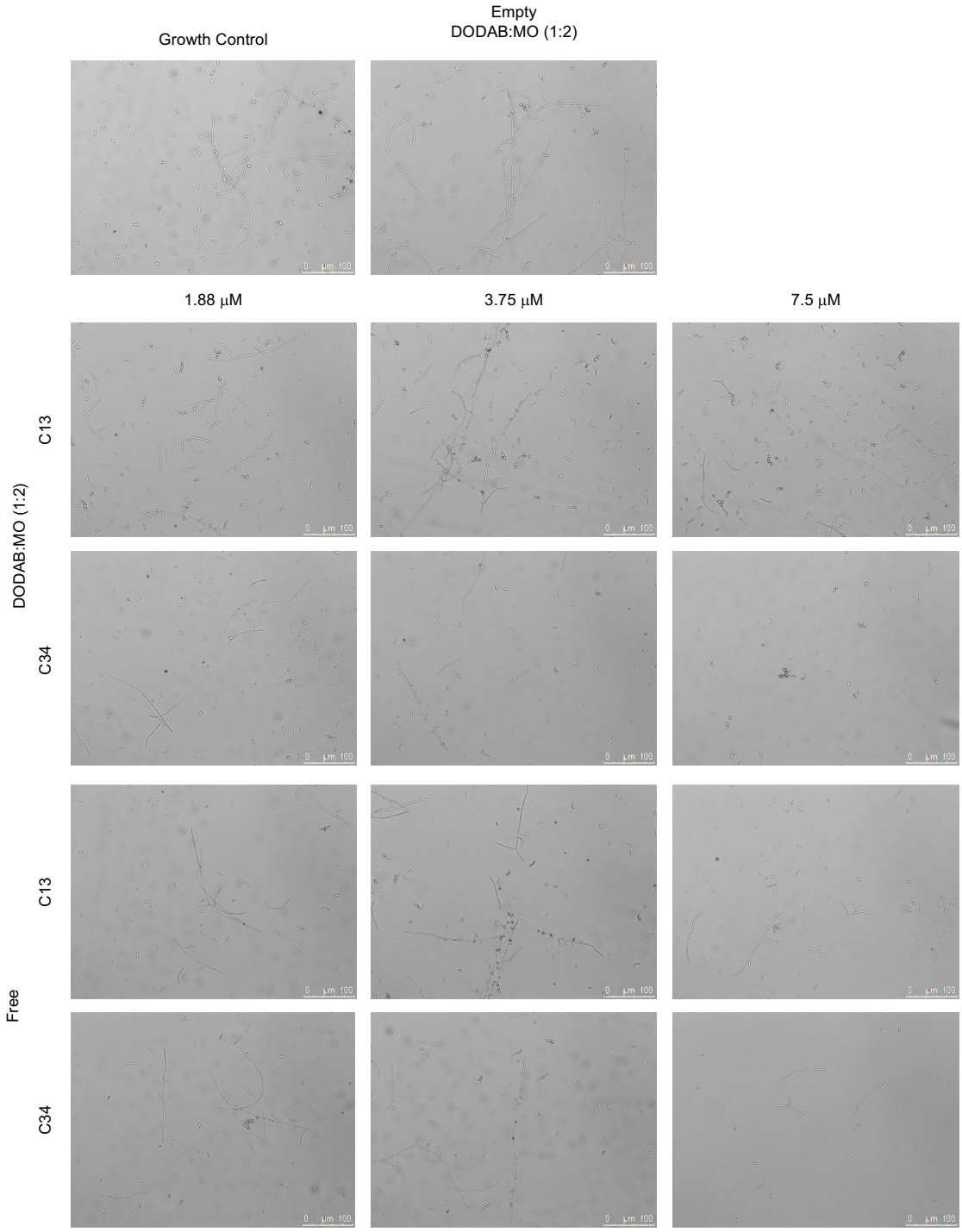


Figure 5.6 Representative microscopy images of *Candida tropicalis* cells after 24 h incubation in RPMI with empty liposomes (71 μg/mL), C13 and C34 nanoformulations and free C13 and C34, at 1.88, 3.75 and 7.5 μM.

In *C. albicans*, after 24h of incubation in RPMI (control), 40% of all cells presented filaments (Figure 5.7). This percentage significantly decrease (P <0.001) in cells incubated with encapsulated or free C34 (Figure 5.7), in a dose-dependent mode. The lower percentage of filaments, less than 10%, was observed in all incubations with 3.75 and 7.5 μM of C34 (Figure 5.7). The percentage of filaments in *C. albicans* cells incubated with C13, encapsulated or free, also decreased significantly in comparison with the control (P <0.001). However, contrary to C34, increasing concentrations of the compound did not increase inhibition of hyphal transition. No significant differences (P >0.05) were observed comparing encapsulated and free compound in all conditions tested for both compounds.

Curiously, incubation with empty liposomes also inhibited *C. albicans* filamentation (P <0.0001) comparing with the control. Thus, differences in the percentage of *C. albicans* filaments, comparing incubations with C13 or C34 with empty liposomes, were only significant at 3.75 and 7.5 μM of C34, in both forms, free and encapsulated.

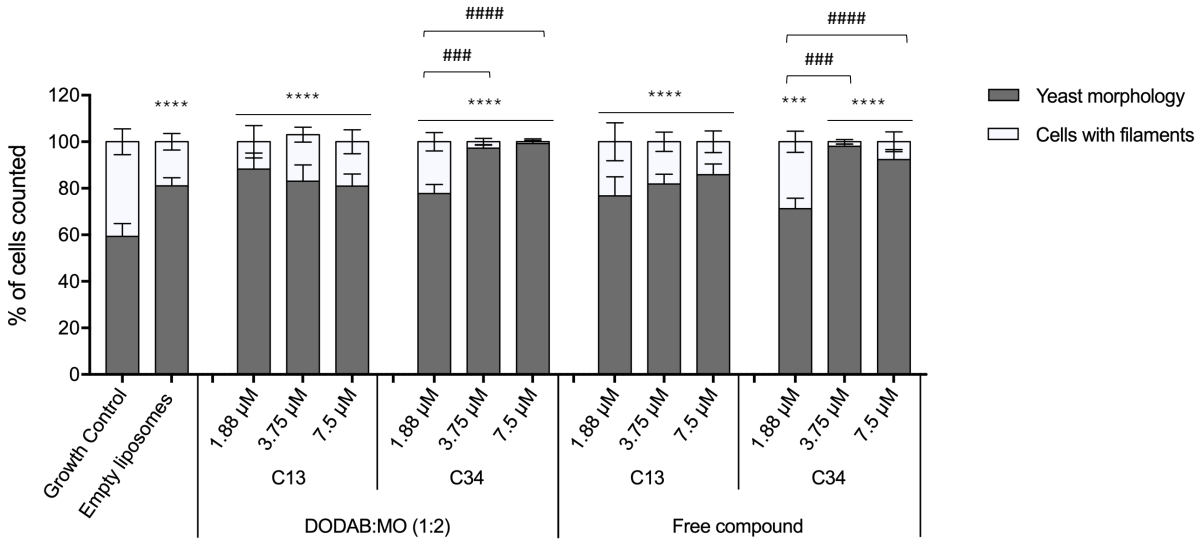


Figure 5.7 Percentage of *C. albicans* cells in yeast phase and in hyphal phase, in different growth conditions, namely the use of different concentrations of free and encapsulated C13 and C34. No less than 300 cells were counted in each condition. Significant differences between the different conditions and the growth control are represented by: ***P <0.001 and ****P <0.0001. Significant differences between different concentrations in each conditions are represented by: ###P <0.001 and ####P <0.0001.

Previous work demonstrated that genetically altered strains, unable to filament, were avirulent in a murine model of *C. albicans* invasive infection [50]. In this work, free and encapsulated C34 at 3.75 and 7.5 μM

did not present antifungal activity against *C. albicans*, however, were able to inhibit yeast filamentation. These results suggest C34 can still be exploited in aiding control the fungal infection progression, if not through inhibition of growth, through inhibition of an important virulence factor, filamentation.

The effect of C13 and C34 in hyphae size was also evaluation (Figure 5.8). This analysis showed that almost 50% of filamented cells from the growth control presented hyphae sizes higher than 100 µm. Comparing the hyphae size of control cells with cells incubated with empty liposomes or with 1.88 µM of free or encapsulated C13 or C34, no significant differences were observed (P>0.05). Increasing concentration of compounds, either free or encapsulated, showed to decrease the size of filaments (P<0.05), except for C34 encapsulated. However, with DODAB:MO C34, the hyphae sizes were already very small. No filaments, with more than 100 µm, were observed in cells incubated with encapsulated or free C34 at 3.75 and 7.5 µM, or C13 at 7.5 µM. These results indicate that these compounds affect both the number and size of filaments.

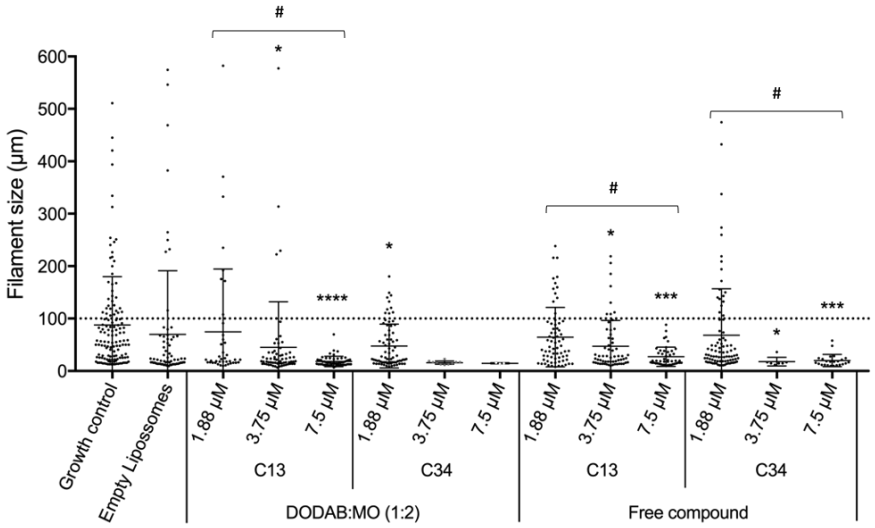


Figure 5.8 *C. albicans* filaments size (µm) after incubation with DODAB:MO encapsulated or free C13 and C34. Each dot represents a filament measured. No less than 300 cells were counted in each condition. Significant differences between the different conditions and growth control are represented by: *P <0.05, **P <0.01 and ***P <0.001. Significant differences between different concentrations, in each condition, are represented by: #P <0.05.

C. albicans hypha, when compared with yeast-like cells, present a different cell wall composition, hyphal cells contain twice as much chitin as the yeast cells do [51]. Malfunction of ER leads to defects in the cell wall biosynthesis and defects in hyphal development [52, 53]. Benzo[*a*]phenoxazines accumulate at the perinuclear membrane of the endoplasmic reticulum (ER), suggested as a potential target [30]. This

capacity may explain the inhibition of filamentation by C34, however, more tests are needed to confirm this mechanism in *C. albicans*.

Figure 5.9 presents results obtained with *C. tropicalis* filamentation tests. However, results indicate no significative differences in phenotypic witching (P >0.05) when *C. tropicalis* control cells were compared with cells incubated with the different compounds, free or encapsulated (Figure 5.9).

Unlike in *C. albicans*, the role of filamentation in *C. tropicalis* pathogenesis is controversial. Jiang *et al.* (2016) [54] suggested that hyphal formation increased *C. tropicalis* ability to invade and damage epithelial cell in murine models. However, recent studies demonstrated that phenotypic switching in *C. tropicalis* and *C. parapsilosis* is associated with reduced pathogenicity and reduced expression of genes involved in pathogenicity-related processes [55].

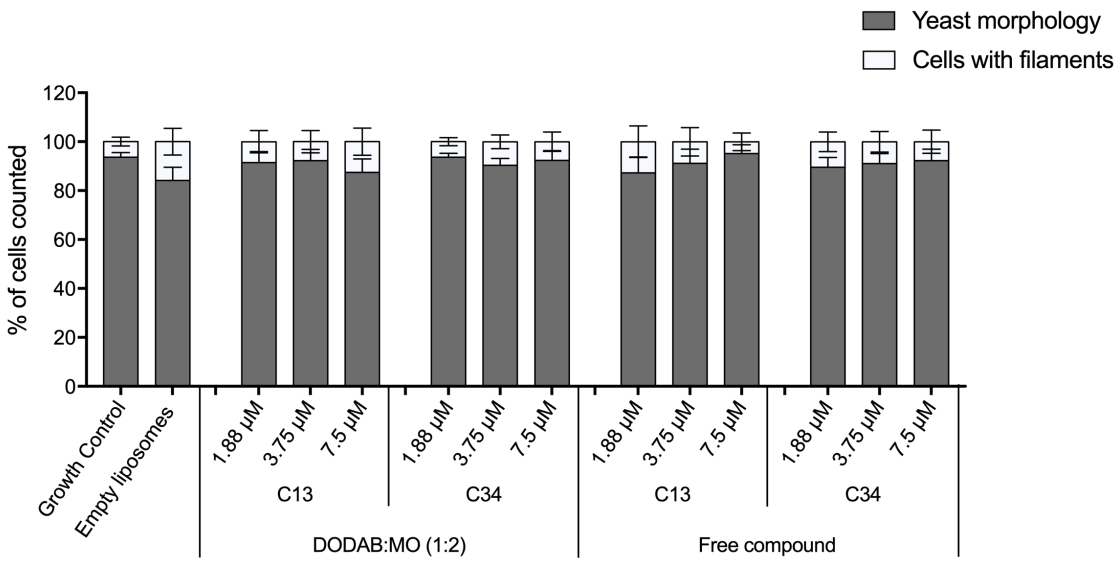


Figure 5.9 Percentage of *C. tropicalis* cells in yeast phase and hyphal phase, according to different growth conditions, namely the use of 1.88, 3.75 and 7.5 µM of free and encapsulated C13 and C34. No less than 300 cells were counted in each condition.

5.4.6 Effect of Benzo[α]Phenoxazine Derivative C34 in *Candida* Adhesion

Adherence of fungal cells to host surfaces is crucial in *Candida* pathogenesis, for initiating colonization and establishment of the disease. Moreover, adhesion to surfaces as catheters or prostheses contributes to biofilm formation and allow *Candida* cells access to the bloodstream and internal organs [56].

The benzo[*a*]phenoxazine derivative C34 presented the highest antifungal activity against the *Candida* species tested, and showed to reduce *C. albicans* filamentation and hyphae size. As such, the influence of free and encapsulated C34, at 1.88 and 3.75 μM, in *Candida* sp. adhesion was tested (Figure 5.10). C34 at 7.5 μM is not presented since the activity at this concentration did not allow sufficient cell growth to observe potential differences.

Results indicate that less than 30% of *C. auris* control cells adhered to the well polystyrene surface, and no significant differences were observed in adhesion of cells inoculated with empty liposomes, or with free or encapsulated C34 (Figure 5.10). This result is in agreement with findings showing that *C. auris* exhibits a minimal ability to adhere to plastic surfaces [57].

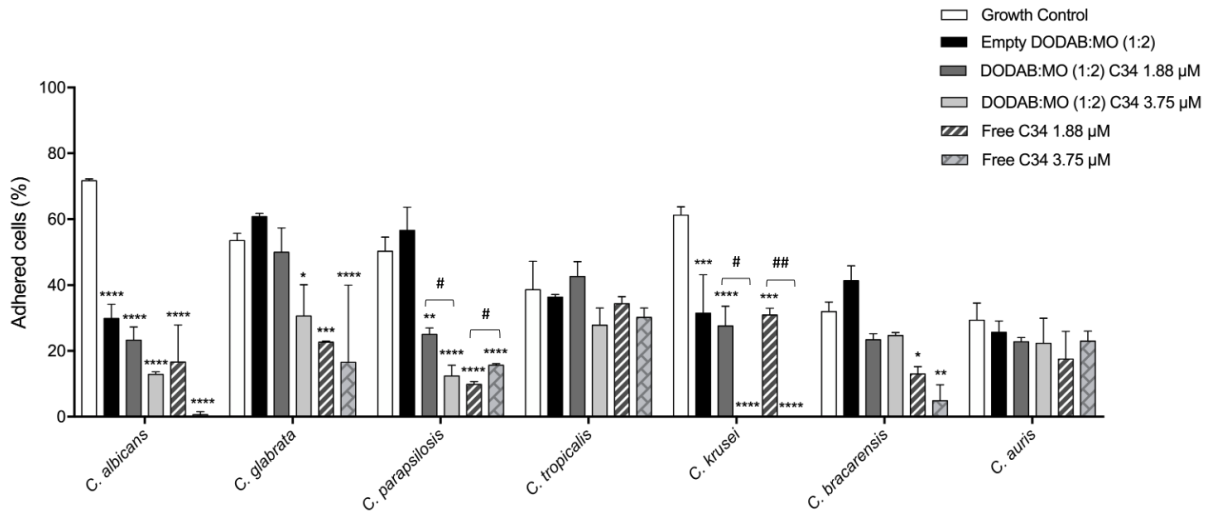


Figure 5.10 Percentage of adhered *Candida* cells after incubation with free and encapsulated C34 at 1.88 and 3.75 μM. All values shown are the average values from independent experiments ± SD. Significant differences in the adherence comparing with growth control are represented by: *P <0.05, **P <0.01, ***P <0.001, **** P <0.0001. Significant differences between the different concentrations of C34 are represented by: #P <0.05 and ##P <0.01.

Although *C. tropicalis* presented a higher % of adherence than *C. auris,* incubation with the compounds also did not affect adhesion. This result is in agreement with the lack of effect of these compounds in filamentation (Figure 5.9). *C. tropicalis* can adhere to silicone and latex catheters [56] thus, the higher % of adherence observed in this study.

C. glabrata, C. parapsilosis, and *C. krusei* are described as species with ability to adhere to surfaces and produce biofilms, although at different levels [56, 57]. This feature was confirmed, by the percentage of adhered cells during control growth, 53.6%, 50.3% and 61.2%, respectively. As expected, growth control

of *C. albicans* presented 71.7% of cells adhered. *C. albicans* presents a set of specialized adhesins that confer the highest capacity to adhere, to biotic and abiotic surfaces [60, 61].

In cells incubated with empty liposomes, *C. albicans* and *C. krusei* presented significant differences in adherence when comparing with growth control ($P < 0.0001$ for *C. albicans* and $P < 0.001$ for *C. krusei*). Incubations with free or encapsulated C34 1.88 and 3.75 μM decreased the percentage of adherence of *C. albicans*, *C. glabrata, C. parapsilosis*, *C. krusei* and *C. bracarensis* ($P < 0,05$). This reduction in cell adhesion was most evident in *C. albicans* cells, in which the percentage decrease to less than half.

Except for *C. tropicalis* and *C. auris*, we can conclude that overall, a decrease in adhesion, with increasing concentrations of C34, is observed, and that the free compound is more active in than the corresponding liposomal formulation, except for *C. krusei.* The activity of C34 in *C. bracarensis* adhesion was lost, after encapsulation.

As referred before, proteins located in ER are important for *Candida* adhesion, filamentation and pathogeny [51] and the perinuclear membrane ER is suggested as a potential target of benzo[a]phenoxazines [30]. Thus, impaired activity in ER due to the benzo[a]phenoxazine derivative C34 may explain cell adhesion reduction. However, this hypothesis needs to be explored.

5.5 CONCLUSION

In this study we evaluated the *in vitro* activity of different benzo[*a*]phenoxazine derivatives against *Candida, Aspergillus* and *Rhizopus* species, and selected two compounds to encapsulate in a DODAB:MO (1:2) formulation as an alternative to the use of free antifungals.

The compounds selected, C13 and C34, presented high activity against the most frequently isolated *Candida* species, in both free and encapsulated forms. Moreover, this antifungal activity was also observed when testing fluconazole resistant species/strains.

Liposomes are frequently used to confer protection to active compounds or to reduce the toxicity of drugs, however, the encapsulation can reduce the compounds release as well as inhibit compounds activity. In this study, the antifungal activity observed was similar or higher with encapsulated compounds, according to species tested, allowing to conclude that encapsulation does not affect the activity of compounds, but can improve their activity, particularly of C13.

Filamentation and adhesion ability are important virulence factors, essential for *Candida* species pathogenesis. Free and encapsulated C34 reduced filamentation of *C. albicans* cells and hyphae size, and reduced adhesion capacity of *C. albicans, C. glabrata, C. parapsilosis* and *C. krusei*. These results are important since failures in adhesion and filamentation contribute to the attenuated virulence of these *Candida* species.

In conclusion, the results obtained demonstrated that the benzo[*a*]phenoxazine derivative C34 could be a potential antifungal drug, due to the high activity against all fungal species tested, including the multidrug resistant *C. auris*. However, further studies are needed to study cytotoxicity of the compounds, and the effect of their encapsulation.

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CHAPTER 6

Benzo[a]phenoxazine DODAB:MO liposomes enhances the antifungal effect of fluconazole on fluconazole-resistant *Candida* species

Adapted from:

Benzo[*a*]phenoxazine DODAB:MO liposomes enhances the antifungal effect of fluconazole on fluconazole-resistant *Candida* species

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6.1 ABSTRACT

The increasing incidence of fungal infections represents a major problem for immunocompromised patients, being associated with high morbidity and mortality rates. *Candida* species are the most frequently isolated etiological agents, being associated with a variety of infections. Current treatments for fungal infections face some problems due to the drug toxicity, pathogen resistance, low drug bioavailability and late diagnosis. The loss of antifungal drug's efficacy represents a potential risk of spreading of resistant strains, so the development of new antifungal strategies is crucial.

In the previous studies (Chapter 5), the benzo[a]phenoxazine chloride derivative C34 presented good antifungal properties in the free form and encapsulated in DODAB:MO liposomes, inhibiting both, *Candida* species growth and filamentation. So, in this work the *in vitro* activity of C34 liposomes in macrophage yeast killing was evaluated. Several C34 formulations were tested against clinical isolates from *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. bracarensis* and *C. auris*, including fluconazole-resistant strains and the Minimum Inhibitory Concentration (MIC) values were determined. Cytotoxicity was assessed by the MTT assay and the cell line J774 was used to evaluate yeast killing in the presence of C34 formulations.

DODAB:MO (1:2) liposomes were prepared with C34 concentrations ranging from 2.9 to 93.8 μM. Liposomes prepared with concentrations <23.4 μM were more unstable, particularly immediately after preparation, but tended to stabilize after the first week of storage. After stabilization liposome mean size was around 400.0 to 120.9 nm. Encapsulation efficiency ranged from 50.6% for liposomes with 46.8 μM of C34 to 96.2% for liposomes with 2.9 μM. Encapsulating C34 reduced the MIC values in a species dependent-manner, in *C. glabrata*, *C. bracarensis* and *C. auris* and some strains of *C. krusei* and *C. tropicalis*. DODAB:MO (1:2) liposomes prepared with 23.4 μM of C34 were selected, in order to obtain a final concentration of 36 μg/mL of lipid and 0.94 μM of C34, which corresponded to the highest C34 non-cytotoxic concentration. These liposomes presented low mean sizes (120.9 ± 25.5 nm) and PDI values (<0.40), were positively charged (+52 mV) and presented an encapsulation efficiency around 56%. This non-cytotoxic formulation was combined with fluconazole to evaluate their interaction and results showed a reduction of the combined MIC in comparison with C34 and fluconazole alone for the fluconazole-resistant strains, independently of the species. Moreover, macrophage yeast killing in the presence of C34 compound was much more efficient than macrophages alone. The decreased

percentage of yeast survival was mainly due to the presence of C34 and was independent of the liposome's lipids or fluconazole.

These results suggest that use of free or encapsulated C34 in combination with fluconazole could be considered for the development of an antifungal strategy, particularly for fluconazole resistant strains. Given the important role of immune innate response in the control of fungal infections and that C34 was able to enhance macrophage killing, the use of C34 could be consider in an effective antifungal strategy.

Keywords: *Candida* sp., benzo[a]phenoxazines, C34, liposomes, fluconazole.

6.2 INTRODUCTION

The increased populations' lifespan, the age-related loss of immune-competence and the advances in medical and surgical therapies, like solid organ transplantation, use of new chemotherapeutic agents or immunomodulatory agents, are considered the main reasons for the increasing number of immunocompromised patients [1]. These individuals present predisposing risk factors for opportunistic invasive fungal infections, which are associated with high morbidity, mortality rates and hospital costs [2, 3].

The ubiquitous *Candida* species remain the most common cause of serious fungal infections, being responsible for 10-15% of all hospital-acquired infections, and the incidence will likely continue to increase in the coming decades [4]. Several *Candida* species are considered pathogenic, but infections are mainly caused by *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* or *C. krusei* [4, 5]. These species are present in the commensal flora of healthy individuals, but in dysbiosis or compromised immune defenses, they can cause opportunistic infections, named candidiasis.

The interaction of *Candida* cells with the host is crucial in the pathogenesis of invasive infections. To establish infection, the opportunistic pathogen has to colonize a host, penetrate the surface, survive and divide in the host cell, and avoid the immune response. Therefore, the severity of infection depends on the ability of *Candida* cells to overcome host immune defenses [6]. Macrophages play an important role in the innate immune response. They mobilize into infectious sites and are involved in the elimination of *Candida* cells during the infection process, mostly through phagocytosis [7]. In phagocytosis, after engulfment, the pathogen is trapped within an intracellular vesicle and targeted for destruction by a complex set of digestive enzymes and toxic reactive oxygen (ROS) and nitrogen (NOS) species [8].

In severe infections, antifungal drugs can complement the host immune defenses to control the fungal infection. Since the 1990s, fluconazole is used as a prophylactic agent in the treatment of invasive fungal infections [9]. Although it is the most frequently used antifungal, several reports showed the loss of clinical efficacy of fluconazole due to the increasing incidence of resistant species, such as *C. krusei*, or the development of resistance by species such as *C. glabrata* [10]. Moreover, the occurrence of outbreaks caused by new multi-resistant species, such as *C. auris*, also represents a problem, and express the need to develop new antifungal strategies [11, 12].

Benzo[*a*]phenoxazine chlorides are organic fluorophores with reported antifungal activity against *Saccharomyces cerevisiae.* [13–15]. Our previous study tested the antifungal activity of five

benzo[a]phenoxazine compounds with proven activity against *Saccharomyces cerevisiae*, with Minimum Inhibitory Concentrations (MICs) between 1.56 and 25 μM. For that, we tested several pathogenic *Candida, Aspergillus* and *Rhizopus* species, and obtained a MIC range of 3.75-60 μM. As observed previously in *Saccharomyces cerevisiae* (MIC of 1.56 μM), C34 showed to be the compound with the lowest range MIC (3-75-15 μM) and presented high antifungal activity against all species tested, in both free form and encapsulated in DODAB:MO (1:2) liposomes. Previous results also showed that free and encapsulated C34 affect negatively filamentation, which is an important *C. albicans* virulence factor to overcome host immune responses (Chapter 5).

Effective antifungal strategies must act as inhibitory and/or fungicidal agents but also as adjuncts of the immune system [17]. These actions depend on the drug, the immunological status of the host and the fungal pathogen. Some studies have demonstrated that different antifungal compounds can also improve *Candida* phagocytosis and yeast killing [18, 19]. Moreover, synergistic combinations of two antifungal compounds can overcome the problem of toxicity and other side effects associated with high doses of singles drugs, by reducing doses of each compound and access different multi-target mechanisms [16]. The main objective of the present study is to evaluate the synergistic effect of these compounds, alone or encapsulated in DODAB: MO (1:2) liposomes, in enhancing macrophages *C. albicans* killing. This study evaluated if the selected strategy could be used as a potential antifungal strategy.

6.3 MATERIAL AND METHODS

6.3.1 Materials

The benzo[a]phenoxazinium chloride used, C34, was synthetized in the Centre of Chemistry at the University of Minho with purity higher than 95% [15, 20]. C34 stock solutions were prepared in dimethylsulphoxide (DMSO) (Sigma-Aldrich) to a final concentration of 12 mM. Fluconazole was purchased from Sigma-Aldrich-USA with 98% purity, and stock solutions prepared in DMSO to a final concentration of 25600 μg/mL.

Dioctadecyldimethylammonium bromide (DODAB) (Tokyo Kasei) and 1-monooleoyl-rac-glycerol (MO) (Sigma-Aldrich) were used for liposome production. Dulbecco's Modified Eagle's medium (DMEM) (ThermoFisher Scientific) was supplemented with 10% heat inactivated fetal bovine serum (FBS) (Valbiotech), 2 mM L-glutamine and 1 mM sodium pyruvate. Ethanol (high spectral purity) was purchased from Usavol, and Saponine, RPMI 1640 and 3-(N-Morpholino)propanesulfonic acid (MOPS) from Sigma-Aldrich.

6.3.2 Preparation and Characterization of Liposomes

6.3.2.1 Preparation of liposomes by lipid-film hydration method

C34 was encapsulated in DODAB:MO (1:2) based liposomes prepared by film hydration method. Briefly, DODAB and MO were dissolved in ethanol in a round-bottom tube, vortexed and the organic solvent removed by rotary evaporation at 60 °C, safely above the lipids phase transition temperature. The lipid film was prepared at 4 mM total lipid concentration.

Then, the lipid film was hydrated with solutions of C34 at 60 °C, to obtain liposomes with 2.9 μM, 5.9 μM, 11.7 μM, 23.4 μM, 46.9 μM and 93.8 μM of C34 to 2 mM (888 μg/mL) of lipids. Finally, the dispersions were sonicated for 6 minutes. Empty liposomes were also prepared at 2 mM (888 μg/mL) with ultrapure water with 2% DMSO.

6.3.2.2 Size, polydispersity index and ζ -potential

The ζ-potential and the mean size of formulations were determined by dynamic light scattering (DLS) at 25 °C in the Malvern ZetaSizer Nano ZS particle analyzer. The Malvern Dispersion Technology Software

(DTS) was used with multiple narrow mode (high resolution) data processing to determine the mean size (nm), polydispersity index (PDI) and error values. The liposomes surface charge was measured indirectly by ζ-potential analysis, using electrophoretic light scattering (ELS) at 25 °C. DTS with monomodal mode data processing was used to determine average ζ-potential (mV) and error values. All values were acquired in quintupled.

6.3.2.3 Quantification of encapsulation efficiency

All formulations prepared were pelleted by ultracentrifugation (100 000 g during 1 hour at 4 °C) and the supernatant removed. The concentration of non-encapsulated C34 in the supernatant was determined by spectrophotometry at 645 nm, using a standard curve of C34 previously determined.

6.3.3 Antifungal Activity of Nanoformulations

A total of fourteen *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. bracarensis* and *C. auris* isolates were used in this work, including reference strains of *C. albicans* SC5314, *C. glabrata* IGC2418, *C. parapsilosis* ATCC22019, *C. krusei* ATCC6258 and *C. bracarensis* NCYC5133. All these isolates are conserved at the collection of Biology Department of Minho University in 30% glycerol at −80 °C. Before testing, yeast strains were grown at 30 °C for 48 hours on YPD agar medium (Yeast extract 1%, Bactopeptone 1%, Glucose 2% and Agar 2%). The activity of all nanoformulations against *Candida* strains was evaluated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocols for yeasts, previously described (EUCAST definitive document E.DEF 7.3.1). 16 µl of each liposomal formulation prepared were added to the test wells (total volumes 200 µl), and so the final concentrations of C34 tested were 0.23, 0.47, 0.94, 1.88, 3.75 and 7.5 µM, and of lipids 71 µg/mL. Empty liposomes and free compounds were also tested, as controls. These formulations were tested in triplicate, in three independent assays.

6.3.4 *In vitro* Combination of Fluconazole and DODAB:MO C34 Formulation

In vitro interaction of fluconazole and selected DODAB:MO: C34 formulation (36 µg/mL : 0.94 µM) against all yeast strains selected was determined by the EUCAST protocols for yeasts previously described.

Briefly, serial dilutions of fluconazole were performed and added to 96-well plates, to obtain the intended final concentrations. Fluconazole concentrations tested ranging from 0,125 to 128 µg/mL. Then, 8 µl of DODAB:MO C34 23.4 µM were added to each well.

After cultured on YPD agar medium for 24 hours at 30 °C, *Candida* cells were suspended in sterile water and cellular density adjusted to 1-3 x 10^6 cell/mL. A final concentration of 0.5-1.5 x 10^5 cell/mL was used. Sterility control and growth control were included in each microplate.

After 24 h of incubation at 30 °C, the absorbance was measured at 530 nm using a microplate absorbance reader. The Minimum Inhibitory Concentration (MIC) of each compound was considered as the lower drug concentration with growth inhibition of ≥80% after 24 h in comparison with the growth control. MICs obtained were compared with MICs of fluconazole alone. The activity of free C34 at 0.94 µM and empty liposomes at 36 µg/mL were also tested. EUCAST clinical breakpoints values were used to classify *Candida* strains as susceptible or resistant.

6.3.5 Cytotoxicity Assay

In vitro studies were performed using the murine macrophage-like cell line J774 as previously described [21]. The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FBS), 1% glutamine and 1% sodium pyruvate, at 37 °C in a 5% CO_2 atmosphere. After confluent growth, macrophage cells were recovered, washed and resuspended in DMEM to the desired final concentration.

Macrophages were plated on 96-well tissue culture plates (ThermoFisher Scientific) at 1 x 10^4 cells/well and incubated overnight at 37 °C in a 5% CO_2 atmosphere. In a first assay, all DODAB:MO liposomes loaded with C34, were tested by adding 16 µl of each liposomal formulation prepared. In a second test, different volumes of all the prepared formulations were added to the cells, to obtain different lipid: C34 combinations. Three concentrations of empty liposomes, 18, 37, 72 µg/mL, and four concentrations of free C34, 0.23, 0.47, 0.94 and 1.88 µM, were tested as controls.

Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [22] following 24 h and 48 h incubation with the different formulations, at different concentrations. MTT formazan was solubilized by adding DMSO:ethanol (1:1) solution and absorbance measured at 570 nm. Control of viability (100%) was performed using untreated cells, and of cytotoxicity using cells treated

with Tris-HCl. All tests were performed in triplicates. Results were expressed as percentage of viability, according to Carneiro *et al.* (2015) [23].

6.3.6 Macrophage Killing Assay

To evaluate the influence of free and encapsulated C34 in the ability of macrophages cells to kill *Candida albicans* a previously described protocol was used with some adaptations [24]. Briefly, macrophages were plated onto 96-well tissue culture plates (ThermoFisher Scientific) at 1 x 10^4 cells/well and incubated overnight at 37 °C in a 5% CO_2 atmosphere. Free and encapsulated C34, empty liposomes and fluconazole were added into the wells at the desired concentration. A suspension of *C. albicans* SC5314 cells (5 x 10^4 cells/well) at a ratio of 5:1 (yeast:macrophage) was added, and cells incubated for 1h at 37 °C in a 5% CO_2 atmosphere. After incubation macrophages were lysed, with 10% saponin solution, and serial dilutions of the suspensions plated on YPD agar medium. After 24 h of incubation at 37 °C CFUs were determined.

6.3.7 Statistical Analysis

Statistical analyses were performed with GraphPad Prism (5.0 version, GraphPad Software, USA). Comparative analyses of different groups were made using analysis of variance (ANOVA) followed by the Bonferroni test. Unless otherwise stated, results shown are from at least three independent experiments with three replicates. In all the cases, $P < 0.05$ values were considered statistically significant.

6.4 RESULTS

6.4.1 Characterization of DODAB:MO (1:2) Liposomes Loaded with C34

In previous studies we tested the antifungal activity of DODAB:MO (1:2) liposomes loaded with benzophenoxazinium[a]chloride C34 and observed growth inhibition of different strains of *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. bracarensis* and *C. auris*. In the present study DODAB:MO (1:2) C34 formulations were produced and tested for their ability to act in synergy with fluconazole to enhance macrophage killing.

Nanoformulations were prepared using 2.9, 5.9, 11.7, 23.4, 46.8 and 93.8 μM of C34 and 888 μg/mL (2mM) of DODAB:MO (1:2). Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS) were used to measure liposomes mean size (nm), polydispersity (PDI) and zeta potential (mV) after compounds incorporation. Figure 5.1 presents the properties of empty DODAB:MO (1:2) liposomes and all six formulations prepared.

The empty DODAB:MO (1:2) liposomes presented a mean size of 132.9 ± 8.6 nm, PDI values of 0.21 ± 0.01 and a high net positive charge (+65 mV), as expected. After storage at 4 °C significative differences were only observed in the superficial charge, after 2 and 3 weeks.

Liposomes loaded with low concentrations of C34 (2.9, 5.9 and 11.7 μM) presented increased mean sizes and PDI values, in comparison with empty liposomes ($P < 0.0001$). Although, after only 1 week, the mean size and PDI values of these formulations decreased significantly, the values still higher than empty liposomes (Figure 6.1). Increasing C34 concentrations to 23.4, 46.8 and 93.6 μM, C34-liposomes presented mean sizes, (120.9 ± 25.5, 138.9 ± 7.4 and 205.2 ± 29.1 nm, respectively), and polydispersity values (PDI ≤0.40) similar to the empty liposomes. No significant differences ($P > 0.05$) were observed after storage at 4 °C.

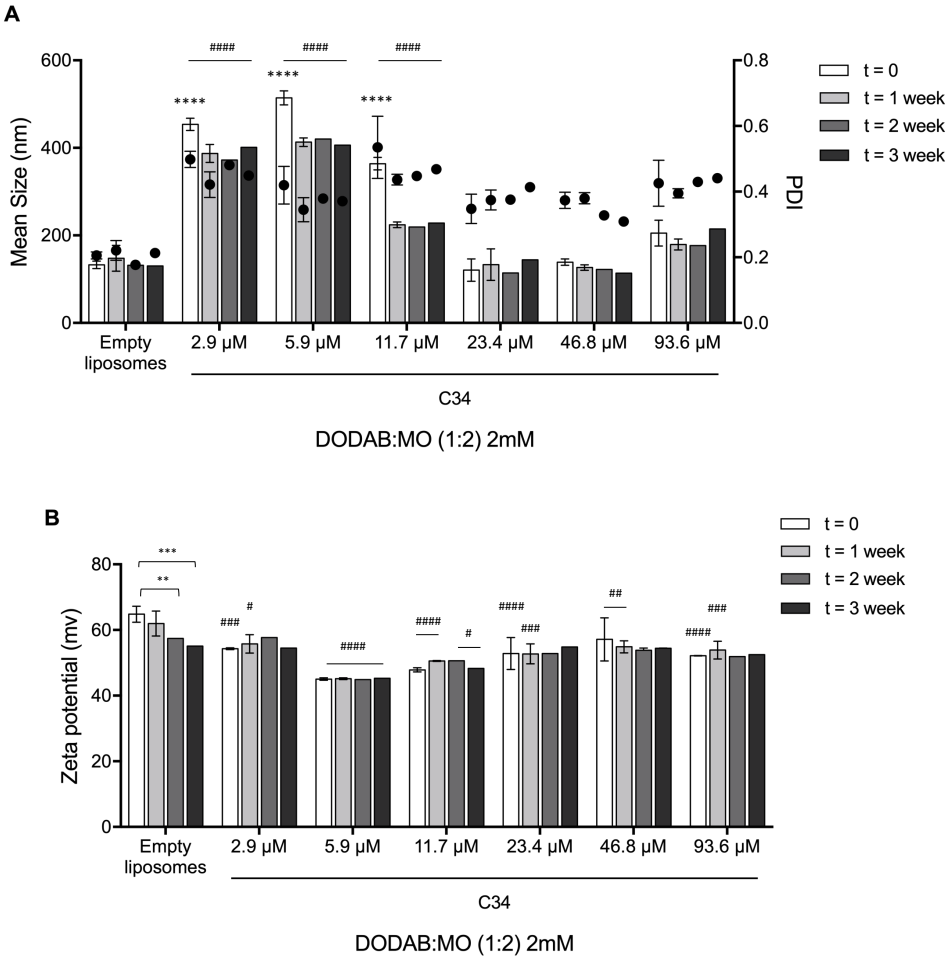


Figure 6.1 Size (A bars), PDI (A spherical symbols) and ζ-potential (B bars) of DODAB:MO (1:2) nanoparticles prepared with 2.9, 5.9, 11.7, 23.4, 46.9 and 93.8 µM of C34, measured in the day of production, and after 1, 2 and 3 weeks of storage at 4 °C. All values shown are the average values from three independent experiments ± SD. Significant differences between the different weeks within each condition, are represented by: *P <0.05, **P <0.01, ***P <0.001 and ****P <0.0001. Significant differences between empty liposomes and liposomes loaded with C34 are represented by: #P <0.05, ##P <0.01, ###P <0.001 and ####P <0.0001.

All formulations prepared with C34 were also positively charged, varying between +45 mV for liposomes loaded with 5.9 µM of C34 to +57 mV for liposomes loaded with 46.8 µM of C34, and no significant differences were observed during storage. However, in comparison with empty liposomes a reduction on the surface charge was observed for all liposomes after 1 and 2 weeks of storage (P >0.05) (Figure 5.1). Liposomes with 5.9 µM and 11.7 µM of C34 showed differences in the surface charge in comparison with empty liposomes at all time points. Surface charge returned to values similar to the empty liposomes when higher concentrations of C34 were used. No significative differences (P >0.05) were observed after 1, 2 and 3 weeks of storage at 4 °C.

These results suggest that the amount of C34 affected significantly liposomal formulations. Adding low concentrations, 2.9-11.7 μM, clearly enhances DODAB:MO liposomes mean size, and polydispersity, but with concentrations of C34 >11.7 μM, C34-liposomes showed similar characteristics as the empty liposomes, suggesting that stabilization/organization of C34-liposomes is dependent on C34 concentration (Figure 6.1).

6.4.2 Determination of Encapsulation Efficiency

The encapsulation efficiency of the six prepared formulations was determined, after ultracentrifugation, by spectrophotometry and results presented in Table 6.1. The encapsulation efficiency was higher than 50% for all C34 concentrations used, ranging from 50.6% for liposomes with 46.8 μM of C34 to 96.2% for liposomes with 2.9 μM of C34.

Table 6.1 Encapsulation efficiency of different concentration of C34 in DODAB:MO (1:2) 2mM liposomes.

DODAB:MO (1:2) 2mM	Encapsulation (%)	SD
C34 2.9 μM	96.2	5.4
C34 5.9 μM	60.7	6.3
C34 11.7 μM	51.0	3.2
C34 23.4 μM	55.5	12.8
C34 46.9 μM	50.6	3.5
C34 93.8 μM	70.4	1.7

Results indicate that encapsulation efficiency tends to decrease with increasing concentrations of C34, except for C34 at 93.8 μM.

6.4.3 Confirmation of the Antifungal Activity

The antifungal activity of encapsulated C34 formulations were tested by modification of EUCAST methodology. The activity of empty liposomes and free C34 was also tested. Table 6.2 presents the Minimum Inhibitory Concentration (MIC) of free and encapsulated C34 obtained against seven *Candida* species, according to EUCAST methodology. These results showed that the use of encapsulated C34 decreases the C34 MIC in several strains, particularly *C. glabrata*, *C. bracarensis* and *C. auris*.

Table 6.2 Strains tested in this study and respective Minimum Inhibitory Concentrations (MICs) of C34 and C34 encapsulated in DODAB:MO (1:2) liposomes. **Bold** strains with lower MIC when tested with encapsulated C34.

	MIC (μM)	
Isolates	**Free C34**	**Encapsulated C34***
C. albicans SC 5314	15	15
C. albicans SER 7	15	15
C. glabrata IGC 2418	7.5	**3.5**
C. glabrata 182	15	**7.5**
C. parapsilosis ATCC 22019	7.5	7.5
C. parapsilosis 160[a]	7.5	7.5
C. tropicalis 2D	7.5	**3.75**
C. tropicalis 45	7.5	7.5
C. krusei ATCC 6258	3.75	**1.88**
C. krusei H11	3.75	3.75
C. bracarensis 153 MT	15	**7.5**
C. bracarensis NCYC 5133	7.5	**3.75**
C. auris 17-274	7.5	**3.75**
C. auris 17-270	7.5	**3.75**

*Final concentration of 71 μg/mL of DODAB:MO (1:2)

6.4.4 Cytotoxicity Assay

The cytotoxicity of the formulations with proven activity against *Candida* species (Table 6.2), namely liposomes prepared with 23.4, 46.9 and 93.8 μM of C34 (final concentration of 1.88, 3.75 and 7,5 μM, respectively), was evaluated using murine macrophage-like cell line J774, by measuring cell metabolic activity by MTT assay. However, both free or encapsulated C34, at all concentrations were highly toxicity, presenting viability values lower than 30%. So, to determine the highest non-toxic C34 concentration, different volumes of the six liposomal formulations produced were tested (Table 6.3).

Table 6.3 Volumes of DODAB:MO (1:2) C34 formulations used and final concentrations of lipids and C34 tested.

	DODAB:MO (1:2) 2mM (888 µg/mL)					
	C34 2.9 µM		**C34 5.9 µM**		**C34 11.7 µM**	
Volume (µL)	**Lipid** (µg/mL)	**C34** (µM)	**Lipid** (µg/mL)	**C34** (µM)	**Lipid** (µg/mL)	**C34** (µM)
16	71	0.23	71	0.47	71	0.94
8	-	-	36	0.23	36	0.47
4	-	-	-	-	18	0.23

	DODAB:MO (1:2) 2mM (888 µg/mL)					
	C34 23.4 µM		**C34 46.9 µM**		**C34 93.8 µM**	
Volume (µL)	**Lipid** (µg/mL)	**C34** (µM)	**Lipid** (µg/mL)	**C34** (µM)	**Lipid** (µg/mL)	**C34** (µM)
16	71	1.88	-	-	-	-
8	36	0.94	36	1.88	-	-
4	18	0.47	18	0.94	18	1.88

- not tested

Figure 6.2 shows cell viability values obtained for all new conditions tested after 24 and 48 h of co-incubation. Conditions presenting cell viability higher than 80% were considered non-cytotoxic. After 24 and 48 h of incubation, all empty liposome lipid concentrations presented cell viability values above 94%, showing that empty liposomes are not cytotoxic for these cells (Figure 6.2).

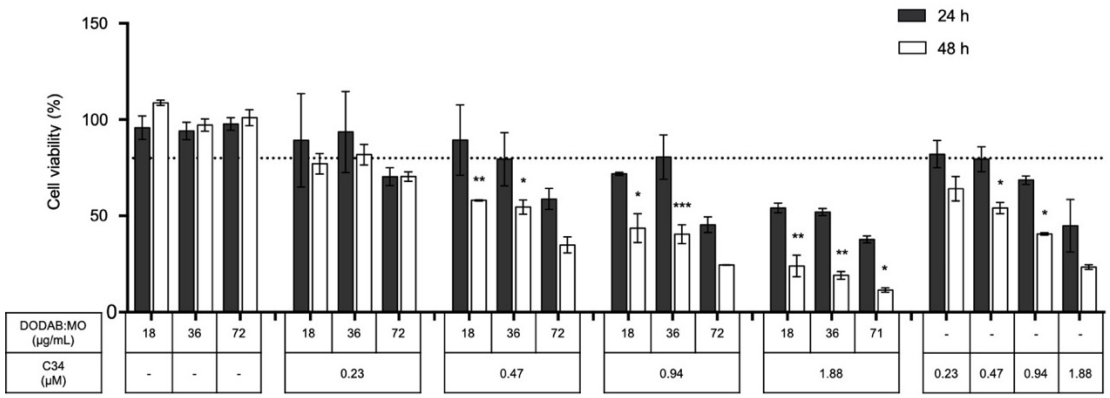


Figure 6.2 Effect of different concentrations of empty liposomes, free C34 and combinations of lipid/C34 on the viability of J771 cells. Viability tests were performed using the MTT assay after 24 h (grey bars) and 48 h (white bars) of incubation. Results indicate the mean ± SD of three independent assays. Significant differences between the two time points of incubation, 24 and 48 h, are represented by: *P <0.05, **P <0.01, ***P <0.001.

Cell viability reduces with increasing concentration of free C34, indicating that its cytotoxicity is dose-dependent. At 24h of incubation, the only nontoxic C34 concentrations tested were 0.23 μM and 0.47 μM, with 82 and 80% of cell viability, respectively. After 48 h of incubation, a significant decrease of cell viability was observed in cells incubated with 0.47 and 0.94 μM of C34 ($P = 0.0352$ and $P = 0.0153$, respectively).

A slight increase in cell viability was obtained with C34-liposomes in comparison with the corresponding free compounds. However, the differences were not significant. Curiously, although all empty liposome concentrations tested were non-cytotoxic, with similar viability values, the C34-liposomes with 71 μg/mL of lipids were consistently more cytotoxic, comparing the same amount of C43 ($P < 0.05$).

After this analysis, only five C34-liposomes presented cell viability ≥ 80%, liposomes with 0.23, 0.47 or 0.94 μM of C34 encapsulated into 16 or 36 μg/mL of lipids. Thus, only these formulations could be tested with macrophage cells to evaluate their potential to help antifungal phagocyte activity. However, these C34 concentrations are far from the MIC values tested for *Candida* cells. To enhance antifungal potential, the non-toxic condition with the highest C34 concentration, C34 at 0.94 μM with 36 μg/mL of lipid was selected to continue the tests with macrophages.

6.4.5 *In vitro* Antifungal Activity of Fluconazole and DODAB:MO C34 Formulation

Since non-toxic concentrations of C34 are far from the MICs for *Candida* species tested, we decided to test the synergistic effect of fluconazole and the selected C34-liposomes (0.94 μM of C34 with 36 μg/mL of lipid). First, the antifungal activity of fluconazole in the presence of this liposomes against *Candida* species was tested by the EUCAST method. The species-specific breakpoints for fluconazole determine that for *C. albicans, C. tropicalis* and *C. parapsilosis,* strains with MICs values ≤2 μg/mL are classified as susceptible (S), MIC values of 4 μg/mL as intermediate (I), and MIC values >4 μg/mL as resistant (R). The breakpoint values for *C. krusei* are not established, due to the intrinsic resistance, and for *C. glabrata* only the resistance value (>32 μg/mL) is established since fluconazole is not considered a good alternative against this specie (The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretations of MICs for antifungal agents, version 10.0, 2020. www.eucast.org/astoffungi/clinicalbreakpointsfroantifungals/). For *C. auris* the same breakpoint values of *C. glabrata* are suggested, since no values were established for this species [26].

Table 6.4 presents MIC values obtained with fluconazole and with fluconazole and the C34-liposomes. Five strains (*C. albicans* SER7, *C. krusei* ATCC 6258 and H11, and *C. auris* 17-274 and 17-270) were

classified as resistant to fluconazole, 3 as intermediate (*C. glabrata* 182 and *C. bracarensis* 153MT and NCYC 5133) and the remaining 6 as susceptible. Combining the selected C34-liposome with fluconazole, MIC values for fluconazole-resistant strains decreased, by two dilutions in three strains, in *C. albicans* SER7 (from >128 to 32 μg/mL), *C. krusei* ATCC 6258 (from 16 to 4 μg/mL) and *C. krusei* H11 (from 32 to 8 μg/mL) and by one dilution in *C. auris* 17-274 (>128 to 64 μg/mL). Only for fluconazole-resistant strain *C. auris* 17-270 the MIC value remained similar. Regarding the susceptible strains tested, the MIC values did not change, except for *C. parapsilosis* ATCC 22019 that decreased from 1 to 0.5 μg/mL.

Table 6.4 List of strains tested in this study and respective Minimum Inhibitory Concentrations (MICs) of fluconazole and fluconazole combined with DODAB:MO (1:2) : C34 (36 μg/mL : 0.94 μM). **Bold** strains with decrease of fluconazole MIC after combination with C34 formulation.

	MIC	
Isolates	**Fluconazole (μg/mL)**	**Fluconazole with DODAB:MO (1:2)/C34* (μg/mL)**
C. albicans SC 5314	0.125	0.125
C. albicans SER 7	**>128**	**32**
C. glabrata IGC 2418	1	1
C. glabrata 182	4	4
C. parapsilosis ATCC 22019	1	0.5
C. parapsilosis 160[a]	0.5	0.5
C. tropicalis 2D	0.125	0.125
C. tropicalis 45	0.5	0.5
C. krusei ATCC 6258	**16**	**4**
C. krusei H11	**32**	**8**
C. bracarensis 153 MT	4	4
C. bracarensis NCYC 5133	4	4
C. auris 17-274	**>128**	**64**
C. auris 17-270	**>128**	>128

*Final concentration of 36μg/mL of DODAB:MO (1:2) and 0.94 μM of C34

No differences in fluconazole MIC values obtained were observed when tested with empty DODAB:MO (1:2) liposomes, showing that the synergistic effect was independent of the lipids. As expected, yeast cell

growth inhibition with the selected C34-liposome alone was lower than 10% against all *Candida* strains, indicating no significant antifungal activity of the compound at 0.94 μM.

These results show that the combination of C34-liposome potentiates fluconazole antifungal effect against resistant *Candida* strains.

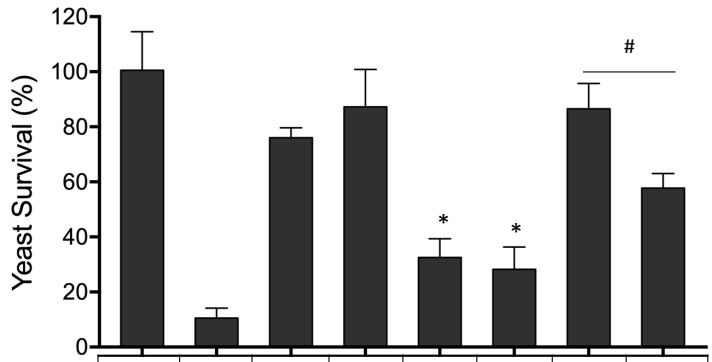
6.4.6 Macrophage Killing Assay

In the previous work (Chapter 5), C34 formulations reduced the number and size of *C. albicans* filaments, which is an important virulence factor for macrophage evasion. Thus, we evaluated the ability of macrophages to kill *Candida* cells in the presence of the selected C34-liposomes (36 μg/mL lipids: 0.94 μM C34) and fluconazole (Figure 6.3).

Fluconazole is known to be a fungistatic agent, but, no information about C34 is available. So, *C. albicans* was incubated with C34 to evaluate if this compound can kill yeast cells. Since mammalian non-toxic concentration of C34 is far from *Candida* MICs, we tested *C. albicans* SC5314 with the C34 at its MIC concentration without macrophage cells (Figure 6.3). Results indicate that one hour of incubation with C34 at its MIC concentration, yeast cell viability reduced by 90%, suggesting that C34 is fungicidal.

Interaction of macrophages with *Candida* reduced by 24.0% yeast survival. Surprisingly, free C34 and C34-liposomes, decreased significantly the percentage of yeast survival by 32.5% ($P = 0.0343$) and 28.3% and ($P = 0.0189$), respectively (Figure 6.3) and no significative differences ($P > 0.05$) were observed between them. This result shows that the use of DODAB:MO liposomes did not affect the activity of C34 in enhancing macrophage yeast killing. This observation is consistent with the fact that empty liposomes only decrease killing by 12.8% however, no significant differences were observed in comparison with macrophages incubated with *C. albicans* ($P > 0.05$).

As expected, macrophages in the presence of fluconazole (0.125 μg/mL) presented a yeast survival of 87%, which was not significant different in comparison with macrophages and *Candida* alone. However, this percentage decreases to 57.5% when fluconazole is combined with C34-liposomes ($P = 0.0361$), confirming that C34-liposomes aids macrophages yeast killing. This result also shows that the presence of fluconazole seems to decrease the effect of C34-liposomes, however statistically this difference is not significative.



C. albicans	+	+	+	+	+	+	+	+
DODAB:MO (µg/mL)	-	-	-	36	-	36	-	36
C34 (µM)	-	15	-	-	0.94	0.94	-	0.94
Flu (µg/mL)	-	-	-	-	-	-	0.125	0.125
			Macrophages J774					

Figure 6.3 Results of macrophage killing assay using the cellular line J774 and the reference strain *C. albicans* SC5314. These graphic compares the yeast survival after incubation with macrophages, macrophages with 36 µg/mL of DODAB:MO (1:2) liposomes, 0.94 µM of C34, and the combination lipid : C34 (36 µg/mL : 0.094 µM). Also compares the synergistic effect of Fluconazole (0.125 µg/mL) and C34 liposomes (36 µg/mL : 0.094 µM). Results indicate the mean ± SD of independent assays. Significant differences with *Candida* cells incubated with macrophages are represented by: *P <0.05. Significant differences between the use of fluconazole and the synergy with C34 liposomes are represented by: #P <0.05.

6.5 DISCUSSION

Candida species are responsible for a variety of infections, from less-severe superficial lesions in mucosa and skin, to life-threatening disseminated mycosis, characterized by the spread of fungi throughout the tissues and blood circulation [1]. The increasing incidence of these infections and the diversity of pathogenic species contributed significantly to the high mortality in immunosuppressed patients [2, 3]. The delayed diagnosis, drug toxicity, antifungal drug resistance, low drug bioavailability and lack of oral or intravenous preparations are some of the factors that contribute to this increasing tendency. To enhance effectiveness of antifungals frequently used, a variety of strategies have been studied. The improvement of the existing agents is a frequent strategy, being Amphotericin B lipid complexes the most frequently studied and tested. Amphotericin B remains the agent with the broadest antifungal spectrum, however, it also presents a variety of adverse effects and a substantial renal toxicity [26]. The use of lipid formulations of Amphotericin B, such as AmBisome or Ampholip, showed to reduce nephrotoxicity and also confer protection to the active compound [27, 28]. Other important strategy is the development of new antifungal drugs with different cellular targets. Benzo[a]phenoxazine derivatives antifungal activity have been suggested to be due to the DNA intercalative binding of their polycycles, and accumulation on the membrane of endoplasmic reticulum, in studies using the model yeast *S. cerevisiae* [13–15]. In our previous work, benzo[a]phenoxazine compounds also showed great antifungal activity against several *Candida* and *Aspergillus* species (Chapter 5). We also showed that these compounds reduced significantly filamentation of *Candida* species, particularly *C. albicans*, which is considered one of the most important virulence factors.

In this study we aimed to evaluate the applicability of these compounds in *in vitro* macrophage killing assays. N,N-dioleyl-N,Ndimethylammonium bromide (DODAB): Monoolein (MO) (1:2) were used to encapsulate the benzo[a]phenoxazine compound as in previous studies [23]. The benefit of MO in the liposome composition is that it acts as an enhancer of solubilization [29, 30] and also a stabilizer, conferring fluidity to liposomes by favoring lipid chain mobility, important to improve the fusion of liposomes with cell membranes [31]. We selected C34 benzo[a]phenoxazine compound because it presented both, the highest antifungal activity and capacity to decrease filamentation.

DODAB:MO liposomes were prepared with concentrations of C34 ranging from, 2.9 to 93.8 μM. It was observed that at low concentrations of C34, liposomes presented high mean sizes and polydispersity, and that, at concentrations above 11.7 μM, the mean size and polydispersity of the liposomes were

similar to empty liposomes. These results indicate that lower concentrations of C34 destabilize the DODAB:MO liposomes. Benzo[a]phenoxazine derivatives are lipophilic and bear a delocalized positive charge, thus its encapsulation is favorable within the lipid phase of liposomes [32]. This interaction will induce a liposomal rearrangement that is dependent on the C34 concentration. This rearrangement is also apparent when comparing mean size and polydispersity after storage, liposomes with C34 concentrations under 11.7 μM are the ones that changed more significantly after one week of storage, meaning that after preparation they need more time to rearrange. After the first week of storage mean size and PDI values did not changed in all formulations, including liposomes with concentrations under 11.7 μM, suggesting that these liposomes are stable.

Analyzing the surface charge of the liposomes it is clear that it does not change significantly with C34 concentration, except for liposomes with 5.9 μM. Only a slight decrease in the ζ-potential is observed in comparison with empty liposomes, particularly after preparation and in the first week of storage, suggesting that the C34 molecules may be located within the liposome.

The encapsulation efficiency ranges from 50.6% for liposomes prepared with 46.8 μM of C34 to 96.2% for liposomes prepared with 2.9 μM of C34. These values were determined after preparation, not accounting for liposomal rearrangements. It is known that rearrangements on liposomes surface may induce release of the cargo [33]. However, this may not be the case, since C34-liposomes that evidenced higher rearrangements were the ones with higher encapsulation efficiencies.

Encapsulation of C34 enhanced the antifungal activity of C34 in strains from *C. glabrata*, *C. krusei*, *C. bracarensis* and *C. auris*, but not in the tested strains of *C. albicans* and *C. parapsilosis*. This observation is curious because *C. auris* and *C. glabrata* are among the most difficult species to treat with the available antifungals, and *C. auris* is a growing concern due to hospital out-breaks [12] it is also interesting to observe that encapsulation of C34 enhances C34 antifungal activity against species belonging to the Whole Genome Duplication clade (*C. bracarensis* and *C. glabrata*) while is not that effective in species from the CTG clade (*C. albicans*, *C. parapsilosis, C. tropicalis* and *C. krusei*). *C. auris*, although within the CTG clade is phylogenetically very distant from that group [34]. However, much more strains of each species should be tested because the observed antifungal activity of the C34-liposomes may be strain related.

To evaluate the applicability of these liposomes in real infection we needed to first evaluate their cytotoxicity. Surprisingly, when these liposomes were tested with mammalian macrophage cells, they were extremely cytotoxic. Thus, we decided to determine the maximal non-toxic concentration of these

compounds. The selected combination was DODAB:MO at 36 μg/mL with C34 at 0.94 μM. This combination was achieved by using 8 μl of C34-liposome, DODAB:MO (1:2) loaded with 23.4 μM C34. As characterized previously, this C34-liposome has the lowest mean size and PDI values (120.9 ± 25.5 and PDI <0.4). When comparing with free C34, a slightly decrease in toxicity was observed, but not statistically significant. This result is probably due to the encapsulation efficiency of this formulation (55.5%), being the remain 44.5% of free C34 responsible for the toxicity. However, even with these concentrations after 48 h of incubation all combinations were considered cytotoxic at some level. Even so we decided to proceed with the remaining tests with the non-toxic formulation, because in vivo there is some degradation of the compounds and the majority of antifungal drugs remains in the organism less than 30 hours, for example the terminal elimination half-life in the plasma of fluconazole is 30 h, caspofungin is 8 h and micafungin is between 13 and 20 h [35].

At the selected concentration, DODAB:MO (1:2) C34 formulation did not present antifungal activity against all *Candida* species tested, showing that the use of these formulation as antifungal strategy, in these conditions, is not an alternative. Fluconazole is frequently used as prophylactic agent and is the main treatment agent of invasive fungal infections, however, a loss of efficacy of this drug has been reported due to the emergence of fluconazole resistant species/strains [10, 36]. Antifungal combinations of two compounds can be benefic in the treatment of infections by resistant organisms. For example, Bidaud *et al.* (2020) [37] evaluated the *in vitro* interaction between colistin and caspofungin against *C. auris* and obtained a stronger antifungal activity. In this study, antifungal activity of fluconazole combined with C34 formulation was tested. This combination decreases the fluconazole MICs but only in fluconazole resistant *Candida* strains.

The combination of two active compounds responds to the biological compensation, reduces doses of each compound and access different multi-target mechanisms, inhibiting the development of resistance [19].

Fluconazole mechanism of action is based on the inhibition of ergosterol biosynthesis, and C34 mechanism of action is still poorly characterized, results with other benzo[a]phenoxazines pointing to binging with fungal DNA and interactions with the perinuclear membrane of endoplasmic reticulum [15]. Point mutations on the main fluconazole target, lanosterol 14-alpha demethylase (*ERG11*), are known to contribute to fluconazole resistance, but other genes also contribute such as, *UPC2,* coding for a transcription factor that regulates the expression of genes in the ergosterol pathway, and transporters from the ATP binding cassette (ABC) and major facilitator superfamily (MFS) classes of efflux proteins

(Cdr1 and Cdr2 and Mdr1) [38] It has been described that Nile red (a benzo[a]phenoxazine dye) was efficiently effluxed by *C. albicans* Cdr1p, Cdr2p, and Mdr1p proteins [39]. C34 is a derivative of benzo[a]phenoxazine with alterations is several regions of the molecule (Chapter 5). Since these transporters are over-expressed in fluconazole resistant strains it is possible that C34 can inhibit fluconazole efflux, either directly by competition or indirectly by affecting the activity of these transporters leading to a higher intracellular concentration of fluconazole and thus high antifungal activity. These results showed that the use of C34 formulations combined with fluconazole may be an important alternative as antifungal strategy.

The ability to convert from unicellular yeast to filamentous forms is an important virulence factor. In some cases, the large size of hyphae or pseudohyphae can prevent phagocytosis [40]. This process allows *Candida* cells to evade from macrophages antifungal activity and cause infection [41]. Previous results suggested that C34 reduces the filamentation of *C. albicans* cells (Chapter 5), in this way we decided to assess if the use of the selected C34-liposome would help in macrophage yeast killing. Results obtained showed a surprisingly significant decrease in yeast viability in macrophages treated with free and encapsulated C34. This decrease is apparently independent of the DODAB:MO liposomes, since no differences were observed between free and encapsulated C34, and the use of empty liposomes did not affect yeast survival. This C34 concentration did not presented any antifungal activity against all *Candida* species tested, including *C. albicans*. However, in the presence of macrophages it was able to reduce *C. albicans* cell viability from 76% to 32.5% and 28.2% in the presence of C34 and C34-liposomes, respectively. In *C. albicans*, the Cap1 transcription factor is the major regulator of the oxidative stress following exposure to neutrophils. Direct targets of Cap1 include many key antioxidant genes, such as *CTA1* and *TRX1*, and gene involved in the response to drugs, such as *MDR1* [42]. Thus, considering the hypothesis that C34 may interfere with Cdr1p, Cdr2p, and Mdr1p transporters, a C34-impaired *MDR1* response under macrophage oxidative stress could account for these results. Also, this effect may result of the observed impairment in filamentation induced by C34, facilitating macrophage killing. The influence of C34 formulations was also confirmed by the decrease of yeast survival in cells treated with fluconazole and C34-liposome in comparison with cells treated with fluconazole only. The yeast survival of macrophages treated with the combination of fluconazole and C34-liposomes was not statistically different from cells treated with C34 alone ($P = 0.33$) or with C34-liposomes ($P = 0.62$). These results suggest that, together with fungistatic nature of fluconazole, the decrease in yeast survival after one hour of incubation in mainly due to the C34 compound. In addition, the killing assay was performed after one

hour of incubation, thus, well within the non-toxic period of the liposomes. Thus, the decreased yeast survival can be attributed to the compound specific action.

Taking together these results suggest that the use of free or encapsulated C34 in combination with fluconazole could be considered for the development of an antifungal strategy. Although no substantial differences were observed between free and encapsulated C34 in both the cytotoxicity and antifungal activity the hypothesis that liposomes may confer protection to C34 and affect bioavailability should not be discarded. Moreover, given the important role of immune innate response in the control of fungal infections, C34 may be also considered as alternative. More studies are needed, with more resistant and susceptible strains to validate our hypothesis, as well as to determine the targets of C34.

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CHAPTER 7

Concluding Remarks and Future Perspectives

7.1 CONCLUDING REMARKS

The development of medicine has caused an evident increase in the number of immunocompromised individuals, being cancer chemotherapy, organ transplantation, antimicrobial therapy or abdominal surgery the main risk factors predisposing for invasive fungal infections (IFI). Fungal pathogens represent the major eukaryotic agents of nosocomial and invasive infections, being associated with high morbidity, mortality rates and hospital costs.

A number of etiological agents have been associated with fungal infections, being species of the genus *Candida* and *Aspergillus* the most frequently found. *Candida albicans* is the main causative agent of fungal infections, however other species such as *Candida tropicalis, Candida glabrata, Candida parapsilosis, Candida krusei* or *C. auris* have been increasingly recognized as pathogens with a wide distribution. Regarding *Aspergillus* sp., *A. fumigatus* is the dominant species, being *A. flavus, A. niger* and *A. terreus* also largely associated with invasive aspergillosis in severely immunocompromised individuals. Although *Candida* and *Aspergillus* species are the most frequently isolated in clinical practice, outbreaks of Mucorales infections have been reported in some developed countries, particularly caused by *Rhizopus arrhizus* (*Rhizopus oryzae*).

The rapid and correct identification of infecting species is crucial for the selection of the appropriate antifungal treatment, since species differ in their susceptibility to antifungal agents. Moreover, the fact that the risk of developing deep organ involvement and the severity of clinical manifestations differs depending on the infecting species, enhances the significance of the correct diagnosis. Species identification is also important for epidemiological purposes, for example, repeated identification of a particular species in a given hospital ward or locate may indicate a point outbreak source.

The main objective of this thesis was to contribute to the combat of fungal infections. Different approaches were used to address two main problems in this fight, the diagnosis and the antifungal treatments.

To address the first problem, a new multiplex molecular methodology was developed to identify the clinically important *Candida, Aspergillus* and *Rhizopus* species. The method is very similar with the methodologies commonly used for paternity tests, forensic analysis or diseases characterization, and is based in a PCR multiplex assay followed by capillary electrophoresis. Two panels were designed, the *Candida Panel* to identify *C. albicans, C. parapsilosis, C. glabrata, C. krusei* and *C. tropicalis* and the Filamentous Fungi *Panel* to identify *A. fumigatus, A. flavus, A. terreus, A. niger* and *R. arrhizus.* The methodology developed is able to discriminate the ten most frequent fungal species, comprising more

than 90% of all IFI. The main advantages in comparison with the other molecular methods are the great specificity, easiness, ability to identify mixed infections, the good limit of detection (1-10 pg) and results are easy to interpret. Although this methodology was not suitable to analyze bronchoalveolar lavages, it presented 89% of sensibility and 100% specificity when clinical samples from sterile places were analyzed, showing its potential to be used in the fungal identifications of IFI in clinical practice (Figure 7.1). Moreover, this method also presents high flexibility, since different fungal panels can be designed according to the laboratory needs. The fact that it uses equipment already available in microbiology laboratories in hospitals and medical centers, makes it easily adaptable to the clinical laboratory practice.

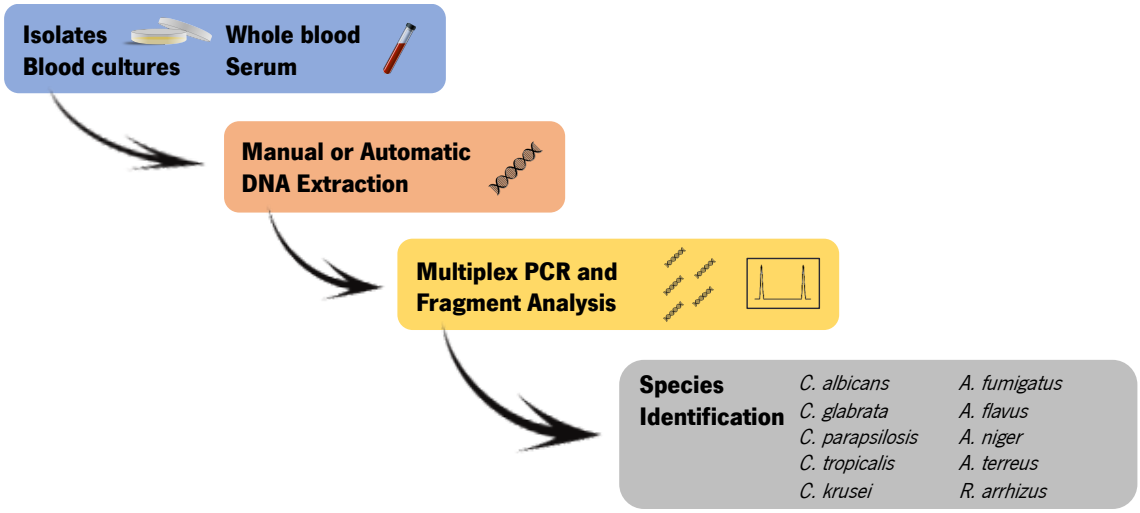


Figure 7.1 Schematic representation of the multiplex PCR methodology developed in this work.

The efficiency of the current treatments to combat fungal infections are limited by the drug toxicity and pathogen resistance as well as by the delayed diagnosis. At the moment, only three groups of antimycotics are commonly used in systemic infections, namely the polyenes, azoles and echinocandins. The loss of clinical efficacy of these drugs creates a potential risk of the spreading of resistant strains, through the emergence of clones with high pathogenic potential. Recurrent vulvovaginal candidiasis (VVC) is a mucosal infection repeatedly treated with antifungals from the azole family. Under constant selective pressure, the emergence of clones with antifungal resistance and pathogenic potential is high. In the literature, several studies showed that specific genotypes in Chinese VVC isolates were correlated with severe infection. These genotypes were identified within the *RLM1* gene, that codes for a transcription factor involved in the regulation of the yeast cell wall, in CAI locus. In this context, and since the identification of virulence factors can also aid in the definition of therapeutic strategies, we determined if

the specific genotypes correlated with severity of the infection were also present in Western populations, including in Portugal. Our results showed that VVC isolates from other geographic regions presented similar CAI alleles genotypes, which could confer a higher fitness in the vaginal environment, but not those specific from the Chinese population. Thus, no clear correlation of variability at *RLM1* gene with high pathogenic potential of strains was identified in Western populations.

In the last years, antifungal treatment recommendations have been challenged with changes in fungal epidemiology and emergence of new species and strains with a multi-resistant profile. This illustrates the need to develop new therapeutic alternatives, namely by the use of new compounds or by the combination of new compounds with the existent antifungals. In this way, to address this problem, in the present work we studied benzo[a]phenoxazine chlorides as potential new antifungal options. These compounds are organic fluorophores used for biological assays, however, they also show antiproliferative properties, having been tested as anti-tumour and antimicrobial agents and raising increased interest in the last years.

In this work, we tested the antifungal activity of five benzo[a]phenoxazine derivatives with differences in the substituents of the phenoxazine core, against the fungal species most frequently isolated from IFI, namely, seven *Candida*, four *Aspergillus* and one *Rhizopus* species. Two of the tested compounds, C13 and C34, revealed high antifungal activity against all species and strains tested, even against species intrinsically resistant to fluconazole, such as *C. krusei* and *C. auris,* displaying MIC values from 3.75 to 15 μM.

Liposomes have been used as drug delivery systems to overcome some limitations that can arise when free compounds are used, such as drug toxicity or limit bioavailability. In this way, in order to evaluate the potential interest of using the compounds in this form, we encapsulated C13 and C34 in cationic DODAB:MO liposomes testing different formulations. DODAB:MO molar ratio (1:2) encapsulation formulations presented the best characteristics, with low mean size (<200 nm) and low polydispersity (<0.4) and high positive charge (>50 mV), which favour the interaction with fungal cells. In general, encapsulation enhanced the antifungal activity of C13, but this effect was less evident with C34. Determination of MIC values for this compound, showed that encapsulation decreases the MIC in several strains, particularly *C. glabrata*, *C. bracarensis* and *C. auris*. Furthermore, free and encapsulated C34 reduced the filamentation ability of *C. albicans*, and inhibited adhesion capacity not only of *C. albicans* but also of *C. glabrata, C. parapsilosis* and *C. krusei*, two effects that may contribute to virulence attenuation and decreased pathogenesis. Besides inhibiting cell growth, C34 could also induced severe

loss of cell viability in *C. albicans* at MIC concentration, showing that its effect is not only cytostatic but markedly fungicidal.

Since C34 exhibited the best antifungal activity, this compound was further tested in combination with fluconazole, against different *Candida* species and *in vitro* in a macrophage killing assay with *C. albicans*. For these assays C34 was used encapsulated and at a much lower concentrations to avoid toxicity to the macrophages. Interestingly, the use of a non-toxic C34 formulation combined with fluconazole improved the fluconazole antifungal activity against fluconazole-resistant strains, no differences being observed for the non-resistance strains. Moreover, C34 at this low concentration (0.94 μM), induced a significative decrease of the survival of *C. albicans* during the phagocytosis process that was not dependent of its encapsulation nor on the presence of fluconazole. This effect may be related with the observed effects of the compound on filamentation, affecting the virulence ability of this specie and facilitating the immune response, but can also be due to the high fungicidal effect of C34 that may further accumulate inside the macrophages. Concluding, C34 presents potential as an antifungal alternative, especially when combined with other antifungals, deserving further studies (Figure 7.2).

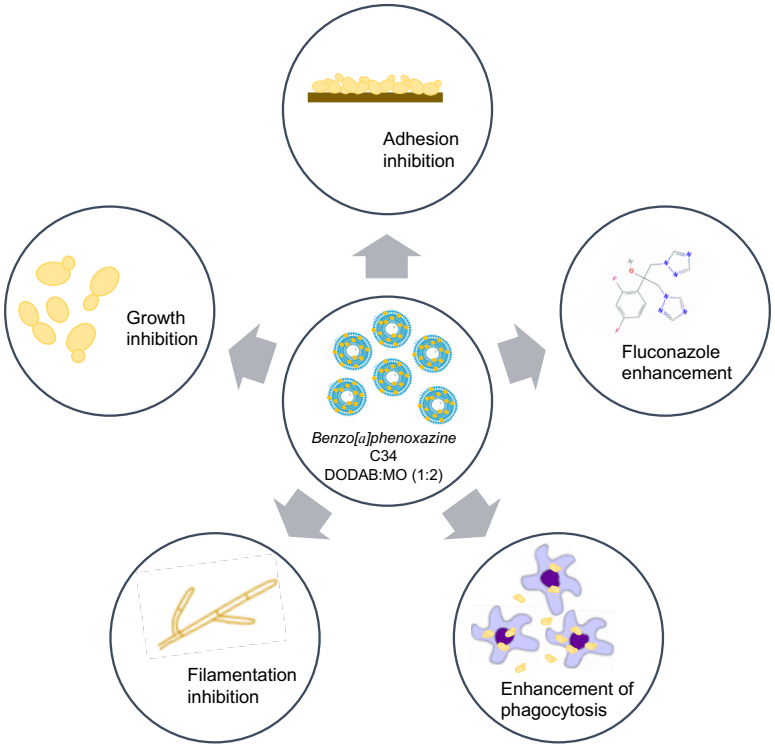


Figure 7.2 Schematic representation of the effects on fungal cells of the benzo[*a*]phenoxazine C34 studied in this work.

7.2 FUTURE PERSPECTIVES

The rapid and accurate identification of the species involved in fungal infections is of extreme relevance for the development and application of the correct therapeutic strategies. Modern molecular approaches can be used with advantage to improve identification procedures, as was the case of the PCR based methodology developed in this Thesis.

This multiplex PCR based methodology showed to be able to identify DNA of different *Candida, Aspergillus* and *Rhizopus* species isolated from different body sources and also directly from blood samples, however the number of clinical samples tested needs to be increased, including samples from patients with proved infection by all 10 selected species. *Aspergillus and Rhizopu*s species frequently affect respiratory tract, so the optimization of this methodology for the identification of fungal DNA from bronchoalveolar secretions and lavages is also of extreme importance. To solve these problems a protocol between our group and Hospital de Braga has been established, promoting the exchange of clinical samples.

Although the methodology developed identifies fungal DNA of the 10 most frequent species causative of fungal infections, other are frequently isolated, such as *Candida auris*, *Cryptococcus neoformans, Pneumocystis jirovecii, Fusarium oxysporum* or *Rhizopus microsporus*. Therefore, it is important to search for specific regions of these specie and include the specific markers in the different panels, or even design different specific panels.

Several studies on different fungal species demonstrated that genomes can accumulate gene mutations that result in phenotypic resistance to antifungals following drug exposure. The identification of genetic markers of drug-resistance is also possible with this methodology. In this way, it should be interesting to adapt the methodology developed for the identification of resistance markers, designing specific markers for resistance genes such as *FKS1* or *FCY2*.

The loss of clinical efficacy of the frequently used antifungal drugs creates a potential risk of the spreading of resistant strains, the development of new antifungal strategies being crucial. In this thesis, we showed that the benzo(*a*)phenoxazine deviate C34 presented good antifungal activity against different fungal species, however, also present high toxicity levels against macrophages cell lines. Unfortunately, encapsulation in DODAB:MO (1:2) liposomes did not decrease the toxicity levels. In this way, we intent to perform changes in C34 molecular structure and test if they affect the toxicity of this compound. We also consider important to test C34 toxicity using keratinocytes and oral/vaginal epithelial cell lines, to assess the potential use of this compound in the treatment of superficial infections. The toxicity may be assessed

using MTT assay, but also by the lactate dehydrogenase (LDH) release, which was not performed in the present work. Toxicity tests should also be performed using the compound C13. Bearing in mind the potential application of the compounds in VVC infections, it would also be important to understand the mechanism underlying the loss of the activity of the compounds at acidic pH values.

The knowledge about the targets and the cellular processes affected by C34 is extremely important to understand its mechanism of action and prevent the development of fungal resistance. To study the cell response to C34 it will be interesting to use the *S. cerevisiae* knock-out haploid mutant collection and identify the genes involved in protection or mediation of C34 antifungal action.

Co-cultures of macrophages with *C. albicans* type strain SC5314 were tested in the presence and absence of a non-toxic C34 concentration to evaluate the control of yeast proliferation (by CFU analysis) in the presence of immune cells. The results showed a decrease of *C. albicans* cells viability, probably due to the influence of this compound in yeast filamentation. However, it would be important to confirm this effect, but also to determine the inflammatory profile that C34 induces by evaluation of cytokines levels, such as TNF- a, IL-6 and IL-10, secreted by macrophages. Alternatively, C34 can induce alteration inside the macrophages that can induce the loss of cell viability of *C. albicans,* for example, by enhancing ROS production, as has been described for other benzophenoxazines by Alberti *et al*. (2003).

The combination of C34 and fluconazole showed that C34 decreases the fluconazole MICs in intrinsically resistant strains. In the future, it would be interest to understand the link between C34 activity and fluconazole resistance, namely by investigating the involvement of the efflux proteins Cdr1 and Cdr2 and Mdr1 generally associated with fluconazole resistance. Additionally, combinations should also be tested using echinocandins, such as micafungin or caspofungin.

Finally, it will be important the perform *in vivo* test of the optimized formulations or free C34, using *C. albicans* i.v. mouse model of systemic infection, to observed if our formulation is able to control the fungal burden.