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Amphotericin B fungicidal activity:

a comprehensive analysis of fungal recovery

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À minha família

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“Aqueles que passam por nós, não vão sós, não nos deixam sós. Deixam um pouco de si, levam um pouco de nós.”

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“Nada acontece por acaso.
Não existe a sorte.
Há um significado por detrás de cada pequeno acto.
Talvez não possa ser visto com clareza imediatamente, mas sê-lo-á
antes que passe muito tempo.”

(Richard Batch)

LIST OF PUBLICATIONS

Ao abrigo do artigo 8° do Decreto-Lei **n.o 388/70**, fazem parte desta dissertação as seguintes publicações:

Manuscripts

I. **Teixeira-Santos R.**, Rocha R., Moreira-Rosário A., Monteiro-Soares M., Cantón E., Rodigues A. G., Pina-Vaz C. (2012). Novel method for evaluating *in vitro* activity of anidulafungin in combination with amphotericin B or azoles. J Clin Microbiol. 50(8):2748-54. doi: 10.1128/JCM.00610-12.

II. **Teixeira-Santos R.**, Ricardo E., Gomes-Guerreiro S., Costa-de-Oliveira S., Rodrigues A. G., Pina-Vaz C. (2015). New Insights Regarding Yeast Survival following Exposure to Liposomal Amphotericin B. Antimicrob Agents Chemother 59: 6181-6187. doi: 10.1128/AAC.00575-15.

III. **Teixeira-Santos R.**, Ricardo E., Branco R. J., Azevedo M. M., Rodrigues A. G., Pina-Vaz C. (2016). Unveiling the Synergistic Interaction Between Liposomal Amphotericin B and Colistin. Front Microbiol 7:1439. doi: 10.3389/fmicb.2016.01439.

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LIST OF ABBREVIATIONS

5-CFDA	5-Carboxyfluorescein diacetate
5-FC	5-flucytosine
AIDS	Acquired immunodeficiency syndrome
AMB	Amphotericin B
AND	Anidulafungin
ATB	Automated Topology Builder
AUC	Area under the curve
AZM	Azithromycin
BMD	Broth microdilution method
BSI	Blood stream infection
CBP	Clinical breakpoint
CFU	Colony forming unit
CI	Confidence interval
CL	Clearance
CLR	Clarithromycin
CLSI	Clinical and Laboratory Standards Institute
C_{max}	Maximum (or peak) plasma concentration
CSF	Caspofungin
CST	Colistin
DAPI	4',6-diamidino-2-phenylindole
DC	Depolarized cells
DCF	2',7'- dichlorodihydrofluorescein
DCFH-DA	2,7-dichlorofluorescin diacetate
$DiBAC_4(3)$	Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol
DNA	Deoxyribonucleotide Acid
ECMM	European Confederation of Medical Mycology
ECV	Epidemiological cutoff value
ERG	Ergosterol
EUCAST	European committee on antimicrobial susceptibility testing
FC	Flow cytometry
FI	Fluorescence intensity
FICI	Fractional inhibitory concentration index
FLU	Fluconazole
FUN-1	2-chloro-4-(2,3-dihydro-3-methyl-[benzo-1,3-thiazol-2-yl]-methylidene)-1
HIV	Human immunodeficiency virus
Hsp	Heat shock protein
IA	Invasive Aspergillosis

ICU	Intensive care unit
IFI	Invasive fungal infection
L-AMB	Liposomal amphotericin B
LPS	Lipopolysaccharide
LZD	Linezolid
MCF	Micafungin
MD	Molecular dynamics
MIC	Minimal inhibitory concentration
MIF	Mean intensity of fluorescence
NPT	Isothermal-isobaric
nwt	non-wild-type
PA	Proportion of agreement
PBS	Phosphate-buffered saline
PI	Propidium iodide
PMT	Photomultiplier
POS	Posaconazole
RIF	Rifampicin
RNA	Ribonucleic acid
RNAP	RNA polymerase
ROS	Reactive oxygen species
SDA	Sabouraud dextrose agar
SI	Staining index
TET	Tetracycline
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VOR	Voriconazole
wt	wild-type
YPD	Yeast extract-peptone-dextrose

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ABSTRACT

Invasive fungal infections (IFIs) have increased significantly over the last decades. Amphotericin B (AMB) represents a very important therapeutic option for the treatment of these infections. Interestingly, despite more than 50 years of use in clinical setting, emergence of resistance to AMB is an extremely rare event. However, although the often confirmed *in vitro* susceptibility, the clinical response to AMB is sometimes reduced. While AMB is effective as a fungicidal drug, several factors can impair its clinical utility, namely underlying diseases, other concomitant administered drugs, timing of antifungal therapy, as well as AMB pharmacokinetics and distribution. The present study sought to understand this long-standing question in a biological perspective. It was evaluated the cascade of functional changes occurring in yeast cells, induced by exposure to liposomal amphotericin B (L-AMB), in a variable concentration simulating the human plasma concentration, along 24 h. Indeed, under such condition, yeast cells developed compensatory responses at distinct levels like membrane permeability, metabolic activity, and production of endogenous reactive oxygen species (ROS) during the first 6 h after the exposure to high plasma concentrations (20 to 5 mg/L); in the remaining 18 h, when exposed to much lower L-AMB concentrations (at or below minimal inhibitory concentration [MIC]), cells revealed almost full recovery with no evidence of fungicidal activity. These results highlighted the importance of monitoring and maintaining L-AMB at sufficient concentrations in plasma and tissue to ensure it produces its fungicidal effect.

Different strategies aiming to improve the clinical efficacy of AMB were also explored. Combined antifungal therapy could be a promising approach for the treatment of IFIs. However, late diagnosis and poor clinical response to antifungal monotherapy frequently promote the use of empirical antifungal combination as salvage therapy, even without scientific base. Since the methodologies available for *in vitro* evaluation of drug associations are very laborious, and thus not compatible with the daily routine of the microbiology laboratory, it was developed a novel assay using flow cytometry, based upon the classic checkerboard method. A cytometric algorithm was determined for the classification of the association between anidulafungin (AND) and AMB or azoles against yeasts as synergistic, indifferent, or antagonistic interaction. This novel protocol exhibited high agreement with the traditional checkerboard method, having the advantage of

providing quantitative results in less than 2 h. A large number of *Candida* isolates with distinct susceptibility patterns to AND, AMB and azoles, was evaluated using this new protocol. The association of AND with AMB or azoles was synergistic for a vast amount of the isolates tested. However, an indifferent effect was frequently found, and a few cases of antagonism occurred. Although this therapeutic approach seems to be promising, these results stress that the association of antifungal drugs should be conveniently evaluated *in vitro* before its clinical use.

Likewise, the combination of antifungals with antibacterial agents deserved a particular attention. The combination of L-AMB with several antibacterial agents was investigated. The association of compounds that act by inhibiting RNA/protein synthesis, namely rifampicin, azithromycin, clarithromycin, and tetracycline with L-AMB was synergistic against *Candida* spp. and *Aspergillus fumigatus*. The most effective association involved L-AMB and colistin (CST), a drug that acts by disrupting the cell membrane of gram-negative bacteria. This association was investigated at a functional and molecular level. Molecular dynamics studies demonstrated that CST forms a stable complex with L-AMB acting together in the fungal membranes. The formation of this complex enhanced and catalyzed the fungicidal activity of L-AMB, as demonstrated by functional studies.

In summary, this dissertation presented a comprehensive analysis of the AMB fungicidal activity and the fungal cell recovery mechanisms, that may justify the fungal survival and treatment failure. In addition, it elucidated about the potential beneficial effects of the combined therapy, either involving antifungal or antibacterial compounds.

RESUMO

As infecções fúngicas invasivas (IFIs) aumentaram significativamente nas últimas décadas. A anfotericina B (AMB) representa uma opção terapêutica muito importante para o tratamento destas infecções. Curiosamente, após mais de 50 anos de uso na prática clínica, a emergência de resistência à AMB é um evento extremamente raro. Apesar de ser frequentemente confirmada susceptibilidade *in vitro*, a resposta clínica à AMB é por vezes reduzida. Embora a AMB seja um fármaco fungicida, vários factores podem comprometer a sua eficácia clínica, nomeadamente doenças subjacentes, outros fármacos concomitantemente administrados, assim como o seu perfil farmacocinético. O presente estudo procurou compreender esta questão numa perspectiva biológica. Foi avaliada a cascata de alterações funcionais que ocorreram nas células de levedura, induzidas pela exposição à anfotericina B lipossómica (L-AMB), numa concentração variável que simula a concentração plasmática humana, ao longo de 24 horas. De facto, nestas condições, as células de levedura desenvolveram respostas compensatórias a níveis distintos, tais como permeabilidade de membrana, actividade metabólica e produção endógena de espécies reactivas de oxigénio (ROS), durante as primeiras 6 horas após a exposição a concentrações plasmáticas elevadas (20 a 5 mg/L); nas restantes 18 horas, quando expostas a concentrações de L-AMB muito menores (iguais ou inferiores à concentração mínima inibitória), as células mostraram recuperação quase completa, não havendo evidência da actividade fungicida do fármaco. Estes resultados destacaram a importância de monitorizar e manter a L-AMB em concentrações suficientes no plasma e nos tecidos, para garantir que esta exerce a sua actividade fungicida.

Foram também exploradas diferentes estratégias com o objectivo de melhorar a eficácia clínica da AMB. A terapêutica antifúngica combinada poderá ser uma abordagem promissora para o tratamento das IFIs. No entanto, o diagnóstico tardio e a pobre resposta clínica à monoterapia antifúngica promovem frequentemente o uso da combinação empírica de antifúngicos como terapêutica de salvação, mesmo sem base científica. Uma vez que as metodologias disponíveis para a avaliação *in vitro* das associações de fármacos são muito trabalhosas e, por isso, incompatíveis com a rotina diária de um laboratório de microbiologia, foi desenvolvido um novo ensaio por citometria de fluxo, baseado no método clássico *checkerboard*.

Foi determinado um algoritmo citométrico para a classificação da associação entre a anidulafungina (AND) e a anfotericina B ou azoles contra leveduras como uma interação sinérgica, indiferente ou antagonista. Este novo protocolo revelou uma elevada concordância com o método tradicional *checkerboard*, tendo a vantagem de fornecer resultados quantitativos em menos de 2 horas. Um grande número de isolados do género *Candida* com diferentes padrões de susceptibilidade à AND, AMB e azoles, foi avaliado usando este novo protocolo. A associação da AND com AMB ou azoles foi sinérgica para grande parte dos isolados testados. No entanto, foi frequentemente encontrado um efeito indiferente e raros casos de antagonismo. Embora esta abordagem terapêutica pareça ser promissora, estes resultados salientam que a associação de fármacos antifúngicos deve ser convenientemente avaliada *in vitro* antes da sua utilização clínica.

Do mesmo modo, a combinação de antifúngicos com agentes antibacterianos mereceu uma atenção particular. A combinação da L-AMB com vários agentes antibacterianos foi investigada. A associação de compostos que actuam pela inibição da síntese de RNA/proteínas, nomeadamente rifampicina, azitromicina, claritromicina e tetraciclina com L-AMB foi sinérgica contra *Candida* spp. e *Aspergillus fumigatus*. A associação mais eficaz envolveu L-AMB e colistina (CST), um fármaco que actua na membrana celular de bactérias gram negativo. Esta associação foi investigada a nível funcional e molecular. Estudos de dinâmica molecular demonstraram que a CST forma um complexo estável com a L-AMB, atuando em conjunto na membrana da célula fúngica. A formação deste complexo potenciou e catalisou a actividade fungicida da L-AMB, como demonstrado pelos estudos funcionais.

Em resumo, esta dissertação descreve uma análise abrangente da actividade fungicida da AMB e dos mecanismos de recuperação da célula fúngica, os quais podem justificar a sua sobrevivência e a falha da terapêutica. Além disso, elucida sobre os potenciais efeitos benéficos da combinação de fármacos, envolvendo compostos antifúngicos e antibacterianos.

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Chapter I

AIMS AND OUTLINE OF THE THESIS

AIMS OF THE STUDY

Despite the clinical success of amphotericin B (AMB) an insufficient response is sometimes reported. The aims of the present work address the fungal survival mechanisms in response to decreasing concentrations of AMB, simulating those obtained in human plasma, and to investigate strategies to improve AMB efficacy, namely antimicrobial associations. Flow cytometric studies were performed in order to characterize different physiological statuses induced by single AMB exposure, and in association with other antimicrobial drugs. In addition, computational molecular dynamics studies were carried out, aiming to unveil the underlying mechanisms of the association between AMB and synergic antimicrobials.

The specific aims of the present work were:

i. To investigate the mechanism of yeast "escape" to AMB effect, by studying the different physiological statuses induced by AMB exposure at decreasing concentrations, along a timeframe;

ii. To determine whether cell survival following AMB exposure is a phenomenon transversal through different yeast species, some of which have serious clinical implications;

iii. To develop a new, rapid and convenient method to evaluate antifungal associations, validating its *in vitro* efficacy;

iv. To improve AMB efficacy by proposing antibacterial compounds that may act synergistically with AMB, enhancing its fungicidal effect;

v. To characterize the AMB/most additive antimicrobial association at a phenotypic and functional level, and to unveil the mechanisms of synergism using computational molecular dynamics simulations.

OUTLINE OF THE THESIS

The present dissertation is divided in chapters, which include the different manuscripts published in international peer reviewed journals and works presented throughout the doctoral program in international conferences as poster presentations or abstracts published online.

Chapter I details the aims of the work and presents a brief explanation of the structure of the thesis, in order to facilitate the reading and understanding of the text.

Chapter II corresponds to the introduction section. This chapter presents the most important facts published in the last years related to the present work. It constitutes a theoretical background to support the understanding and the future discussion of the information presented in the following chapters.

Chapter III includes the second paper published in an international peer reviewed journal. It describes, for the first time, the yeast physiological mechanisms of liposomal amphotericin B (L-AMB)-induced action at plasma concentrations and a constant fixed concentration, along a timeframe. The mechanism of yeast survival to L-AMB action was explored and it has been shown that yeast cells can respond to L-AMB following different perspectives; the adoption of different stress responses can allow its full recovery.

Chapter IV includes the first paper published in an international peer reviewed journal. It describes a rapid alternative method to conventional approaches to test antimicrobial drug interactions. The flow cytometric protocol was developed to evaluate critical antifungal associations like amphotericin B or azoles with the echinocandin anidulafungin.

Chapter V includes the third paper, published in an international peer reviewed journal. It describes the improvement of the fungicidal effect of liposomal amphotericin B at subinhibitory concentrations when combined with distinct antibacterial drugs. The most synergic interaction

was scrutinized and the underlying mechanism characterized at a phenotypic, functional and molecular level.

Chapter VI includes a global discussion of the different studies presented in the previous chapters.

Chapter VII presents the general conclusions of this dissertation and describes the future perspectives aiming to establish new research topics based on the current findings for a more comprehensive study of AMB action, at a biological and clinical level.

Chapter VIII lists the bibliography accessed throughout the development of the work and thesis.

Chapter IX includes the publications associated to the present dissertation.

Chapter II

INTRODUCTION

EPIDEMIOLOGY OF INVASIVE FUNGAL INFECTIONS

Over the past two decades, the incidence of invasive fungal infections (IFIs) has increased significantly in nosocomial settings throughout the world (Pfaller et al., 2006; Pfaller and Diekema, 2007; Richardson and Lass-Florl, 2008). The emergence of such infections invariably associated with substantial morbidity and mortality rates, is directly related to: i) the increase of populations at risk, including haematopoietic stem cell transplant, solid organ transplant recipients, patients with neoplastic disease, HIV/AIDS, advanced age (>70 years), intensive care unit (ICU), surgical and burn patients (Pfaller et al., 2006; Lass-Florl, 2009); ii) the increased use of immunosuppressive and antineoplastic agents or broad-spectrum antimicrobials for a better control of underlying diseases (Pfaller and Diekema, 2007; Richardson and Lass-Florl 2008; Lass-Florl, 2009); and iii) the developments in the medical field and the practice of a more interventionist medicine, which resulted in improved survival of individuals with life-threatening illnesses, but has also contributed to a higher risk of acquisition of opportunistic fungal infections (Richardson, 2005; Shao et al., 2007; Lass-Florl, 2009).

Species of *Candida* and *Aspergillus* remain the most common causes of invasive fungal infections. However, the epidemiology of IFIs has shifted in recent years as Zygomycetes, *Fusarium* spp. and *Scedosporium* spp. have become increasingly important pathogens (Lai et al., 2008; Richardson and Lass-Florl, 2008; Oren and Paul, 2014). The reasons for the changing epidemiology of IFIs are not entirely known, but possibly are related to patient demographics and comorbidities, changes in treatment strategies, and the increased use of antifungal prophylaxis (Lass-Florl, 2009).

The complexity of the risk population and the diversity of fungal pathogens taken together with difficult and late diagnosis of IFIs often result in a poor clinical outcome, despite the antifungal treatment.

Candida species

Candida is a common inhabitant of the normal human flora (e.g. skin, gastrointestinal tract, genitourinary tract) and is also found in the environment. Despite the main reservoir of *Candida* (particularly *C. albicans*) is endogenous, infections can also arise from exogenous

sources (Pfaller et al., 2006). Candidosis can manifest as a wide range of clinical pictures, from mucocutaneous to bloodstream infections (BSIs), being the latter the most frequent manifestation of invasive candidosis (Pfaller et al., 2006; Lass-Florl, 2009).

Over the last 20 years, *Candida* spp. have become the fourth leading cause of BSIs, accounting for 8% to 10% of all BSIs acquired in hospital (Pfaller and Diekema, 2007; Oren and Paul, 2014). The annual incidence of *Candida*-associated BSIs ranged from 6 to 23 per 100 000 persons in the USA, and from 2.53 to 11 per 100 000 persons in European countries (Clark et al., 2004; Hajjeh et al., 2004; Pfaller et al., 2006; Tortorano et al., 2006). The European Confederation of Medical Mycology (ECMM) survey conducted between 1997 and 1999, reported rates of 0.20 to 0.38 per 1000 admissions by participating countries (France, Germany/Austria, Italy, Spain, Sweden and UK) (Tortorano et al., 2006). A French study performed between 2001-2002 has described an annual incidence of candidemia of 6.7 per 1000 admissions (Bougnoux et al., 2008). In Spain, the number of candidemia episodes between 2000 to 2009 has increased from 0.57 per 1000 admissions per year in 2000 to 1.52 in 2009 (Fortún et al., 2012); then, decreased to 0.89 per 1000 admissions between 2010 and 2011 (Puig-Asensio et al., 2014). In Portugal, the incidence of fungemia has decreased from 2.7 per 1000 admissions in 2004 to 0.88 in 2012, being 95% of such infections caused by *Candida* spp. (Costa-de-Oliveira et al., 2008; Faria-Ramos et al., 2014).

Population at risk for candidemia includes mostly patients admitted in the ICU, patients with solid or haematological malignancy and those undergoing abdominal surgery mainly involving the colon. Mucous membrane colonization seems to be a requisite for development of IFIs due *Candida* species, being significantly increased among critically ill patients receiving broad-spectrum antibiotics (Lass-Florl, 2009). Other risk factors for acquisition of invasive candidosis include the use of intravascular devices, catheters and parenteral nutrition, neutropenia, and the combination of such risk factors (Lass-Florl, 2009; Oren and Paul, 2014).

Candida albicans is the main cause of candidemia worldwide and was responsible for more than half of cases of infection in several reports (Pfaller et al., 2006; Tortorano et al., 2006; Lass-Florl, 2009). However, its relative frequency is decreasing, while the frequency of the non-*albicans Candida* species such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* is increasing as causative pathogens in BSI (Lass-Florl, 2009; Oren and Paul, 2014). *Candida*

species distribution is influenced by geographical area and patient's characteristics. *C. glabrata* infections are more common in the elderly; *C. parapsilosis* is more common in children and neonates, while *C. tropicalis* in patients with cancer, especially leukemia and among patients with neutropenia. *C. krusei* is frequent in immunocompromised patients and similar to *C. tropicalis*, in patients with neutropenia (Pfaller et al., 2006; Oren and Paul, 2014). In the Candida Surveillance Study conducted between 2004 and 2007, the distribution of *Candida* species in United States was *C. albicans* the most prevalent species with 43.5%, followed by *C. glabrata* with 24.8%, *C. parapsilosis* with 17.8%, *C. tropicalis* with 8.9%, *C. krusei* with 1.9%, *C. lusitaniae* with 1.3%, and other *Candida* species with 1.9% (Lyon et al., 2010). In the ECMM survey, the incidence rates for non-*albicans* species were 14% each for *C. glabrata* and *C. parapsilosis*, 7% for *C. tropicalis* and 2% for *C. krusei* (Tortorano et al., 2006). A Portuguese multicenter survey conducted between 2011 and 2012, has reported the following *Candida* species distribution: *C. albicans* with 40.4%, *C. parapsilosis* with 22.9%, *C. glabrata* with 13.3%, *C. tropicalis* with 6.3%, and *C. krusei* with 5% (Faria-Ramos et al., 2014). In several reports, the use of prophylactic fluconazole is described as a risk factor for non-*albicans* species, e.g. *C. krusei* and *C. glabrata* (Lass-Florl, 2009; Pfaller et al., 2014).

The reported mortality rates from *Candida* fungaemia range from 28% to 42% in United States, and from 28 to 59% in European surveys, and depend on the species and geographical location (Tortorano et al., 2006; Lass-Florl, 2009; Ha et al., 2012).

Aspergillus species

Aspergillus species are ubiquitous in water, soil, food and decaying materials. Their spores are frequently inhaled by humans (Shao et al., 2007; Gregg and Kauffman, 2015; Gautier et al., 2016). Aspergillosis encompasses a broad spectrum of diseases, including allergic bronchopulmonary aspergillosis, aspergilloma, chronic necrotizing aspergillosis, and invasive aspergillosis (IA) (Perfect et al., 2001). Invasive aspergillosis is a serious opportunistic infection and the second most common IFI, its incidence having increased over the last 20 years (Meersseman and Wijngaerden, 2007; Oren and Paul, 2014). The IA incidence is probably underestimated due to limitations in the diagnosis of this infection; it varies according with patient population and their environment, reaching rates of 7% in Europe (Lass-Florl, 2009; Meersseman

et al., 2004). The main affected population involves neutropenic patients with haematological malignancies (5 - 24%) and/or those receiving haematopoietic stem cell transplantation (2 - 26%) (Weber et al., 2009; Nicolle et al., 2011); patients with chronic granulomatous disease (25 – 40 %) (Denning et al., 1998), patients receiving solid organ transplantation (1 – 15%) or advanced acquired immunodeficiency syndrome (0.5 - 10 %) (Denning et al., 1998; Tong et al., 2009; Weber et al., 2009). Risk factors for IA including neutropenia, antibiotic therapy, corticosteroid therapy, cytotoxic chemotherapy, and other immunosuppressive agents (Maschmeyer et al., 2007; Shao et al., 2007; Lass-Florl, 2009; Gregg and Kauffman, 2015).

Although *Aspergillus fumigatus* is the most common cause of aspergillosis, non-*fumigatus Aspergillus* species such as *A. flavus*, *A. niger* and *A. terreus* are becoming more frequent (Shao et al., 2007; Lass-Florl, 2009). According with an international multicenter study conducted between 2000 and 2011 in critically ill patients, the incidence rates for *Aspergillus* species were: 92% for *A. fumigatus*, 3% for *A. flavus*, 1% for *A. niger* and 3% for another *Aspergillus* spp. (Taccone et al., 2015).

Mortality rates of IA are very high (50-95%), partly due to diagnostic difficulties, limited antifungal treatment options, underlying diseases of patients at risk, and also due to the lack of understanding of virulence factors involved in fungal pathogenicity and possible interaction of the pathogen with the host immune system (Binder and Lass-Florl, 2013).

Zygomycetes

Zygomycetes have emerged as increasingly important pathogens associated with high mortality rates (up to 90%) (Petrikkos et al., 2014). Microorganisms belonging to the order Mucorales (e.g. *Rhizopus*, *Mucor*, *Rhizomucor*) are most frequently implicated in human disease (Lass-Florl, 2009). Mucormycosis is the second most frequent invasive mould infection and its incidence increased from 0.7 per million in 1997 to 1.2 per million in 2006, in Europe (Petrikkos et al., 2014).

The host risk factors for mucormycosis including malignant hematological disease, prolonged and severe neutropenia, poorly controlled diabetes mellitus, iron overload, burns, and prolonged use of corticosteroids (Lass-Florl, 2009; Petrikkos et al., 2014). The clinical

manifestations and outcome of these infections is determined by the underlying diseases of patients (Petrikkos et al., 2012).

ANTIFUNGAL THERAPY

The therapeutic options for invasive fungal infections are narrow, comprising only four chemical classes, divided according to their mechanism of action (Perlin et al., 2015).

The polyenes, the most important member of which is amphotericin B (AMB), bind to ergosterol (the major sterol in the fungal cell membranes), and form complexes that induce membrane damage (Figure 1). The polyene antibiotics have broad antifungal activity against microorganisms ranging from yeasts to filamentous fungi (Carrillo-Muñoz et al., 2006).

The azoles, i.e., fluconazole (FLU), voriconazole (VOR), posaconazole (POS), impair sterol biosynthesis by inhibiting sterol 14α-demethylase, which results in ergosterol depletion and accumulation of toxic methylated sterols (Figure 1). Fluconazole is one of the antifungal agents mostly used in both prophylactic and therapeutic protocols (Tortorano et al., 2006). Its spectrum of activity includes *C. albicans*, most strains of *C. tropicalis*, and *C. parapsilosis*. Conversely, *C. krusei* is intrinsically resistant, and *C. glabrata* demonstrates reduced susceptibility to FLU (Tortorano et al., 2006). Voriconazole is indicated for the primary treatment of aspergillosis, for salvage therapy in case of severe fungal infections due to *Fusarium* sp. and *Scedosporium* sp., and in patient's refractory or intolerant to other antifungals. It exhibits a large spectrum of activity against *Candida* spp., including *C. krusei* and *C. glabrata* (Johnson and Kaufman, 2003; Kontoyiannis et al., 2005). Posaconazole has a broad spectrum of activity towards yeasts, filamentous and dimorphic fungi. However, it is less active *in vitro* against FLU-resistant *Candida* spp., especially *C. glabrata* and *C. krusei* (Nagappan and Deresinski, 2007).

The echinocandins, i.e., anidulafungin (AND), caspofungin (CSF), micafungin (MCF), target the 1,3-β-D-glucan synthase, an enzyme necessary for synthesis of 1,3-β-D-glucan, an important constituent of the fungal cell wall, impairing cell wall synthesis. This antifungal class exhibits good *in vitro* and *in vivo* activity against a range of *Candida* species and is recommended as an alternative for *Aspergillus* infections (Perlin, 2011).

Finally, the pyrimidine analog flucytosine, which is taken up into the cell by a specific transporter and then converted into 5-fluoro-uridine monophosphate by the sequential action of the enzymes cytosine deaminase and uracil phosphoribosyltransferase, causes the production of toxic nucleotides and disruption of DNA and protein synthesis (Figure 1). 5-Flucytosine (5-FC) remains an option for the treatment of *Candida* infection which are life threatening or in the cases where drug penetration may be problematic (Hope et al., 2004). However, monotherapy with 5-FC is limited due to the frequent development of resistance and its narrow spectrum of activity (Vermes et al., 2000).

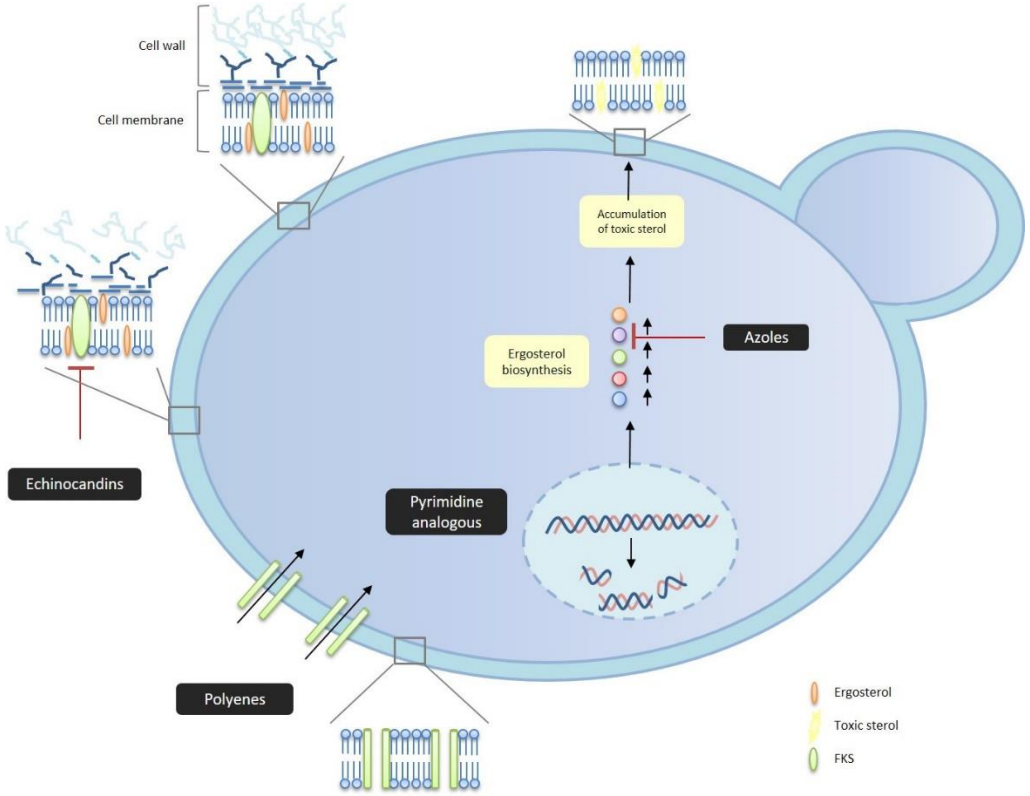

Figure 1 - Mechanism of action of antifungal drugs available for the treatment of invasive fungal infections. Polyenes bind to ergosterol, a main component of the cell membrane, forming pores in the membrane. Azoles antifungals inhibit the enzyme lanosterol 14α-demethylase, involved in the synthesis of ergosterol. This inhibition leads to the production of toxic compounds altering cell membrane structure and permeability. Echinocandins inhibit the enzyme 1,3-β-D-glucan synthase leading to alterations in the cell wall structure. Pyrimidine analogues induce the production of toxic compounds that interfere with the nucleic acid and protein synthesis.

However, many of these antifungal drugs demonstrate reduced clinical efficacy, high toxicity, and are prone to the development of resistance, what limit the antifungal options, and result in a poor clinical outcome (Zeidler et al., 2013).

AMPHOTERICIN B: A MILESTONE IN ANTIFUNGAL THERAPY

Despite the availability of new antifungal drugs over the last thirty years, amphotericin B (AMB) has remained as the last line of defense in the treatment of life-threatening systemic fungal infections (Gray et al., 2012; Anderson et al., 2014). AMB is produced naturally by *Streptomyces nodosus* and was first isolated in 1955 by Gold et al. (Gold et al., 1955). Its molecular structure is characterized by a lactone ring with 38 carbon atoms, encompassing a hydrophilic and a heptaenic chains parallel to each other (Figure 2). The hydrophilic chain of AMB contains several hydroxyl groups; in the "polar head" of the molecule are situated carboxyl and mycosamine groups (C16 and C19, respectively); in the hydrophobic portion of the molecule at position C35 another hydroxyl group is located, which confers amphiphilic properties to the AMB molecule (Figure 2) (Sternal et al., 2004; Mouri et al., 2008). This polyene has been one of the most widely used antifungal drug worldwide in the clinical practice, due to its broad spectrum of fungicidal activity, and extremely rare acquisition of resistance. Its use along more than 50 years is a case study in clinical medicine. However, its use is somewhat hampered by the incidence of side effects such as nephrotoxicity. Additionally, AMB exhibits hepatotoxic activity, and in higher doses neurotoxic and hemolytic activities (Gagós and Arczewska, 2010). Such faits render the conventional amphotericin B deoxycholate formulation unsuitable for treatment. In an attempt to improve the delivery of the drug and the therapeutic index of amphotericin B, three lipid-associated formulations were developed, including liposomal amphotericin B (L-AMB), amphotericin B lipid complex, and amphotericin B colloidal dispersion. The lipid composition of all three of these preparations differs considerably and contributes to different pharmacokinetic parameters (Hamill, 2013).

Mechanism of action

Studies on amphotericin B mechanism of action revealed that its antifungal activity is very complex. For decades, the prevailing mechanism of action has been that AMB binds to ergosterol, inserting into the fungal cytoplasmic membrane, and forms pore-like structures, resulting in osmotic instability, loss of membrane integrity, metabolic disruption, ultimately killing fungal cells (Gray et al., 2012; Anderson et al., 2014; Gonçalves et al., 2016). Additional studies also propose

the hypothesis that AMB induces common oxidative damage death pathways (Belenky et al., 2013).

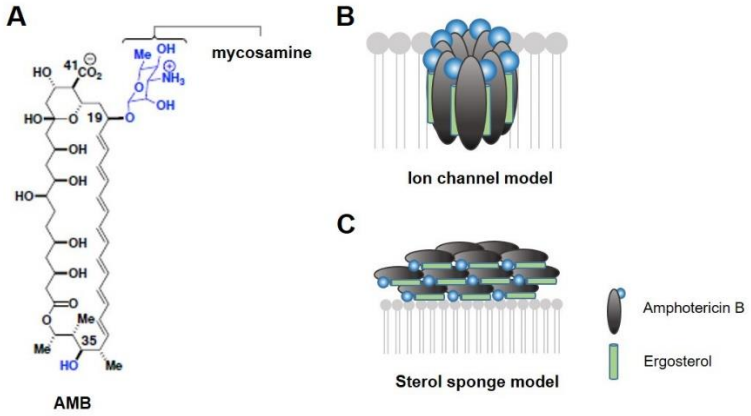

Figure 2 - Models for the structure and function of amphotericin B. (A) Molecular structure of AMB. (B) The classic ion channel model for the structure and function of AMB. (C) A new sterol sponge model, in which AMB primarily exists in the form of large extramembranous aggregates that extract ERG from lipid bilayers (Anderson et al., 2014).

Transcriptomic studies supported the classic AMB action; it was demonstrated that AMB induced alterations in expression of genes involved in cell stress, membrane reconstruction, transport, and cell wall integrity (Zhang et al., 2002; Agarwal et al., 2003; Liu et al., 2005; Ko et al., 2009). Proteomic analysis has been also used to unveil the adaptive response of *Candida* to AMB. Hoehamer et al., have demonstrated that exposure to AMB impaired the abundance of 43 proteins, including those associated with oxidative stress, osmotic tolerance, and carbohydrate metabolism (Hoehamer et al., 2010). The changes of these functional classes of genes/proteins are consistent with the mechanism of action previously proposed. According to this model, the cytotoxic activity of AMB is dependent on the ion channel-forming (Anderson et al., 2014). Importantly, it has long been known that lipid membranes containing sterols are extremely vulnerable to permeabilization by AMB, being the AMB affinity higher for ergosterol (ERG) compared with its affinity for cholesterol; however, there was the doubt whether AMB effect was due to indirect sterol-mediated general changes in membrane properties or direct sterol binding.

Recent structural, molecular and biophysical studies, including computer modeling, spectroscopy, microscopy, show that the channel forming capacity of AMB is not required for fungicidal activity, whereas binding ergosterol is crucial (Bolard, 1986; Baginski et al., 2002;

Milhaud et al., 2002; Volmer et al., 2010). Indeed, the ergosterol plays an important role in fungal cell physiology such as vacuole fusion, cell division, endocytosis, cell signaling, membrane compartmentalization, and functional regulation of membrane proteins. Therefore, ergosterol binding prevents its participation in multiple cellular functions, thus leading to fungal cell death. These studies suggest that AMB primarily kills fungal cells by simply binding ergosterol, and the membrane permeabilization via channel formation represents a second complementary mechanism that further increases drug potency and the rate of yeast killing. Anderson et al. have explored the primary structure and function of AMB in the presence or ERG-containing phospholipid membranes and reported that AMB exists primarily in the form of large, extramembranous aggregates, which kill fungal cells by extracting ergosterol from lipid membranes (Figure 2) (Anderson et al., 2014). The membrane-inserted ion channels are relatively minor contributors, both structurally and functionally, to the antifungal action of this natural product. Moreover, the ion channels formation is strongly dependent on the molecular organization of AMB; only AMB dimers and N-aggregates can cross through the lipid membrane (Gagós and Arczewska, 2010).

Pharmacokinetics and distribution

The effectiveness of the treatment of fungal infections depends on the drug pharmacokinetics and distribution and its adequate penetration and retention at the sites of infection. Liposomal amphotericin B (AmBisome) is a lipid formulation that consists in amphotericin B in small, unilamellar lipossomes (Bekersky et al., 2002). Amphotericin B lipid formulation is safer than conventional AMB (amphoterin B deoxycholate), having the advantage to alter the disposition of AMB in the body and thereby decrease the toxic side effects. The reduced toxicity of L-AMB allows to increase the dose and may contribute to improved antifungal effectivity (Heinemann et al., 1997). Data on the pharmacokinetics of L-AMB are scarce in the literature. However, studies in animals and humans have shown that liposomal amphotericin B results in higher drug levels in plasma than other formulations as well as higher levels of amphotericin B in tissue (Walsh et al., 1998; Bekersky et al., 2002). This is a result of the unique composition of the L-AMB liposomes, which contain rigid, charged phospholipids and cholesterol to retain amphotericin B within the bilayer membranes of the circulating liposomes. Liposomal

AMB remains unchanged in the circulation and distributes as intact liposomes to tissues (Adler-Moore and Proffitt, 1993, 1998). Therefore, the pharmacokinetics of L-AMB differ significantly from those of conventional AMB. According with a study performed by Bekersky et al. in healthy volunteers, the plasma concentrations during the first 24h are 8- to 16-fold higher with L-AMB (C_{max}, 22.9 ± 10 mg/L) than with amphotericin B deoxycholate (C_{max}, 1.4 ± 0.2 mg/L); although the two formulations had similar half-lives, the urinary and fecal clearances (CL) of L-AMB are 10-fold lower than those of AMB deoxycholate (Bekersky et al., 2002). Another study involving critically ill patients has demonstrated that when the patients were administrated L-AMB in a dose 3-fold higher than AMB deoxycholate, the median C_{max} and AUC values were 8.4-fold (14.4 versus 1.7 mg/L) and 9-fold (171 versus 18.65 mg · h/L) higher, respectively, for L-AMB than for AMB deoxycholate. This study also has demonstrated that the median V (volume of distribution) and CL were lower for L-AMB than for AMB deoxycholate (Heinemann et al., 1997). Walsh et al., in a study with neutropenic patients obtained similar results (Walsh et al., 1998). The authors have suggested that the low volume of distribution of L-AMB occurs due to a decreased interaction of AMB with proteins and/or membrane cholesterol, which contribute to a greater peak plasma concentrations and AUC values; the reduced clearance might be related with the fact that L-AMB is eliminated from plasma mainly by the reticuloendothelial system (Heinemann et al., 1997; Walsh et al., 1998; Bekersky et al., 2002). It is also important to stress that most fungal infections are located in the tissue, such as the lung, kidney, liver, and spleen. In these organs, AMB levels given as L-AMB are maintained above the MIC for many species for at least 1 week and longer, depending of the tissue (Adler-More and Proffitt, 2003; Smith et al., 2007).

Spectrum of activity

Amphotericin B exhibits a broad spectrum of activity. It is active against most *Candida* species and *Cryptococcus neoformans* as well as many molds, including *Aspergillus* spp., *Fusarium* spp. and Zygomycetes (Chandrasekar, 2011). Most *C. albicans* and *C. parapsilosis* might be considered to be fully susceptible to AMB, although there have been occasional reports of resistant clinical isolates (Ellis, 2002). While *C. tropicalis* and *C. guilliermondii* are also considered susceptible, can *C. glabrata* and *C. krusei* exhibit decreased susceptibility to AMB (Ellis, 2002; Pfaller and Diekema, 2007). Despite some strains of *C. lusitaniae* have been reported

to be resistant to AMB, most strains appear to be susceptible when tested in laboratory. Regarding the molds, most species of *Aspergillus* are susceptible to AMB; however, a reduced susceptibility to amphotericin B has been described among isolates of *A. flavus* and *A. terreus* (Barchiesi et al., 2013; Gregg and Kauffman, 2015).

Development of resistance

After more than 50 years of use as monotherapy, the acquisition of amphotericin B resistance remains extremely rare, contrary to azoles and echinocandin drugs. Resistance to AMB is an uncommon phenomenon in *Candida* (1-3%, particularly in *C. lusitaniae*), and *Aspergillus*, although a considerable proportion of *A. terreus* and *A. flavus* has higher MIC values (McClenny NB et al., 2002; Pfaller and Diekema, 2007; Cuenca-Estrella, 2014, Gonçalves et al., 2016). Because the lack of clinical strains with resistance to amphotericin B, few studies addressing the molecular resistance mechanisms have been reported. It has been published that resistance to amphotericin B is associated with mutants with low levels of ergosterol and/or prior exposure to azole antifungals, and disturbance of the levels and composition of the phospholipids in the membrane. Some of these changes have been associated with mutations in genes involved in ergosterol biosynthesis ERG2, ERG3, ERG5, ERG6, and ERG11 (Vincent et al., 2013). Furthermore, it was described that perturbations in ergosterol biosynthesis can lead to general increases in the accumulation of diverse small molecules involved in stress response. One of the most powerful and highly conserved adaptation mechanisms comprises the heat shock proteins (Hsps). Hsps function as molecular chaperones, which repair and adapt to cell damage caused by aggregated proteins and ensure proper folding of newly synthesized proteins (Blatzer et al., 2015). It is already described the critical role of Hsp90 in the evolution of AMB resistance in *Candida* and *Aspergillus* (Vincent et al., 2013; Blatzer et al., 2015). Hsp90 also promotes the maturation of a diverse array of metastable signal transduction proteins (known as Hsp90 clients) that function in many stress response pathways. Normally, these client proteins are nonessential stress responses; however, they become essential in AMB-resistant strains. Vincent et al., has demonstrated that Hsp clients, calcineurin and protein kinase C pathways are required to tolerate the stresses imposed by resistance mutations. In turn, the MAP-Kinase Hog1 only is required to tolerate the stress induced by AMB exposure (Vincent et al., 2013; Cohen, 2014). In addition, the

polyenes induce oxidative stress in fungal cells that lead to development of robust stress responses to counteract the antimicrobial action. It was described that the less susceptible isolates can have higher levels of antioxidative enzymes and/or alterations in the production of free radicals (Cohen, 2014; Cuenca-Estrella, 2014; Gonçalves et al., 2016). According to Vincent et al., every mutation that can confer robust AMB resistance came at great cost to the pathogen, and therefore these phenotypes do not become prevalent in the clinic (Vincent et al., 2013). Therefore, its expression at a clinical level may ultimately be somewhat reduced and such strains are not prone to thrive and colonize the human host.

Antifungal susceptibility testing

In recent years we have seen major advances in susceptibility testing for fungi. The need for reproducible and clinically relevant antifungal susceptibility testing has been encouraged by the increasing number of IFIs, the expanding use of antifungal agents, and the development of antifungal resistance (Ellis, 2002; Pfaller and Diekema, 2012). There are two internationally recognized standard methods for the performance of antifungal susceptibility testing of yeast and molds, both using broth microdilution (BMD): the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2008a,b, 2012) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST 2017a,b). Although the CLSI method has provided reliable and reproducible results, it generates a restricted range of amphotericin B minimal inhibitory concentrations (MICs), precluding reliable discrimination between susceptible and resistant isolates of *Candida* species and preventing the development of clinical interpretive breakpoints (CBPs) for AMB *in vitro* testing (Park et al., 2006). Analyses of both clinical trial data and clinical and microbiological data from population-based surveillance studies for *Candida* species also have failed to establish any clinical correlation between amphotericin B MICs, as determined by CLSI BMD and clinical outcome (Pfaller and Diekema, 2012). While for the CLSI protocol there are no CBPs defined for the *Candida* species, the epidemiological cutoff value (ECV) for AMB is 2 mg/L for all species. Whenever the MIC value is ≤ 2 mg/L the strain is wild-type (WT, without mutational or acquired resistance mechanisms); whenever the MIC value is > 2 mg/L the strain is non-WT, having mutational or acquired resistance mechanisms (Pfaller and Diekema, 2012). Regarding *Aspergillus* spp., the ECVs for the CLSI method are 2 mg/L for *A. fumigatus* and *A. flavus*, and 4

mg/L for *A. terreus*; whereas the CBP is 1 mg/L for all *Aspergillus* species (Elefanti et al., 2014). The EUCAST method has already determined AMB clinical breakpoints for *Candida* spp.; *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* are S whenever MIC ≤ 1 mg/L and R when MIC > 1 mg/L (Lass-Florl et al., 2011). For *Aspergillus* spp., the ECVs are 1 mg/L in case of *A. fumigatus* and 4 mg/L for *A. flavus* and *A. terreus*; in turn, the susceptible breakpoint is 1 mg/L for all species (Arendrup et al., 2012; Elefanti et al., 2014). In addition to lack of consensus between CLSI and EUCAST methods, its essential agreement is often poor for some species of *Candida*, namely *C. albicans* and *C. glabrata* (Pfaller et al., 2014). The standardization of reference methods by CLSI and EUCAST has led to the development of several automated or semiautomated commercial systems like the Sensitrite YeastOne, Etest, and Vitek 2. The first is a broth microdilution method that determines the susceptibility profile of yeast and filamentous fungi; the Etest is a susceptibility method using a strip with a predefined concentration gradient of the antimicrobial agent that provides the MIC value, which is difficult to interpret for both yeast and molds; Vitek 2 is an automated commercial method that allows both yeast identification and MIC determination (Ellis, 2002; Canton et al., 2009; Cuenca-Estrella et al., 2010). Since they all dependent of cell growth, all these methods are time consuming, and often provide contradictory results. Recently, flow cytometry (FC) has demonstrated to be a valuable tool for evaluation of antifungal susceptibility testing in yeasts, since it can be used to detect different cellular physiological status induced by the antifungal action by means the use of appropriate fluorescent dyes (Chaturvedi et al., 2004; Czechowska et al., 2008; Pina-Vaz and Rodrigues, 2010). FC susceptibility testing to azoles, amphotericin B, and echinocandins has already been described (Kirk et al., 1997; Ramani et al., 1997; Pina-Vaz et al., 2001a, 2001b and 2010; Rudensky et al., 2005). This new methodology has the advantage of providing timely results (4h *versus* 24 or 48h of available methods). Several authors have considered that routine antifungal susceptibility testing can serve as an adjunct in the treatment of IFIs (Collins et al., 2007; Perkins et al., 2005); however, susceptibility testing is not always performed routinely because the available methods are cumbersome and the time to results is high, what means that patients are often treated empirically. In addition, the lack of well-established clinical breakpoints for AMB and the consequently poor correlation with the clinical outcome may impair the clinical success.

Clinical outcome

Despite the confirmed *in vitro* susceptibility (0.125 to 1 mg/L), *in vivo* response to liposomal amphotericin B is somewhat reduced and an unfavorable outcome is reported in about 40% of the treated patients (Ullmann et al., 2006; Moen et al., 2009). The exact reasons for such dismal response remain unclear. Some authors do not associate this response failure with target modification as it happens with other antifungal drugs, but with inappropriate concentration of amphotericin B at the infection site (Liao et al., 2003; Shapiro et al., 2011; Vincent et al., 2013).

Although the underlying diseases, immunosuppression, concomitant therapies, toxicity, and timing of antifungal therapy affect the mortality of these infections, pathogen susceptibility and AMB plasma and tissue concentrations also represent crucial contributing factors for the success of therapy, that deserve future elucidation in order to obtain an improved clinical response (Elefanti et al., 2014).

COMBINATION THERAPY: THE EFFECT OF AMPHOTERICIN B IN ASSOCIATION WITH OTHER COMPOUNDS

Monotherapy regimens are often ineffective against IFIs, resulting in an unfavorable outcome. Combination therapy may thus represent a beneficial therapeutic option, although there are few scientific studies showing the potential advantages of such approach.

i. With antifungal compounds

The association of AMB with anidulafungin was described as having a synergic interaction against *Candida* spp. (Rosato et al., 2012; Valentin et al., 2012). Several studies have also described that the combination of AMB with caspofungin, another echinocandin, resulted in a synergistic effect against *Aspergillus* spp., *Fusarium* spp. and *C. glabrata* (Arikan et al., 2002; Kiraz et al., 2009; Liu et al., 2012). Nishi et al., have described that the combination of micafungin and AMB also had a benefic effect against *C. glabrata* (Nishi et al., 2009). Likewise, the combination of 5-fluorocytosine and AMB has showed to improve the antifungal effect upon *Cryptococcus neoformans*, even in flucytosine-resistant isolates (Schwarz et al., 2007). Recently, Gazzoni et al., have described that voriconazole in association with AMB results in a synergistic interaction against *C. neoformans* (Gazzoni et al., 2012).

Although the association of antifungal drugs with different mechanisms of action seems to be benefic for the treatment of IFIs, in the cited studies, the effect of drug association was often dependent on the tested isolate. It should be stressed that the methods for evaluation the *in vitro* drugs interaction are cumbersome and difficult to interpret, and sometimes provide contradictory results.

ii. With antibacterial compounds

Since 1970s synergistic effects between tetracycline (TET) and AMB against *C. albicans* and other pathogenic fungi were described (Oliver et al., 2008). Tetracycline has a direct effect upon the mitochondrial function in fungi. According to the authors, the inhibition of the mitochondria function in fungal cells by TET, impairs the sterol metabolism, resulting in lower sterol levels. Thus, cells exposed to TET are more susceptible to AMB due to a higher ratio AMB/ergosterol at the cell surface (Oliver et al., 2008). Clancy and Nguyen, have showed that the combination of AMB and azithromycin (AZM) improved the antifungal effect, reducing AMB minimal inhibitory concentration in *Fusarium* spp. (Clancy and Nguyen, 1998). Azithromycin acts in bacterial cells by inhibiting protein synthesis. While the mechanism of synergism between these two compounds remains unclear, it has been postulated that AMB, by damaging the fungal cell membrane, may facilitate the entrance of AZM into the cells; once inside the cells AZM might act by inhibiting fungal protein synthesis (Clancy and Nguyen, 1998). Several studies have demonstrated that the combination of AMB with rifampicin (RIF) results in a synergistic effect (Ansehn et al., 1976; Srimuang et al., 2000; El-Azizi, 2007). This effect could be related to the fact that AMB binds to the sterols in the fungal cell membrane, thus increasing the antibacterial permeability and facilitating the RIF action on RNA synthesis (Azevedo et al., 2015). A recent study has also described synergistic effects between AMB and clarithromycin (CLR) (Del Pozo et al., 2011). Fungal cell membrane damaged by AMB action, may allow the entrance of CLR, inhibiting the protein synthesis (Azevedo et al., 2015). Recently, Zeidler et al., have reported the synergism of colistin with AMB (Zeidler et al., 2013). Attending to such studies, the association between antibacterial agents and AMB could represent a very interesting therapeutic approach.

Chapter III

NEW INSIGHTS REGARDING YEAST SURVIVAL FOLLOWING EXPOSURE TO LIPOSOMAL AMPHOTERICIN B

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ABSTRACT

In vitro resistance to amphotericin B is an extremely rare event among pathogenic yeasts. However, *in vivo* response is sometimes reduced, resulting in an unfavorable outcome. Such adverse outcomes might be related to subfungicidal plasma concentrations. We aimed to clarify the mechanisms of liposomal amphotericin B (L-AMB, AmBisome)-induced lesions and the mechanisms responsible for yeast cell recovery following exposure at plasma concentrations. The physiological statuses developing following exposure to L-AMB at simulated plasma concentrations (20 to 0.1 mg/L) and at a constant concentration (3 mg/L) were assessed in a 24 h time course assay. Time-kill experiments were also carried out under the same L-AMB treatment conditions.

Our results suggest that yeast cells develop compensatory responses related to membrane polarization, metabolic activity, and reactive oxygen species (ROS) production after exposure to high plasma concentrations (20 to 5 mg/L) during the first 6 h; in the remaining 18 h, when exposed to lower concentrations, cells reveal almost full recovery with no evidence of fungicidal activity. In contrast, whenever cells are exposed to a constant concentration above the MIC, despite initially exhibiting compensatory stress responses, soon afterwards they exhibit membrane depolarization, a decrease of metabolic activity, increasing ROS production, and lastly, programmed cell death and necrosis, thus succumbing to L-AMB fungicidal effect.

This study may represent a step forward in the support of L-AMB use for clinical treatment of invasive fungal infections, since it demonstrates the importance of maintaining the levels of L-AMB above the MIC in the plasma and the tissues to ensure it produces its fungicidal effects.

BACKGROUND

The understanding of how fungal organisms respond to antimicrobial therapy is a relevant question both in terms of evolutionary biology and for treatment of invasive fungal infections. During recent decades, fungi have emerged as major human pathogens; *Candida albicans* represents the fourth most common agent of all hospital-acquired infections (Pfaller and Diekema, 2007).

Despite over 50 years of use as monotherapy, amphotericin B (AMB) still represents an important therapeutic alternative for the treatment of systemic fungal infections, particularly when infection persists despite treatment with alternative drugs (Mora-Duarte et al., 2002). Amphotericin B belongs to polyene drug class and exhibits a broad-spectrum fungicidal activity. For decades, the prevailing mechanism of action has been that AMB primarily binds to ergosterol, inserts into the cytoplasmic membrane, and forms pore-like structures; the result is osmotic instability, loss of membrane integrity, metabolic disruption, and ultimately cell death (Zhang et al., 2002; Gray et al., 2012). Recently, Anderson et al. proposed a new mechanism of action of AMB. Accordingly, amphotericin exists primarily in extramembranous aggregates that kill yeast cells by extracting ergosterol from the plasma membrane. Consequently, membrane ergosterol depletion will interfere not only with cell membrane integrity but also with other cellular processes which highly depend on membrane ergosterol (Anderson et al., 2014).

The development of genetic resistance to AMB among *Candida* species remains extremely rare, in contrast to what is observed with other drugs in the triazole or echinocandin classes (Martel et al., 2010; Walker et al., 2010). However, in spite of the observed high *in vitro* susceptibility (0.125 to 1 mg/L), the *in vivo* response to AMB is somewhat reduced in about 40% of treated patients (Ito and Hooshmand-Rad, 2005; Park et al., 2006; Larsen et al., 2011). The exact reasons for this lower-than-expected response still remain unclear. Some authors do not associate response failure with target modification, as has been observed with other antifungal drugs (Shapiro et al., 2011), but with inappropriate concentrations of AMB at the infection site (Liao et al., 2003). In accordance with this hypothesis, cells exposed to amphotericin B may exhibit different physiological conditions which are related to drug concentration and exposure time (Liao et al., 2003). In addition, studies of yeast apoptosis have revealed the occurrence of a

programmed cell death or a cellular necrotic response depending on the AMB concentration (Phillips et al., 2003; Al-Dhaheri and Douglas, 2010).

Considering the clinical relevance of liposomal amphotericin B (L-AMB) and based on plasma levels described previously (Walsh et al., 1998), this study aims to explore yeast survival mechanisms in response to L-AMB in order to identify different physiological conditions following exposure to decreasing concentrations in a time course assay. In addition, we intend to determine whether cell survival following exposure to L-AMB is a common phenomenon among different yeast species, some of which are relevant clinical pathogens.

Our results provide novel insights regarding mechanisms by which yeast cells can escape to L-AMB fungicidal action depending upon the time of exposure and the concentration of the drug.

MATERIAL AND METHODS

Yeast strains and growth conditions

A broad range of pathogenic and nonpathogenic yeasts were grown in yeast extract-peptone-dextrose (YPD) liquid medium (1% yeast extract, 2% peptone, 2% dextrose; Formedium, Norfolk, United Kingdom) at 35 °C with shaking (150 rpm) until exponential growth phase (details are presented in Table 1) (Costa-de-Oliveira et al., 2013). All of the strains were subjected to antifungal susceptibility testing, cell viability assays, and membrane potential evaluation. For membrane integrity, metabolic activity, reactive oxygen species (ROS) production, and apoptotic assays, only *Saccharomyces cerevisiae* BY4741 was used as model organism. Prior to experiments, yeasts were subcultured twice in YPD agar to ensure the purity of cultures.

Liposomal amphotericin B susceptibility

The MIC of L-AMB (provided by Gilead Sciences, Inc, San Dimas, California) was determined according to the M27-A3 protocol and M27-S3 supplement of the Clinical and Laboratory Standards Institute (CLSI) (Wayne PA, a2008, b2012). MIC values were determined after 24 and 48 h of incubation with L-AMB; MIC was the lowest concentration that prevented any discernible growth. Due to a lack of established clinical breakpoints for AMB, the *Candida* isolates were considered “wild-type” (wt) whenever MIC ≤ 2 mg/L and “non-wild-type” (nwt) when MIC > 2 mg/L, according to the epidemiological cutoff values (ECVs) proposed by Pfaller and Diekema. For non-*Candida* genera, it is only displayed the MIC value, since amphotericin B ECVs and clinical breakpoints remain yet undefined (Pfaller and Diekema, 2012). *C. albicans* ATCC 90028 reference type strain was used as recommended by the CLSI protocol.

Evaluation of cell viability

Yeast suspensions (10^6 yeast cells/mL) were exposed to L-AMB, in accordance with the plasma levels established by Walsh et al. (Walsh et al., 1998). Briefly, cell suspensions were treated with L-AMB 10 mg/L for 30 min; afterwards, cells were harvested by centrifugation at 1,610 x *g* for 10 min at room temperature (Universal 320R; Hettich). The same cells then were resuspended in fresh culture medium and exposed sequentially to serial concentrations of L-AMB:

20 mg/L (for 30 min), 10 mg/L (for 2 h), 5 mg/L (for 3 h), 1 mg/L (for 6 h) and 0.1 mg/L (until 24 h) (detailed in Figure 1A). Another cell suspension was treated with 3 mg/L L-AMB for 24 h. During that period at 1, 3, 6 and 12 h of treatment, those cells were harvested (Universal 320R; Hettich) and resuspended in fresh medium containing L-AMB 3 mg/L, similar to the protocol developed for the cells exposed to various dosages of L-AMB (plasma conditions). Following 1, 3, 6 and 24 h from start of incubation, the number of viable cells was determined by plating on YPD agar and incubating at 35°C for 24 h; the number of CFUs was determined and compared with plate control (not exposed to L-AMB). Before being plated, cells were washed and resuspended in fresh medium in order to prevent antifungal carryover.

Functional characterization of liposomal amphotericin B-induced action

The physiological status of yeast cells which developed following L-AMB exposure to decreasing concentrations or a constant concentration of L-AMB in a time course assay were assessed by flow cytometry. A cell suspension corresponding to 10^6 yeast cells/mL was used in all assays described below. Yeast cells were incubated with various concentrations of L-AMB, in accordance with the scheme previously proposed (Figure 1A). At 1, 3, 6 and 24 h, aliquots were collected and tested; all cytometric evaluations were performed in a standard FACSCalibur cytometer (BD Biosciences, Sydney, Australia) equipped with 3 photomultipliers (PMTs), standard filters, and a 15-mW 488-nm Argon laser using CellQuest Pro software (version 4.0.2). All trials were performed in triplicate.

Assessment of membrane integrity

Cell membrane integrity was assessed with propidium iodide (PI; Sigma-Aldrich, Munich, Germany) staining. After antifungal treatment, yeast cells were stained with 1 mg/L of PI for 30 min at 35°C at 150 rpm in the dark (Pina-Vaz et al., 2005). The fluorescence intensity (FI) was measured at FL3 (630 nm). The amount of injured cells in each sample was defined as the percentage of PI-positive cells.

Assessment of membrane potential

The effect of L-AMB exposure on cell membrane potential was evaluated using Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol [$DiBAC_4(3)$: Sigma-Aldrich, Munich, Germany] as described by Teixeira-Santos et al. (Teixeira-Santos et al., 2012). After L-AMB treatment, cells were incubated for 15 min in the dark at 35 °C, at 150 rpm, with 0.5 mg/L of $DiBAC_4(3)$. The FI was registered at FL1 (530 nm).

Assessment of metabolic activity

Metabolic changes induced by L-AMB in *S. cerevisiae* were evaluated using 5-Carboxyfluorescein diacetate (5-CFDA; Sigma-Aldrich, Munich, Germany), at 10 µM final concentration. Antifungal-treated cell suspensions were stained with 5-CFDA and incubated for 45 min, at 35 °C, at 150 rpm in the dark (Liao et al., 2003). The mean intensity of fluorescence (MIF) was registered at FL1 (530 nm).

Assessment of endogenous ROS production

ROS production was assessed as previously reported (Yan et al., 2009). In brief, the cell suspension was incubated with 20 mg/L of 2,7-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich, Munich, Germany) for 30 min at 35°C at 150 rpm. Yeast cells were washed (2,655 x *g* for 5 min at room temperature; 5417R, Eppendorf) and resuspended PBS (Sigma-Aldrich, Munich, Germany); afterwards, cells were treated as described above. As control, cells were treated with H_2O_2 0.4 mM (PanReac, Castellar del Valles, Spain). The FI was determined at FL1 (530 nm). ROS production was calculated by subtracting the FI value of cells treated with simple antifungal agent from that of cells treated with both antifungal agent and DCFH-DA.

TUNEL assay

DNA strand splitting was demonstrated by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) with the *in situ* cell death detection kit, fluorescein, from Roche (Mannheim, Germany). Yeast cells were fixed with 3.7% (vol/vol) formaldehyde (Applichem, Darmstadt, Germany) for 30 min at room temperature and washed thrice with PBS (2,655 x *g* for 5 min at room temperature; 5417R, Eppendorf). Afterwards, cell

walls were digested with 24 mg/L of lyticase (Sigma-Aldrich, Munich, Germany) at 37°C for 60 min. Cells were rinsed with PBS, incubated in permeabilization solution (0.1% [vol/vol] Triton X-100 and 0.1% (wt/wt) sodium citrate) for 2 min on ice, and rinsed twice with PBS (5417R, Eppendorf). For a positive control, cells were treated with DNase I enzyme (Roche, Mannheim, Germany) for 10 min and then washed twice in PBS (5417R, Eppendorf). Yeast cells subsequently were incubated with 15 µL of TUNEL reaction mixture, for 60 min at 37°C and then washed twice in PBS (5417R, Eppendorf). Finally, cell suspensions were submitted to flow cytometric analysis, and the percentage of apoptotic cells was determined at FL1 (530 nm).

Simultaneously, 10 µL of the treated cell suspension was placed in a microscope slide, and the protocol for 4′,6-diamidino-2-phenylindole (DAPI; Roche, Mannheim, Germany) nuclear staining was carried out as previously described, using 1 mg/L of DAPI (Almeida et al., 2007), for further analysis under fluorescence microscopy with a Carl Zeiss Axiovert inverted microscope and laser wavelengths of 405 nm (DAPI) and 488 nm (TUNEL).

Statistical analysis

Results are presented as mean value and the respective standard deviations. A comparison of results was performed using paired-samples Student's *t* test. $P < 0.05$ was considered significant. For calculations of all measures, the SPSS program (version 19.0) was used.

RESULTS

Liposomal amphotericin B effect on cell viability

Our primary goal in this study was to determine the effect of therapeutic plasma concentrations of L-AMB upon cell viability. We focused on L-AMB effects within the first 24 hours of exposure, a time period corresponding to the first drug infusion. The effect of L-AMB on yeast cells was evaluated by colony forming units count following incubation with decreasing concentrations of L-AMB (Figure 1B). As detailed in Figure 1B, a slight reduction of viability was observed for up to 6 h of exposure; however, after 6 h of exposure to decreasing concentrations, cells recovered their replication ability with this effect being most marked after 24 h of exposure (10^6 to 10^9 cells/mL). Conversely, in the case of cells exposed to L-AMB 3 mg/L for 24 h, a growth reduction was consistently registered throughout the 24 h period, and the cells were unable to replicate. At 24 h, a significant difference between both experimental conditions was registered ($p < 0.001$).

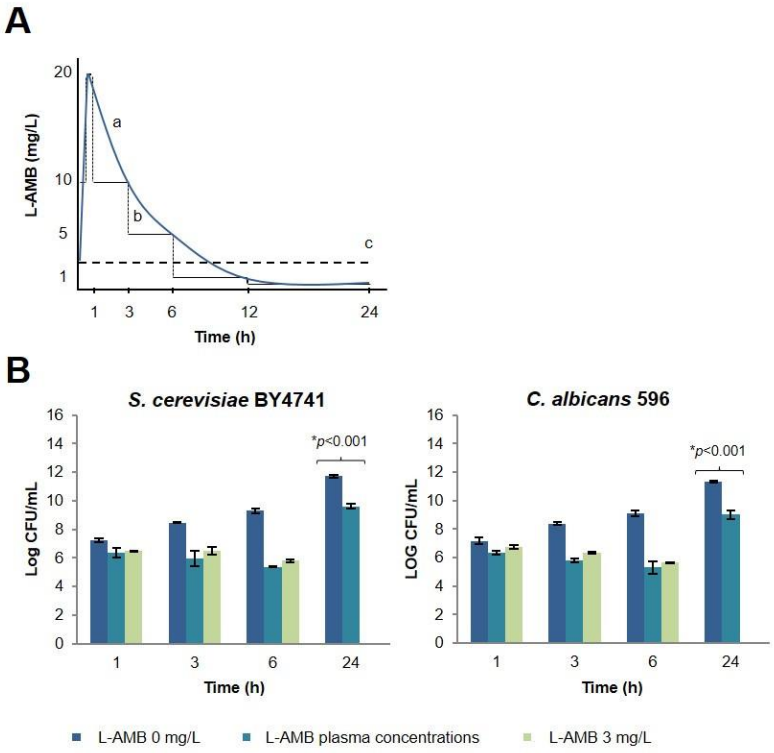

Figure 1 - **Effect of liposomal amphotericin B on *S. cerevisiae* BY4741 and *C. albicans* 596.** (A) Study design scheme. (a) Plasma concentration-time curve obtained after a first infusion of L-AMB at 3 mg/L. (b) The scheme proposed in accordance with L-AMB plasma levels described by Walsh et al. (Walsh et al., 1998); (c) Dashed line represents the

treatment with a constant concentration of 3 mg/L L-AMB during 24 hours. (B) Viability assessment by CFU enumeration of *S. cerevisiae* BY4741 and *C. albicans* 596 cells exposed to treatment conditions (b) and (c). Data at respective time points are given as mean ± standard deviations. An asterisk indicates significant differences between the two treatment conditions.

Overall, the killing kinetics were similar among all species studied (Table 1). *S. cerevisiae* BY4741 and *C. albicans* 596 are representative examples (Figure 1B). When comparing such results with the assessment of cell viability with PI (Figure 2A), we observed some discrepancies. While after 1, 3 and 6 h of incubation with L-AMB under both drug treatment conditions there was a reduction of colony forming units per milliliter, the cells were not permeable to PI. Only after 24 h was any PI uptake observed, but it was seen in only about 50% of the cells treated with L-AMB 3 mg/L.

Liposomal amphotericin B-induced alterations on membrane potential

To assess the effect of L-AMB on the cytoplasmic membrane potential of yeast cells, we used $DiBAC_4(3)$ staining. $DiBAC_4(3)$ enters only depolarized cells, where it binds reversibly to intracellular components, resulting in an increased fluorescent signal. The results obtained regarding membrane depolarization of *S. cerevisiae* BY4741 are depicted in Figure 2B. Viable nontreated cells stained with $DiBAC_4(3)$ exhibited a small percentage of depolarized cells (≈ 5%), which remained constant over time. Cells treated with L-AMB plasma concentrations displayed an increase of depolarized cells (DC) up to 6h of incubation; the percentage of DC at 1, 3 and 6 h of incubation was 13,01%, 20,13% and 45,83%, respectively. Interestingly, after 24 h of incubation, membrane yeast cells seemed to repolarize, corresponding to a DC final value of 19,67%. Conversely, treatment of yeast cells with L-AMB 3 mg/L resulted in a time-dependent increase of depolarized cells. Figure 2B shows that following 24 h of incubation, cells treated with 3 mg/L L-AMB displayed higher membrane depolarization than cells treated with plasma concentrations; the percentage of depolarized cells at this time point was 80,45%.

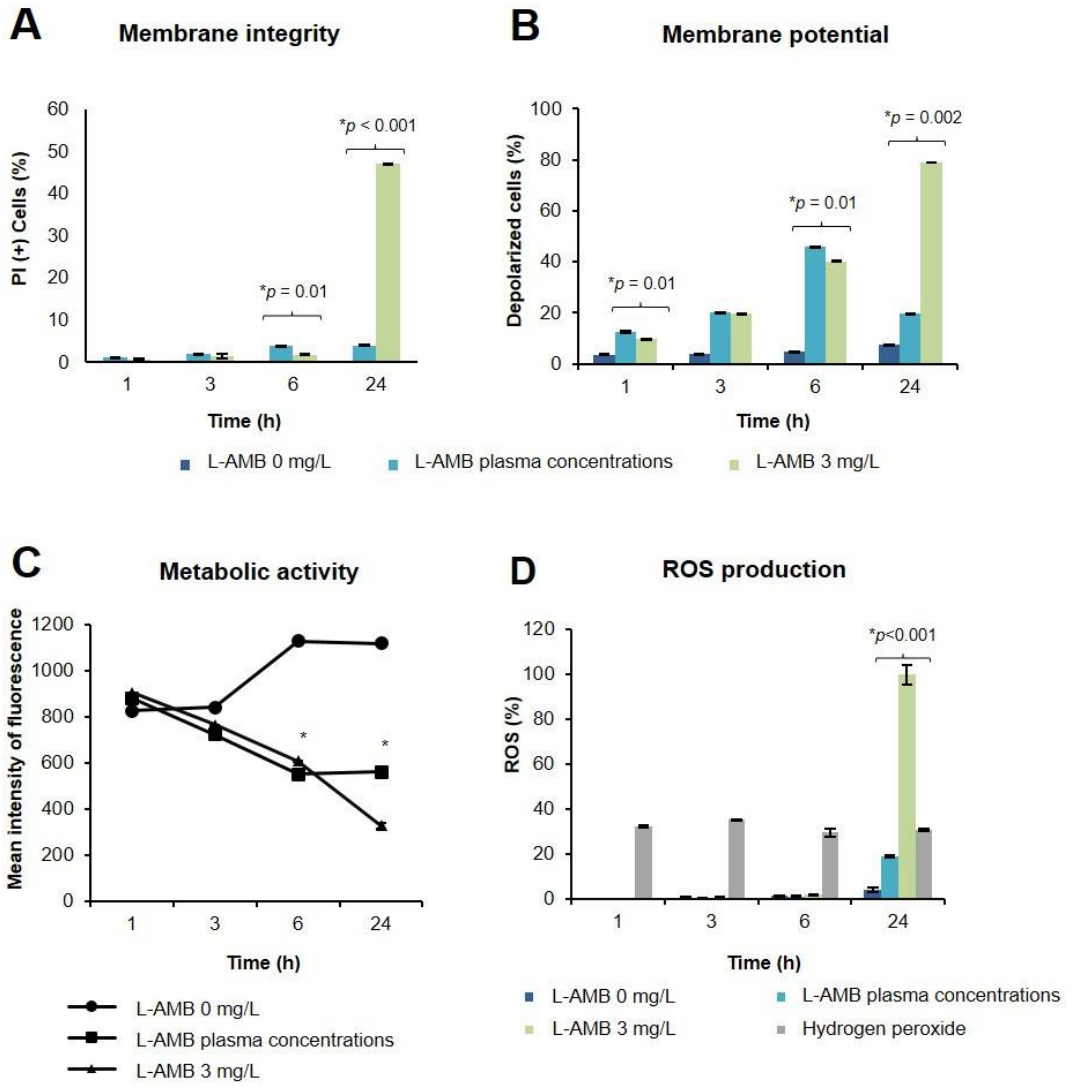

Figure 2 - Effect of liposomal amphotericin B on *S. cerevisiae* BY4741 physiological parameters. (A) Cell membrane integrity was assessed with propidium iodide (PI). (B) Cell membrane potential was evaluated using $DiBAC_4(3)$. (C) Metabolic activity was determined by 5-CFDA staining. (D) Endogenous reactive oxygen species (ROS) production as determined by DCFH-DA staining. An asterisk indicates significant differences ($P < 0.05$) between the two treatment conditions.

To evaluate whether this mechanism of cell salvage is conserved in the presence of decreasing concentrations of L-AMB during a 24 h period among distinct yeasts, a broad range of yeast species with different phylogenetic relationships (see Table 1) were assessed by flow cytometry regarding the respective cell membrane potential. The species studied showed different susceptibility profiles to L-AMB with all wild-type *Candida* strains (MIC, ≤ 2 mg/L), except *C. krusei* with a MIC of 8 mg/L. Non-*Candida* yeast genera also showed MIC values of < 2 mg/L. Regardless of the susceptibility profile, all species studied revealed depolarization of cell

membrane after 6 h of incubation with L-AMB plasma concentrations, followed by repolarization of the cell membrane by 24 h (Table 1). However, cells treated with 3 mg/L L-AMB exhibited a time-dependent increase of membrane depolarization (data not shown).

Table 1 – Yeast strains used in this study. Liposomal amphotericin B susceptibility test results and membrane potential of yeast cells treated with L-AMB plasma concentrations after 3, 6 and 24 h, expressed as percentage of depolarized cells.

ID	Species	Source	MIC (mg/L)	% of depolarized cells		
				3 h	6 h	24 h
ATCC 90028	*Candida albicans*	American Type Culture Collection	0.5	61.21	77.42	26.66
596	*Candida albicans*	Clinical isolate	0.5	33.62	40.16	21.72
590	*Candida glabrata*	Clinical isolate	1	28.19	37.51	20.04
597	*Candida parapsilosis*	Clinical isolate	0.5	34.62	45.98	9.57
120	*Candida krusei*	Clinical isolate	8	31.08	43.29	19.81
514	*Candida tropicalis*	Clinical isolate	1	48.65	77.92	21.54
479	*Candida dubliniensis*	Clinical isolate	0.06	21.64	36.73	25.57
520	*Candida lusitaniae*	Clinical isolate	0.25	30.18	33.21	22.94
D1	*Cryptococcus neoformans*	Clinical isolate	0.5	28.14	35.79	19.31
BY4741	*Saccharomyces cerevisiae*	American Type Culture Collection[a]	0.5	20.13	45.83	19.67
PYCC 6480	*Debaryomyces hansenii*	Portuguese Yeast Culture Collection[a]	1	43.78	89.90	30.47
PYCC 4166	*Kluveromyces lactis*	Portuguese Yeast Culture Collection[a]	0.06	27.89	38.89	5.63
CBS 732	*Zygosaccharomyces rou*xii	CBS-KNAW Fungal Biodiversity Center[a]	0.06	14.85	34.71	12.38

[a]kindly provided by Prof. Catarina Prista, Instituto Superior de Agronomia, Lisboa, Portugal.

Metabolic alterations triggered by L-AMB exposure

To assess metabolic effects of L-AMB on *S. cerevisiae* cells, we performed 5-CFDA staining. 5-CFDA is a cell-permeant esterase substrate; it measures both enzymatic activity and cell membrane integrity. The results obtained for MIF displayed by cells stained with 5-CFDA are detailed on Figure 2C. The MIF displayed by viable cells not treated with L-AMB following 1 h of treatment was 820.05; it increased after 6 h of incubation (MIF = 1,127.35) and remained constant up to 24 h. After 1 h of incubation, yeast cells treated with plasma concentrations of L-AMB displayed a MIF slightly higher than viable cells (901.01). However, following 3 and 6 h, the MIF decreased to values of 721.33 and 549.78 respectively; afterwards, the MIF remained constant until 24 h of treatment. When yeast cells were treated with 3 mg/L L-AMB over time, the MIF increased after 1 h of incubation (MIF = 923.54), indicating that the cells were metabolically active. However, after 3 h of incubation, the MIF decreased over time; at 24 h the MIF was 327.49. This event may be related to a lower enzyme activity or cell membrane injury.

Induction of endogenous ROS production by L-AMB

Reactive oxygen species production was assessed by DCFH-DA staining. DCFH-DA is oxidized to highly fluorescent 2',7'- dichlorodihydrofluorescein (DCF) by ROS. Exposure to L-AMB plasma concentrations resulted in reduced ROS formation in *S. cerevisiae* cells (≈ 18%) (Figure 2D). In contrast, prolonged and constant exposure to 3 mg/L L-AMB resulted in high formation of ROS (p <0.001); after 6 h of incubation, a significant increase of the number of fluorescent cells (reaching 100%) was registered. This assay reveals that 3 mg/L L-AMB leads to intracellular accumulation of ROS, which is associated with oxidative damage and possibly is involved in induced programmed cell death.

DNA damage and nuclear fragmentation

DNA fragmentation was measured by TUNEL assay. The percentage of *S. cerevisiae* cells that exhibited TUNEL-positive nuclei after exposure to plasma concentrations of L-AMB for 3 and 6 h was about 20% (Figure 3). There were significant differences between cells exposed to L-AMB plasma concentrations and to 3 mg/L L-AMB (p <0.001); following exposure to L-AMB at a constant 3 mg/L concentration, TUNEL-positive cells increased over time, indicating apoptotic-like DNA-fragmentation; after 24 h of incubation with 3 mg/L of L-AMB, 60.8% of yeast cells exhibited TUNEL-positive nuclei, twice the proportion of TUNEL-positive cells following exposure to L-AMB plasma concentrations (Figure 3A, 3B). During apoptosis, *S. cerevisiae* cells exposed to L-AMB exhibited evidence of nuclear fragmentation associated with DNA damage (Figure 3C), including irregular shaped DNA, as previously described (Wu et al., 2010).

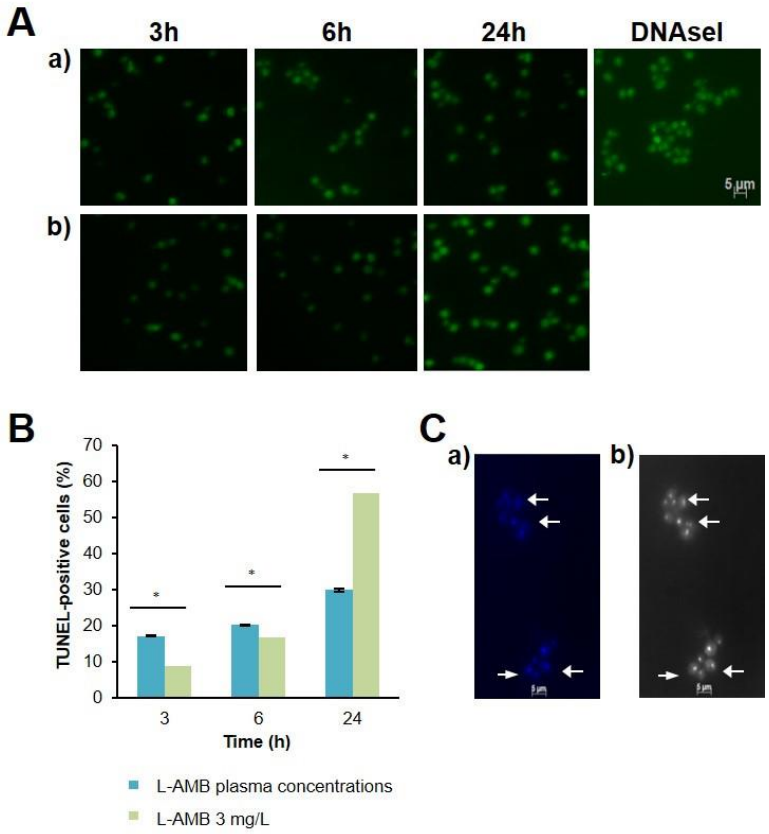

Figure 3 - **TUNEL staining of *S. cerevisiae* BY4741 cells exposed to liposomal amphotericin B.** (A) Fluorescence microscopy imaging showing TUNEL-positive cells after treatment with L-AMB plasma concentrations (a) and with 3 mg/L (b) after 3, 6 and 24 h. (B) Percentage of cells exhibiting damaged DNA (i.e., cells positive by TUNEL) after treatment with L-AMB, as assessed by flow cytometry. An asterisk indicates significant differences ($P < 0.05$) between the two treatment conditions. (C) Nuclear fragmentation as shown by DAPI staining. Fluorescence microscopy imaging with (a) and without (b) DAPI filter. *S. cerevisiae* cells exposed to L-AMB exhibit an irregular shape and fragmented DNA, two findings in accordance with DNA damage during apoptosis.

DISCUSSION

Our study clearly demonstrated that exposure to L-AMB plasma concentrations induces compensatory responses at distinct levels, like replicative ability, membrane potential, metabolic activity, endogenous ROS production, and DNA damage. In the treatment regimen with plasma concentrations, yeast cells are exposed to high concentrations of L-AMB (5-20 mg/L) over a short time period (6 h), which can trigger a stress response. Consequently, the yeast cells initially compensate by upregulating their physiological responses to minimize this stress; afterwards, over the remaining 18 h they are exposed to a much lower L-AMB concentration, which allows its recovery. This may explain why the yeast cells exposed to the simulated plasma concentrations are less L-AMB susceptible. Accordingly, no evidence of fungicidal activity was found, which may be responsible for fungal infection persistence in the blood of these patients. Conversely, following constant exposure to 3 mg/L, cells develop compensatory mechanisms for survival initially; however, as the concentration is kept constant for 24 h, the cells succumb to the drug effects. In addition, in interpreting this data, one also has to consider the fact that most fungal infections localize in the tissues, such as the lungs, kidneys, liver and spleen. In these tissues, concentrations of L-AMB reach well above the MIC and are maintained at these levels for at least 1 week and longer depending upon the tissues (Adler-Moore and Proffitt, 2003; Smith et al., 2007).

An important challenge for cell physiology and microorganism survival is a successful, balanced growth when confronted with environmental imbalances. A variety of cellular processes and physiological changes have to be coordinated to allow yeast cells to reproduce, grow, and respond to environmental stresses (Brauer et al., 2008). We demonstrated that yeast cells use distinct time-programmed mechanisms to respond to L-AMB-induced stress. When yeast cells were exposed initially to high concentrations of L-AMB (5 to 20 mg/L) during the first 6 h, followed by much lower levels of drug, closer to their MIC (simulated plasma concentrations), the loss of replication competency seems to be a relatively early event that could be easily overcome, provided that the yeast then were exposed for longer periods of time to drug concentrations at or below their MICs (Figure 1B). Previous studies have shown that some yeast cells exposed to amphotericin B demonstrate a capacity for resuscitation, although they are unable to replicate

(Liao et al., 1999 and 2003). It is notable that increasing and then decreasing L-AMB concentrations rapidly evoke compensatory responses by yeast cells, including the recovery of replication ability. Interestingly, such cells appear to exhibit an intact cell membrane, as shown by PI staining (only about 5% of cells are PI positive after 24 h) (Figure 2A). Conversely, yeast cells constantly exposed to 3 mg/L L-AMB lose their viability, as confirmed by CFU determination, also with clear evidence of cell membrane injury (50% of cells PI positive at 24h).

Regarding membrane potential, the phenomenon of cell salvage was found to be conserved among different yeasts (pathogenic and nonpathogenic) with different phylogenetic relationships and antifungal susceptibility profiles, suggesting that this recovery is related to insufficient L-AMB exposure. An essential aspect of environmental adaptation is the equilibrium of ion concentration, which determines cell membrane potential (Ke et al., 2013). It is well known that amphotericin B increases fungal cell membrane permeability to ions (Milhaud et al., 2002). Yeast cells initially exposed to high L-AMB concentrations exhibited evidence of plasma membrane depolarization soon after 1, 3 and 6 h of incubation, as shown by $DiBAC_4(3)$ staining; however, in such cells membrane potential was restored afterwards during the time that the cells were exposed to levels of drug close to or below their MICs (Figure 2B). Our results suggest that with initial exposure to high L-AMB levels, there is a cellular response characterized by recovery of plasma membrane potential which is maintained when the cells are then incubated with low levels of L-AMB (at or below MICs). In contrast, yeast cells exposed to 3 mg/L L-AMB exhibited a marked time-dependent impairment in membrane potential (Figure 2B). These results suggest that yeast cell recovery after exposure to high levels of L-AMB is related to subsequent exposure to nonfungicidal concentrations of L-AMB.

Cell metabolic activity was significantly reduced by L-AMB plasma concentrations at 3 and 6 h of incubation, remaining constant up to 24 h. In contrast, yeast cells continuously exposed to L-AMB at constant concentration (3 mg/L) initially displayed a high metabolic activity after 1 h of incubation, suggesting a different metabolic stress response. This fact is in accordance with the hypothesis that yeast cells exposed to antifungal pressure reprogram their metabolism in response to an environmental stress (Belenky et al., 2013). A previous study involving yeast genome-scale microarrays demonstrated that amino acid metabolism, phosphate metabolism, and carbohydrate metabolism genes are upregulated after treatment with 2.5 mg/L of

amphotericin B (Zhang et al., 2002). After 3, 6 and 24 h of incubation with 3 mg/L L-AMB, the fluorescence resulting from CFDA cleavage decreased (Figure 2C); this finding can be explained by membrane pore formation, which may lead to the loss of fluorescence, or by an extremely reduced metabolic activity (Breeuwer et al., 1995).

Previous studies have shown that amphotericin B induces *C. albicans* apoptosis (Phillips et al., 2003; Al-Dhaheri and Douglas, 2010). According to our results, endogenous ROS production was induced following 6 h of L-AMB exposure (Figure 2D); this finding also was corroborated by results for DNA damage (Figures 3A and 3B). Notably, apoptosis was more evident when *S. cerevisiae* was exposed to 3 mg/L L-AMB than when exposed to L-AMB plasma concentrations, as documented by both type of assays. Interestingly, L-AMB at plasma concentrations did not kill *S. cerevisiae* cells by necrosis; in fact, cells exposed to such concentrations displayed no evidence of plasma membrane damage, as shown by PI staining.

In conclusion, our results using *S. cerevisiae* as a model organism clearly demonstrate that yeast cells can respond in two ways to L-AMB: (i) expression of compensatory responses and survival when, in the plasma, the yeast are initially exposed to high concentrations of L-AMB and then much lower concentrations of the drug for extended periods of time; and (ii) induction of programmed cell death and/or necrosis, occurring at constant high concentrations. These findings provide important insights regarding L-AMB antifungal activity and may ultimately lead to the need to review therapeutic regimens.

Chapter IV

NOVEL METHOD FOR EVALUATING *IN VITRO* ACTIVITY OF ANIDULAFUNGIN IN COMBINATION WITH AMPHOTERICIN B OR AZOLES

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ABSTRACT

Combination of antifungal drugs with distinct targets has been used as salvage therapy, although without sound scientific support. *In vitro* studies validating such combinations are still scarce and the methodologies are very laborious and time-consuming. This study proposes a flow cytometric (FC) protocol to evaluate the effect of the combination of anidulafungin (AND) with amphotericin B (AMB) or azoles (fluconazole and voriconazole), tested upon 39 and 36 *Candida* strains, respectively. The concentration assayed in the combination was 0.5×MIC of each antifungal drug. The membrane potential marker $DiBAC_4(3)$ was used for AND-AMB association, and the metabolic marker FUN-1 was used for AND-azoles association. Drug interaction effect was determined by calculating a staining index (SI) using the sum of the percentage of depolarized cells (DC) after treatment with drugs combination divided by the DC of the single antifungal drug in the case of $DiBAC_4(3)$; the sum of the mean intensity of fluorescence (MIF) displayed by cells treated with the combination of drugs divided by the MIF of the single antifungal drug in case of FUN-1. A SI value <1 corresponds to antagonism; between 1-4 no interaction, and >4 synergism. The combination of AND and AMB by FC and checkerboard was synergistic for 46 and 43% of isolates, and antagonistic for 5 and 8%, respectively. For the combination of AND and azoles, it was synergistic for 36% and antagonistic for 3% by FC, and synergistic for 44% and antagonistic for 3% by checkerboard. When the FC method was compared to the gold standard checkerboard method, the agreement was 0.91 (95% confidence interval [95% CI] of 0.88 to 0.94), sensitivity was 0.88 (CI 95% of 0.73 to 0.95), and specificity was 0.95 (CI 95% of 0.84 to 1). Thus, FC is a rapid and reliable method (<2 hours) to assess the effect of antifungal drug combinations.

BACKGROUND

Candida species represent an important cause of invasive fungal infections with high morbidity and mortality rates (Richardson, 2005; Shao et al., 2007). Over the past few years, some epidemiological changes have been registered: the incidence of *C. albicans* has been reduced followed by a growing incidence of non-*albicans* species (Erjavec et al., 2009; Pfaller and Diekema, 2007; Tortorano et al., 2006). Late diagnosis and high mortality rates frequently promote the use of empiric antifungal combinations as salvage therapy without a sound scientific basis (Baddley and Pappas, 2005). The availability of new antifungal drugs with novel targets of action has enlivened the interest in combination therapy. Likewise, it is not possible to assume that the simultaneous administration of two or more drugs with distinct mechanisms of action would improve the clinical outcome compared to monotherapy (Chamilos and Kontoyiannis, 2006). It is unknown whether a combination might reduce the effectiveness of each drug or increase the potential for drug interactions or even toxicity, keeping in mind that this carries a significantly increased cost to the health care system without previously proven clinical benefits (Cuenca-Estrella, 2004). Thus, it is important and timely to critically evaluate the role of combination therapy.

The methods available for studying drug combinations are few and cumbersome and often provide contradictory results. The most commonly utilized are the checkerboard method (based on a mathematical model) and the time-kill assay, both impossible to implement in the routine of clinical laboratories because they are very laborious (Lewis et al., 2002; White et al., 1996). Therefore, the Etest was proposed as an alternative; however, it also has serious limitations, including cost, despite its good correlation with the classical method. The Etest is difficult to interpret when dealing with the azoles, due to the inconsistent growth patterns, and at least 24 hours are needed to provide results, since it is based on microbial growth (Lewis et al., 2002). Critical patients need a rapid response.

Flow cytometry (FC) is a valuable tool for studying antifungal susceptibility, since it can be used to detect different physiological cell stages by using the appropriate fluorescent markers (Chaturvedi et al., 2004; Czechowska et al., 2008). FC susceptibility testing to azoles,

amphotericin B (AMB), and echinocandins has already been described (Pina-Vaz et al., 2001a; Pina-Vaz et al., 2001b; Pina-Vaz C, 2010; Ramani et al., 1997; Rudensky et al., 2005).

The goal of this study was to develop an FC protocol to characterize the effects of the combinations of AMB or azoles with the echinocandin anidulafungin (AND) upon *Candida* spp.

MATERIAL AND METHODS

Fungal strains

Thirty-nine *Candida* strains were tested regarding the association between AND and AMB: 14 *Candida albicans*, 8 *C. glabrata*, 9 *C. parapsilosis*, 4 *C. tropicalis*, 1 *C. guilliermondii*, 2 *C. krusei* and 1 *C. lusitaniae*. For the association between AND and azoles (fluconazole [FLU] or voriconazole [VOR]), 36 strains were tested: 16 *C. albicans*, 9 *C. glabrata*, 7 *C. parapsilosis*, 7 *C. tropicalis*, and 1 *C. krusei*. Clinical isolates of *Candida* spp. with a previously characterized antifungal susceptibility phenotype were selected from the collection of the Department of Microbiology, Faculty of Medicine of Porto, Portugal, in order to study all possibilities: susceptible or nonsusceptible to both drugs or susceptible to one and nonsusceptible to the other. *C. albicans* ATCC 90028 was used as the reference strain. Before the initiation of each experiment, the yeasts were subcultured twice on Sabouraud agar (Liofilchem, Teramo, Italy) to ensure both the viability and that the culture was pure.

Drugs and chemicals

Stock solutions of AMB (Sigma-Aldrich, Taufkirchen, Germany), FLU (Sigma) VOR, and AND (Pfizer, New York, USA) were prepared as recommended by the CLSI protocol M27-A3 (CLSI, 2008) and stored at – 80°C. Fluorescent dyes 2-chloro-4-(2,3-dihydro-3-methyl-[benzo-1,3-thiazol-2-yl]-methylidene)-1 (FUN-1), and Bis-(1,3-dibutylbarbituric acid) trimethine oxonol) [$DiBAC_4(3)$] (both acquired from Molecular Probes, Leiden, Netherlands) were prepared in phosphate-buffered saline (PBS) (Sigma) and kept at – 20°C.

Checkerboard microdilution studies

Checkerboard assays were performed for AND plus AMB, and for AND plus azoles (FLU or VOR). MIC values of each antifungal and for the associations were determined after 24 hours of incubation. The concentrations tested for each antifungal ranged between 0.06 to 32 mg/L for FLU, 0.015 to 8 mg/L for VOR and AMB, and 0.06 to 2 mg/L for AND. Endpoints were determined according to values defined by the M27-A3 protocol (CLSI, 2008). The fractional inhibitory concentration index (FICI), which is defined as the sum of the MIC of each drug when used in

combination divided by the MIC of the drug when used alone, was calculated to determine the interaction effect; a FICI ≤ 0.5 represents synergism, >0.5 to 4 represents no interaction, and > 4 represents antagonism (Johnson et al., 2004; Odds, 2003).

Flow cytometry studies

For FC assays, the strains were subcultured in Sabouraud broth and incubated with agitation at 35°C until the exponential growth phase in order to obtain a homogenous population and thus, to correlate the perturbations in cellular parameters observed by FC with the drug action, independently of the growth phase. Then, 0.5 MacFarland density yeast suspensions were prepared in PBS, corresponding to 10^6 yeast cells/mL. The cell suspensions were incubated at 35ºC with subinhibitory concentrations (0.5 x MIC value) of each antifungal alone and in combination as described above. In order to standardize the FC protocol for all strains, the 0.5 x MIC of each drug was chosen since the breakpoints to AND and FLU are being reviewed, and their values are species dependent, and because antagonistic and synergistic classifications usually rely on deviations from additivity (Yeh et al., 2009; Pfaller et al., 2011a, b). According to the Loewe additivity definition, 0.5× MIC of drug A combined with 0.5× MIC of drug B is equivalent to 1 MIC of drug A or 1 MIC of drug B in an additive drug pair (Yeh et al., 2009). Even so, for strains inhibited at high MIC values and that do not present antagonism with the combination of 0.5× MIC, a new test was carried out using the breakpoint of the drug (e.g., 8 mg/L for FLU and 4 mg/L for AND) in order to evaluate its clinical significance.

Following 1 h of incubation, the cells were washed and incubated for 15 min in the dark at room temperature with 0.5 mg/L of $DiBAC_4(3)$, a lipophilic anion able to diffuse across depolarized membranes, in the case of the association of ANB plus AMB, and with 0.5 mg/L of FUN-1, a metabolic marker, in the case of association of AND plus azoles. The intensity of fluorescence of 30,000 cells was registered at FL1 (530 nm) for $DiBAC_4(3)$, and FL2 (575 nm) for FUN-1. The samples were analyzed in a FACSCalibur Cytometer (BD Biosciences, Sydney, Australia) standard model equipped with 3 photomultipliers (PMTs), standard filters, and a 15 mW 488 nm Argon Laser and using cell Quest Pro software (version 4.0.2). Instrument controls followed the standard procedures described by the manufacturer. All trials were performed in triplicate. The evaluation of *in vitro* drug interactions by FC was determined according to the

staining index (SI), which is similar to FICI described above. The SI was calculated as the sum of the percentage of depolarized cells (DC) after treatment with drugs combination divided by the DC of the drug alone for $DiBAC_4(3)$, and the sum of mean intensity of fluorescence displayed by cells treated with drugs combination divided by the fluorescence of the drug alone for FUN-1. Hence, SI = (DC AND+AMB/DC AND) + (DC AND+AMB/DC AMB) for AND-AMB association, and SI = (MIF AND+azole/MIF AND) + (MIF AND+azole/MIF azole) (MIF, ratio between mean fluorescence intensity of fluorescence of treated cells and viable cells) for AND-azoles association. Taking into account the standard classification of the checkerboard results, an association provided by FC was defined as antagonistic (A) for SI < 1, no interaction (NI) for SI between 1 and 4, and synergistic (S) for SI > 4.

Determination of viable cells

The number of viable cells in each FC assay was determined by plating 100 µL of serial dilutions on Sabouraud agar medium and incubating at 35ºC for 24 h. Afterwards, the number of colony forming units (CFU) was determined. No carryover antifungal effect was detected. All assays were performed in triplicate.

Statistical analysis

To evaluate the agreement between the checkerboard and FC studies, the proportion of agreement (PA) and the value of *Kappa* (K) were calculated (Sabin, 2000). In order to check the diagnostic validity of FC to detect the effect of the AND with AMB or azoles, having the checkerboard method as the reference, sensitivity and specificity were calculated (with confidence intervals at 95%). For calculation of all measures, the SPSS program (version 19.0) was used.

RESULTS

A protocol for evaluation of AND and AMB susceptibility by FC using $DiBAC_4(3)$ as a marker was optimized. Our method is indeed able to discriminate for both drugs the susceptible and nonsusceptible *Candida* strains. A typical example of FC analysis for antifungal susceptibility testing is represented in Figure 1. Accordingly, for each strain, the autofluorescence of the cell population in analysis is measured. This value is always represented on the first decade of the log scale of intensity of fluorescence, while the ethanol-treated cells (dead cells, the positive control) showed a high increase in the green fluorescence intensity (FL1 [530 nm]) as expected. The viable nontreated cells stained with $DiBAC_4(3)$ had a slight increase in the fluorescence (2-fold) in comparison with the viable nontreated and nonstained cells (autofluorescence). Treatment of susceptible strains with AND produced a dose-dependent increase for the fourth decade of intensity of fluorescence of cells, which was not observed in the nonsusceptible strains (Figures 1A and 1B).

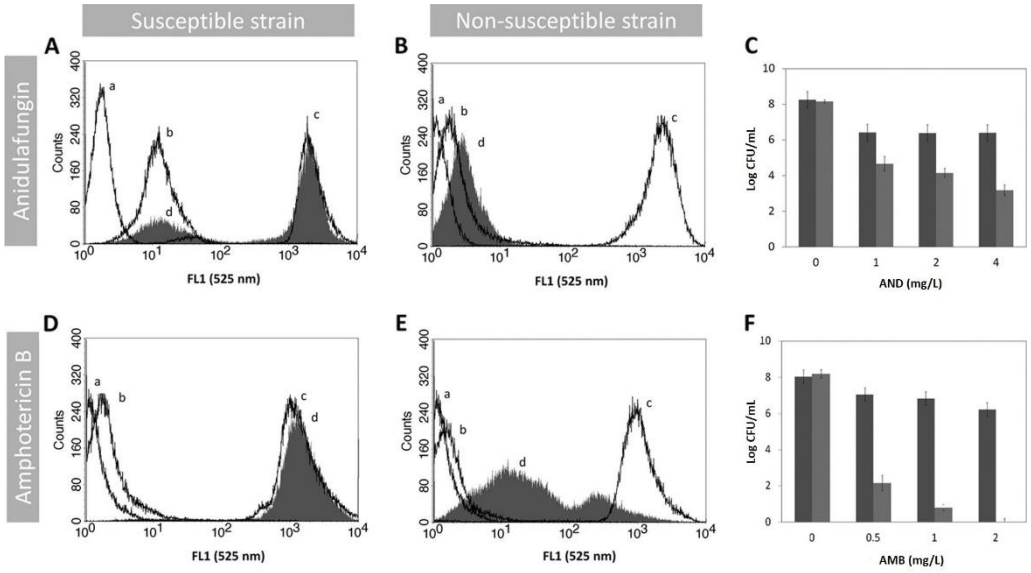

Figure 1 - *In vitro* antifungal activities of anidulafungin and amphotericin B. Distribution of fluorescence intensity of the *C. albicans* 0207 AND-susceptible strain (A), *C. parapsilosis* 0136 AND-nonsusceptible strain (B), *C. albicans* O207 AMB-susceptible strain (D), and *C. lusitaniae* D51 AMB-nonsusceptible strain (E). In each histogram, the autofluorescence is represented by line (a); line (b) represents the fluorescence of untreated cells stained with $DiBAC_4(3)$; line (c) is the fluorescence of cells treated with 70% ethanol and stained with $DiBAC_4(3)$ (positive control); line (d) is the fluorescence of cells treated with 1 mg/L of antifungal

drugs during 1 h and stained with $DiBAC_4(3)$. (C, F) Determination of the number of CFU (CFU/mL) of cell suspensions treated with different antifungal concentrations under conditions identical to those of the flow cytometric assay. The nonsusceptible strain is represented by the dark-gray bars and the susceptible strain by the light-gray bars.

The results obtained after 2 h of incubation with the antifungal were similar to data after 1h (data not shown). This increase in fluorescence intensity corresponds to a decrease in the number of CFU (Figures 1A to 1C).

Likewise, treatment of susceptible strains with 1 mg/L of AMB also induced an increase in fluorescence intensity similar to that of the positive control after 1 h of incubation. In contrast, for strains having higher MIC values, only a slight increase in fluorescence intensity was observed after incubation with AND or AMB, highlighting the fact that plasma membrane depolarization is dependent on antifungal action, and thus dependent on the antifungal susceptibility profile (Figures 1D and 1E). The CFU values determined in similar conditions agree very well with the FC data (PA = 0.92) (Figures 1C and 1F).

Since azole treatment did not result in any increase in the intensity of fluorescence after DiBAC4(3) staining, even after 2 h of incubation, neither in susceptible or in nonsusceptible strains (data not shown), a different fluorescent probe was chosen. FUN-1, a metabolic marker, has already been demonstrated by our group to be an excellent probe for azole susceptibility testing of *Candida* spp. (Pina-Vaz et al., 2001b). Thus, we developed an FC protocol to evaluate FLU, VOR, and AND susceptibility by FC using FUN-1 as a marker (Figure 2). An increase in the intensity of fluorescence was registered only for susceptible strains after 1 h of incubation. Conversely, this increase was not found for nonsusceptible strains as expected (Figures 2B and E). The CFU values were consistent with FC data (PA = 0.86) (Figures 2C and 2F).

Regarding the antifungal association studies, the FC assays for antifungal associations were performed with subinhibitory concentrations (0.5× MIC values) of each drug either alone or in association using the previous optimized conditions (Figures 1 and 2). These values were used to be able to standardize since the MIC varies for each strain. $DiBAC_4(3)$ was used for staining cells treated with AND-AMB and FUN-1 for staining cells treated with AND-FLU or AND-VOR. A typical synergistic interaction of AND-AMB evaluated by FC is represented in Figure 3A. The lack of interaction between AND and FLU association is represented in Figure 3B.

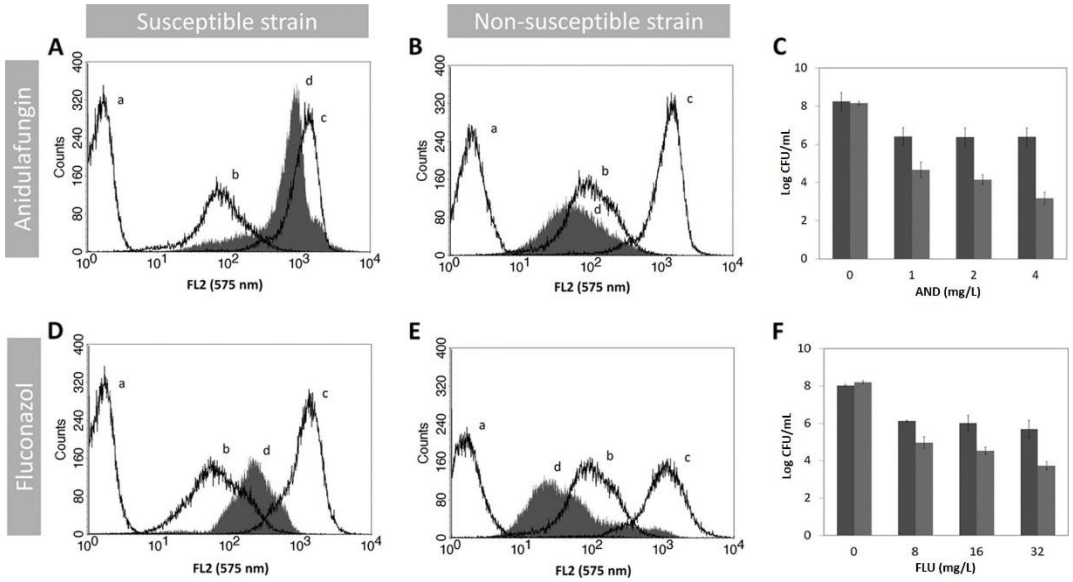

Figure 2 - *In vitro* antifungal activities of anidulafungin and fluconazole. Distribution of fluorescence intensity of *C. albicans* 0207 AND-susceptible strain (A), *C. parapsilosis* 0136 AND-nonsusceptible strain (B), *C. albicans* O223 FLU-susceptible strain (D), and *C. albicans* O216 FLU-nonsusceptible strain (E). In each histogram, the autofluorescence is represented by line (a); line (b) represents the fluorescence of untreated cells stained with FUN-1; line (c) is the fluorescence of cells treated with 70% ethanol and stained with FUN-1 (positive control); line (d) is the fluorescence of cells treated with antifungal drugs (1 mg/L of AND and 16 mg/L of FLU) during 1 h and stained with FUN-1. (C, F) Determination of the number of CFU (CFU/mL) of cell suspensions treated with different antifungal concentrations under conditions identical to those of the flow cytometric assay. The nonsusceptible strain is represented by the dark-gray bars and the susceptible strain by the light-gray bars.

Taking into account the FC data, the association between AND and AMB was synergistic in 46% of cases (18 of 39); there was no interaction in 49% of isolates (19 of 39), and the association was antagonistic in 5% (2 of 39) (Table 1). The association between AND and FLU was synergistic in 36% of cases (13 of 36), there was no interaction in 61% (22 of 36), and the association was antagonistic in 3% (1 of 36) (Table 2). The association between AND and AMB evaluated by the checkerboard microdilution method was synergistic in 43% of strains tested (17 of 39), there was no interaction in 49% (19 of 39), and the association was antagonistic in 8% (3 of 39). Antagonistic interaction was detected only for *C. albicans*. For *C. glabrata* isolates, this

association was quite promising since there was no evidence of an antagonistic effect and “no interaction” was not a frequent event (Table 1).

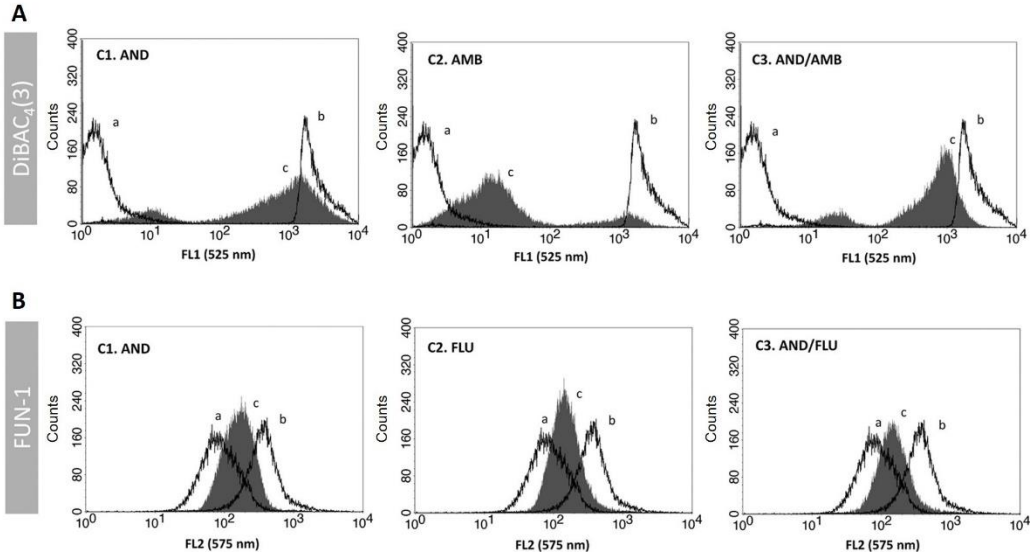

Figure 3 - Evaluation of antifungal combination effect using flow cytometry. (A) Flow cytometric analysis of the combination effect between anidulafungin and amphotericin B on the *C. albicans* O215 strain, an example of synergistic association. Line (a), fluorescence of untreated cells stained with $DiBAC_4(3)$; line (b), fluorescence of cells treated with 70% ethanol and stained with $DiBAC_4(3)$; line (c), fluorescence of cells treated with antifungal drugs and stained with $DiBAC_4(3)$; C1, cells treated with a subinhibitory concentration of AND (0.5× MIC); C2, cells treated with a subinhibitory concentration of AMB (0.5× MIC); and C3, cells treated with a subinhibitory concentrations of both antifungal drugs in association (AND 0.5× MIC + AMB 0.5× MIC). (B) Flow cytometric analysis of the combination effect between anidulafungin and fluconazole on the *C. albicans* OL196 strain, an example of indifferent association. Line (a), fluorescence of untreated cells stained with FUN-1; line (b), fluorescence of cells treated with 70% ethanol and stained with FUN-1; line (c), fluorescence of cells treated with antifungal drugs and stained with FUN-1; C1, cells treated with a subinhibitory concentration of AND (0.5× MIC); C2, cells treated with a subinhibitory concentration of FLU (0.5× MIC); and C3, cells treated with subinhibitory concentrations of both antifungal drugs in association (AND 0.5× MIC + FLU 0.5× MIC).

Table 1 - *In vitro* interaction of anidulafungin and amphotericin B by the checkerboard and flow cytometry methods against 39 *Candida* species.

Fungal strains	Checkerboard MIC (mg/L)				Flow cytometry DC (%)			
	AND	AMB	AND/AMB	FICI (Interpretation)	AND	AMB	AND/AMB	SI (Interpretation)
C. albicans O236	0.25	0.25	0.125/0.03	0.62 (NI)	45.04	13.53	22.42	2.15 (NI)
C. albicans O223	0.25	0.125	0.06/0.015	0.36 (S)	71.23	14.58	69.08	5.71 (S)
C. albicans O216	0.125	0.25	0.06/0.015	0.54 (NI)	49.68	8.24	25.17	3.56 (NI)
C. albicans O189	0.25	0.125	0.06/0.015	0.36 (S)	26.33	1.78	24.77	14.86 (S)
C. albicans OL196	0.03	0.25	0.06/0.015	2.06 (NI)	83.45	27.89	33.56	1.61 (NI)
C. albicans O207	0.015	0.125	0.06/0.015	4.12 (A)	48.96	10.45	8.53	0.99 (A)
C. albicans O245	0.015	0.25	0.06/0.015	4.06 (A)	48.13	37.23	20.21	0.99 (A)
C. albicans O237	0.25	0.25	0.06/0.015	0.30 (S)	42.59	1.57	44.53	29.41 (S)
C. albicans O183	0.015	0.06	0.015/0.015	1.25 (NI)	22.69	10.41	13.20	1.85 (NI)
C. albicans O222	1	0.125	0.06/0.015	0.18 (S)	37.40	8.78	32.65	4.59 (S)
C. albicans O215	0.25	0.125	0.06/0.015	0.36 (S)	68.42	13.05	65.04	5.93 (S)
C. albicans O190	0.25	2	0.125/0.015	0.51 (NI)	19.34	11.08	17.57	2.49 (NI)
C. albicans O195	0.015	0.125	0.06/0.015	4.12 (A)	53.12	12.98	17.89	1.72 (NI)
C. albicans ATCC	0.03	0.06	0.015/0.015	0.75 (NI)	61.34	37.12	53.11	2.30 (NI)
C. glabrata OL158	4	0.25	1/0.03	0.37 (S)	79.44	19.36	75.85	4.87 (S)
C. glabrata O206	0.03	0.25	0.06/0.015	2.06 (NI)	35.48	70.21	50.81	2.16 (NI)
C. glabrata O188	0.50	0.125	0.06/0.015	0.24 (S)	82.83	3.95	58.39	15.49 (S)
C. glabrata OL163	0.25	0.25	0.06/0.015	0.30 (S)	73.94	15.09	57.93	4.62 (S)
C. glabrata OL149	0.25	0.25	0.125/0.03	0.62 (NI)	75.39	36.95	58.23	2.35 (NI)
C. glabrata O175	0.06	0.125	0.015/0.015	0.37 (S)	66.85	10.44	63.11	6.99 (S)
C. glabrata O180	0.03	0.25	0.015/0.015	0.56 (NI)	78.27	3.01	73.13	25.23 (S)
C. glabrata O181	4	0.06	0.015/0.03	0.50 (S)	59.89	5.66	37.48	7.25 (S)
C. guilliermondii 33	1	0.125	0.25/0.03	0.49 (S)	23.28	2.46	21.11	9.49 (S)
C. krusei OL16	0.25	0.25	0.06/0.015	0.30 (S)	29.72	4.81	27.58	6.66 (S)
C. krusei O234	0.125	0.125	0.06/0.015	0.60 (NI)	14.92	2.11	10.53	5.70 (S)
C. lusitaniae D51	0.5	2	0.25/0.25	0.63 (NI)	79.93	49.79	69.93	2.28 (NI)
C. parapsilosis OL143	2	0.25	1/0.03	0.62 (NI)	72.14	23.71	55.46	3.11 (NI)
C. parapsilosis O246	1	0.25	0.5/0.06	0.74 (NI)	67.93	48.06	58.03	2.06 (NI)
C. parapsilosis ATO17	4	0.125	1/0.06	0.73 (NI)	2.94	0.34	0.78	2.56 (NI)
C. parapsilosis OL144	2	0.25	0.06/0.03	0.15 (S)	21.15	0.63	16.10	26.32 (S)
C. parapsilosis O204	4	0.125	2/0.06	0.98 (NI)	82.33	31.18	80.53	3.56 (NI)
C. parapsilosis O136	2	0.125	1/0.03	0.74 (NI)	15.58	14.50	16.98	2.26 (NI)
C. parapsilosis Cpo41	8	0.125	0.06/0.06	0.49 (S)	8.87	5.98	15.06	4.22 (S)
C. parapsilosis O158	2	0.125	0.5/0.03	0.49 (S)	45.08	4.92	21.61	4.87 (S)
C. parapsilosis O56	4	0.125	2/0.03	0.74 (NI)	21.72	11.53	18.67	2.48 (NI)
C. tropicalis OL202	0.06	0.25	0.015/0.015	0.31 (S)	90.22	3.45	89.79	27.02 (S)
C. tropicalis OL205	0.25	0.25	0.06/0.015	0.30 (S)	70.98	23.04	53.85	3.10 (NI)
C. tropicalis OL193	0.03	0.5	0.06/0.015	2.03 (NI)	42.01	11.21	11.01	1.24 (NI)
C. tropicalis 1304	1	0.06	0.5/0.03	1 (NI)	11.54	1.98	3.96	2.34 (NI)

[a]S, synergism; A, antagonism; NI, no interaction; FICI, fractional inhibitory concentration index; DC, percentage of depolarized cells; SI, staining index. DC percentage is based on the use of 0.5× MIC of each drug. FICI = (MIC AND/AMB)/MIC AND + (MIC AMB/AND)/MIC AMB. SI = (DC AND + AMB)/DC AND + (DC AND + AMB)/DC AMB.

The results of AND and FLU association were similar to that for AND and VOR. Synergism was observed in 44% of strains (16 of 36), no interaction in 53% (19 of 36), and an antagonistic effect in 3% (1 of 36). These antagonistic events were detected for only one isolate of *C.*

parapsilosis. Concerning *C. glabrata*, once again no antagonism was observed between AND and the azoles, and no interaction was also a rare event, it being detected only in two strains. For strains that are susceptible-dose dependent or resistant to FLU and those which are nonsusceptible to AND, not showing antagonism with the combination of 0.5× MIC, the results of the assays performed using the breakpoint concentration were similar to those obtained with 0.5× MIC (data not shown).

Table 2 - *In vitro* interaction of anidulafungin and fluconazole by the checkerboard and flow cytometry methods against 36 *Candida* species.

Fungal strains	Checkerboard MIC (mg/L)				Flow cytometry DC (%)			
	AND	FLU	AND/FLU	FICI (Interpretation)	AND	AMB	AND/AMB	SI (Interpretation)
C. albicans O189	0.25	32	0.06/0.06	0.24 (S)	1.10	1.26	2.60	4.44 (S)
C. albicans O190	0.25	64	0.06/0.015	0.24 (S)	0.48	0.48	1.24	5.13 (S)
C. albicans O195	0.015	4	0.015/0.06	1.02 (NI)	2.70	1.62	2.34	2.31 (NI)
C. albicans O205	0.06	2	0.06/0.015	1.01 (NI)	1.68	0.93	0.82	1.36 (NI)
C. albicans O207	0.015	4	0.015/0.06	1.02 (NI)	2.30	1.74	1.55	1.57 (NI)
C. albicans O216	0.25	64	0.06/0.06	0.24 (S)	4.77	3.16	7.72	4.06 (S)
C. albicans O223	0.25	1	0.06/0.06	0.30 (S)	1.75	1.39	4.37	5.65 (S)
C. albicans O236	0.25	16	0.06/0.06	0.24 (S)	1.10	1.03	2.17	4.08 (S)
C. albicans O237	0.25	64	0.06/0.06	0.24 (S)	2.67	1.29	3.50	4.03 (S)
C. albicans O245	0.015	64	0.015/0.06	1 (NI)	1.41	1.27	1.34	2.00 (NI)
C. albicans OL122	0.125	0.5	0.125/0.015	1.03 (NI)	8.00	4.79	5.69	1.90 (NI)
C. albicans OL160	0.03	16	0.015/0.125	0.51 (NI)	0.38	0.38	0.59	3.09 (NI)
C. albicans OL171	0.015	0.5	0.015/0.015	1.03 (NI)	0.48	0.49	0.48	2.00 (NI)
C. albicans OL172	0.03	16	0.015/0.125	0.51 (NI)	1.26	0.91	1.68	3.19 (NI)
C. albicans OL196	0.015	64	0.015/0.06	1 (NI)	1.28	1.41	1.28	1.91 (NI)
C. albicans ATCC	0.03	0.125	0.015/0.015	0.62 (NI)	1.69	1.40	1.79	2.33 (NI)
C. glabrata O158	4	4	0.5/1	0.38 (S)	7.93	1.01	8.90	9.93 (S)
C. glabrata O181	4	4	0.25/1	0.31 (S)	2.48	0.51	2.83	6.72 (S)
C. glabrata O188	0.5	8	0.06/0.06	0.13 (S)	3.77	2.81	7.15	4.44 (S)
C. glabrata O180	0.5	16	0.125/0.06	0.25 (S)	0.46	1.02	1.30	4.09 (S)
C. glabrata O206	0.03	16	0.015/0.06	0.51 (NI)	1.88	1.80	2.04	2.22 (NI)
C. glabrata OL149	0.25	16	0.06/0.06	0.24 (S)	2.08	1.22	3.24	4.20 (S)
C. glabrata OL158	4	8	0.06/4	0.52 (NI)	1.27	1.01	1.14	2.03 (NI)
C. glabrata OL163	0.25	16	0.06/0.06	0.24 (S)	1.78	1.57	1.96	2.35 (NI)
C. glabrata OL164	0.25	8	0.06/0.015	0.24 (S)	0.96	0.62	1.61	4.29 (S)
C. krusei OL16	0.25	64	0.125/0.06	0.51(NI)	1.10	1.06	1.15	2.13 (NI)
C. parapsilosis Cpo41	8	0.125	0.06/0.5	4.01 (A)	1.34	1.11	0.59	0.97 (A)
C. parapsilosis O136	4	2	0.25/2	1.06 (NI)	1.64	0.91	0.92	1.57 (NI)
C. parapsilosis O246	2	0.5	0.25/0.25	0.625 (NI)	1.53	1.72	1.66	2.05 (NI)
C. parapsilosis O56	4	0.5	0.5/0.25	0.625 (NI)	2.33	4.06	3.07	2.07 (NI)
C. parapsilosis OL143	2	2	0.25/0.5	0.38 (S)	3.37	4.37	4.96	2.61 (NI)
C. parapsilosis ATO16	4	0.25	0.5/0.06	0.37 (S)	3.10	3.53	4.31	2.61 (NI)
C. parapsilosis OL144	2	2	0.5/1	0.75 (NI)	1.95	1.18	1.94	2.65 (NI)
C. tropicalis OL193	0.015	2	0.015/0.06	1.03 (NI)	1.12	1.68	1.53	2.27 (NI)
C. tropicalis OL202	0.015	2	0.015/0.06	1.03 (NI)	0.47	0.75	0.46	1.60 (NI)
C. tropicalis OL295	0.25	2	0.06/0.06	0.27 (S)	1.43	0.98	2.57	4.42 (S)

[a]S, synergism; A, antagonism; NI, no interaction; FICI, fractional inhibitory concentration index; MIF, mean intensity of fluorescence; SI, staining index. MIF was determined using 0.5× MIC of each drug. FICI = (MIC AND/FLU)/MIC AND + (MIC FLU/AND)/MIC FLU. SI = (MIF AND + FLU)/MIF AND + (MIF AND + FLU)/MIF FLU.

Our FC assay showed three more cases of “no interaction” than the checkerboard method, and there was one more case of antagonism in the checkerboard method related to one *C. albicans* strain (O195) in comparison with the FC method. The *Kappa* value obtained between both methods was 0.83 (95% CI of 0.79 to 0.87), it being 0.82 (95% CI of 0.76 to 0.88) for AND-AMB association and 0.84 (95% CI of 0.78 to 0.90) for the AND-azoles association. The proportion of agreement calculated was 0.91 (95% CI of 0.88 to 0.94), namely 0.90 (95% CI of 0.85 to 0.95) for the AND-AMB association and 0.92 (95% CI of 0.87 to 0.97) for the AND-azoles association. Regarding sensitivity and specificity of the FC method, considering the checkerboard assay as the reference methodology, sensitivity was 0.88 (95% CI of 0.73 to 0.95) for detection of synergistic effects, and specificity was 0.95 (95% CI of 0.84 to 1). In order to detect an antagonistic interaction, FC sensitivity was 0.75 (95% CI of 0.3 to 0.95), and its specificity was 1.

DISCUSSION

The use of drugs with different mechanisms of action in association may play a key role in treatment of invasive fungal infections (Cuenca-Estrella, 2004; Chamilos and Kontoyiannis, 2006). In the past few years, this combined antifungal therapy has received increased attention. Antifungal interaction involving *Cryptococcus* has been studied; however, reports on the effect of antifungal combinations involving the most frequent *Candida* spp. are less common. Most of these studies have demonstrated synergism, whereas others have reported no interaction and occasionally antagonism (Chamilos and Kontoyiannis, 2006; Rodríguez et al., 2007; Serena et al., 2008; Kiraz et al., 2009; Jones et al., 2010).

Echinocandins are a novel class of antifungals that have the cell wall as their target. The literature addressing the relationship between such drugs and membrane-active drugs such as polyenes or azoles against *Candida* is still somewhat limited (Lewis et al., 2002; Karlowsky et al., 2006; Kiraz et al., 2009). Importantly, drugs with different targets of action could reinforce each other, allowing a decrease in doses and thus reducing side effects for patients (Mukherjee et al., 2005; Karlowsky et al., 2006). AND acts by inhibiting the 1,3-β-D-glucan synthesis, the major component of the fungal cell wall (Pound et al., 2010). AMB and azoles are membrane-active drugs; the former acts by making holes in the membrane and the latter by inhibiting ergosterol synthesis. Echinocandins probably enhance the effect of membrane-active drugs by increasing their access to the target (Baddley and Pappas, 2005; Karlowsky et al., 2006). Nevertheless, it cannot be assumed that the use of two or more effective drugs with distinct mechanisms of action would produce an improved outcome compared to the results seen with a single compound (Cuenca-Estrella, 2004; Chamilos and Kontoyiannis, 2006).

Like in most previously published studies, the association between azoles or AMB with AND resulted, for the majority of the strains, in a synergy or no interaction (Pancham et al., 2005; Kiraz et al., 2009; Nishi et al., 2009). In fact, in our study, antagonism for AND-AMB association was observed only for three strains of *C. albicans* (O207, O245, O195) and one for the AND-FLU association in a strain of *C. parapsilosis* (Cpo41). Each drug alone had very low MIC values for these strains, but they increased following the association, although still remaining low. With regard to echinocandins, most authors found no antagonism between micafungin and azoles or

AMB (Serena et al., 2008; Nishi et al., 2009; Chaturvedi et al., 2011). Barchiesi et al. did not find advantages in associating caspofungin and the polyene, with the exception of *C. parapsilosis*, but the study included a much more limited number of strains (Barchiesi et al., 2007). In some *Candida* strains, echinocandins are highly active, and so the fungicidal activity may be difficult to improve after combination treatment (Nishi et al., 2009). Moreover, we have shown that the drug interaction potential is species- and strain-dependent, which enhances the importance of the novel protocol described here for the first time.

The mathematic model used for the checkerboard method, which allows a quantification analysis after calculating the FICI, has been the most commonly used procedure to characterize the activity of antimicrobial combinations in clinical laboratories. Other methods, such as time-kill assays and Etest, have been used (Kiraz et al., 2009); however, despite their good correlation with the checkerboard assay, all of them take at least 24h to provide results. The terminology used to assign the results into interpretative categories is often a subject of debate and confusion, with difficult resolution (Johnson et al., 2004). Synergism and antagonism have clear and intuitive meanings, although “no interaction” is a somewhat subjective category without a clear clinical relevance (Johnson et al., 2004).

At the moment, few but relevant reports have helped to demonstrate the value of cytometric assays as excellent yet underexplored tools in clinical microbiology (Rudensky et al., 2005; Czechowska et al., 2008; Pina-Vaz and Rodrigues, 2010). Flow cytometry is a powerful high-throughput technology that allows the characterization of several thousands of cells per second, distinguishing between different physiological states. Differentiations between viable, intermediate, and nonviable cells are possible using fluorescent dyes. Using these tools, fast, reliable data could be obtained with great benefit for the patient. Echinocandin antifungal activity was studied using $DiBAC_4(3)$ or FUN-1, although for azoles only FUN-1 could be used after a short incubation time. Regarding AMB, previous studies reported the impossibility of studying its activity with FUN-1 (Pina-Vaz et al., 2011b). Thus, the study of the AND-AMB association was performed using $DiBAC_4(3)$ and that of the AND-azoles association was performed using FUN-1 (Figures 1 to 3). $DiBAC_4(3)$ is a membrane potential marker that can enter depolarized cells, where it binds to intracellular proteins or membranes and exhibits enhanced fluorescence; and FUN-1 is a metabolic activity staining that passively diffuses through yeast cell walls. In

metabolically inactive cells, FUN-1 remains in the cytoplasm, displaying a green fluorescence, while in active cells it is processed, which results in the formation of distinct vacuolar structures that exhibit a red fluorescence, accompanied by a reduction in the green cytoplasmic fluorescence (Lee and Kwak, 1999; Pina-Vaz et al., 2001a). For both markers, an increase in fluorescence intensity [FL1 (530 nm) for $DiBAC_4(3)$ and FL2 (575 nm) for FUN-1] agrees with the reduction of CFU counts, meaning a reduction of cell viability. The FC protocols described show an excellent agreement with the checkerboard method and high sensitivity and specificity, thus allowing the study of different combinations of antifungal drugs in less than 2 h.

Chapter V

UNVEILING THE SYNERGISTIC INTERACTION BETWEEN LIPOSOMAL AMPHOTERICIN B AND COLISTIN

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ABSTRACT

Patients with multiple comorbidities are often administered simultaneously or sequentially antifungals and antibacterial agents, without full knowledge of the consequences of drug interactions. Considering the clinical relevance of liposomal amphotericin B (L-AMB), the association between L-AMB and six antibacterial agents was evaluated against four clinical isolates and one type strain of *Candida* spp. and two clinical isolates and one type strain of *Aspergillus fumigatus.* In order to evaluate such combined effects, the minimal inhibitory concentration (MIC) of L-AMB was determined in the presence of 0.5-, 1-, 2- and 4-fold peak plasma concentrations of each of the antibacterial drugs. Since the L-AMB/colistin (CST) association was the most synergic, viability assays were performed and the physiological status induced by this association was characterized. In addition, computational molecular dynamics studies were also performed in order to clarify the molecular interaction.

The maximum synergistic effect with all antibacterial agents, except CST, was reached at 4-fold the usual peak plasma concentrations, resulting in 2-to 8-fold L-AMB MIC reduction for *Candida* and 2-to 16-fold for *Aspergillus.* For CST, the greatest synergism was registered at peak plasma concentration (3 mg/L), with 4-to 8-fold L-AMB MIC reduction for *Candida* and 16-to 32-fold for *Aspergillus*. L-AMB at subinhibitory concentration (0.125 mg/L) combined with CST 3 mg/L resulted in: a decrease of fungal cell viability; an increase of cell membrane permeability; an increase of cellular metabolic activity soon after 1 h of exposure, which decreased until 24 h; and an increase of ROS production up to 24 h. From the molecular dynamics studies, AMB and CST molecules shown a propensity to form a stable molecular complex in solution, conferring a recognition and binding added value for membrane intercalation.

Our results demonstrate that CST interacts synergistically with L-AMB, forming a stable complex, which promotes the fungicidal activity of L-AMB at low concentration.

BACKGROUND

Fungi are recognized as major pathogens in critically ill patients. *Candida* and *Aspergillus* species are the most common agents of invasive fungal infections (IFIs), although other yeasts and filamentous fungi are becoming emerging pathogens (Gullo, 2009). In the clinical practice, patients at risk for IFIs often receive concomitantly or sequentially antifungal therapy and antibacterial agents, either for prophylactic or therapeutic purposes (Stergiopoulou et al., 2009). However, this procedure is often adopted without full knowledge of the consequences resulting from pharmacological drug interactions.

A recent literature review showed that the combination of amphotericin B (AMB) or fluconazole with antibacterial agents that impair RNA or protein synthesis, such as rifampicin (RIF), azithromycin (AZM), clarithromycin (CLR), and tetracycline (TET), enhances the *in vitro* activity of the antifungal agent against yeast and filamentous fungi (Azevedo et al., 2015). In addition, Zeidler *et al*. demonstrated that colistin (CST), an antibiotic that targets Gram-negative membranes, exhibits a synergistic effect with antifungal agents belonging to the echinocandin class against *Candida* spp. According to the authors, the echinocandin weakens the fungal cell wall facilitating the colistin action upon fungal membranes, and, consequently, this effect enhances the antifungal activity of the echinocandin (Zeidler et al., 2013). These results are particularly promising since the available antifungal panoply is narrow, encompassing only a few classes of agents, and the discovery of new drugs is a slow and exhaustive process (Roemer and Boone, 2013). Moreover, some antifungals demonstrate limited efficacy, high toxicity, and are prone to the development of antifungal resistance. Therefore, the association of compounds that enhance the efficacy of antifungal drugs may contribute to a more effective reduction of fungal burden and minimize the development of resistance (Zeidler et al., 2013).

Although the association of antifungal and antibacterial agents may have beneficial implications in clinical terms, little is known about the pharmacological drug interactions and the underlying mechanisms of synergism. While there are few studies available addressing this topic, it is important to stress that antagonistic or indifferent effects may also be found (Petrou and Rogers, 1988; Stergiopoulou et al., 2009; Venturini et al., 2011). Thus, an extensive study aiming

to evaluate the mechanisms involved in the association between antifungal and antibacterial agents upon fungal growth inhibition is mandatory.

We hereby propose to evaluate the association between liposomal amphotericin B (L-AMB) and several antibacterial drugs against *Candida* spp. and *Aspergillus fumigatus*, as well as to unveil the mechanisms underlying such drug interactions. In the case of synergistic interaction, we have investigated using molecular dynamics (MD) simulations whether (i) the antibacterial agent has an intrinsic activity upon fungal cells; or (ii) the antibacterial agent acts as a facilitator of AMB activity. Molecular dynamics simulations have been widely applied to study the interaction of AMB molecules with the fungal membranes, since the mechanism of action of AMB is not well characterize (Baginski et al., 1997; Sternal et al., 2004; Czub and Baginski, 2006; Czub et al., 2007; Neumann et al., 2009; Cohen, 2010; Foglia et al., 2014). Thus, this approach may be essential to unveil the mechanism of synergism.

Our results demonstrate which antibacterial agents improve L-AMB efficacy and elucidate about the hypothetical underlying mechanism.

MATERIAL AND METHODS

Strains and growth conditions

Four *Candida* clinical isolates and one type strain (C*. albicans* 596, *C. albicans* 38, *C. glabrata* 590, *C. krusei* 120, and *C. albicans* ATCC 90028) and two clinical isolates and one type strain of *Aspergillus fumigatus* (*A. fumigatus* 76, *A. fumigatus* 88, and *A. fumigatus* ATCC MYA-3626) were used in this study. Clinical isolates of *Candida* spp. and *A. fumigatus* were obtained from patients admitted at Centro Hospitalar S. João, Porto, Portugal. Isolates are included in the Collection of fungal clinical isolates deposited at Department of Microbiology, Faculty of Medicine of Porto, Portugal. *Candida* isolates were grown in Sabouraud dextrose agar (SDA; Formedium, Norfolk, United Kingdom) at 35 °C for 24 h (Teixeira-Santos et al., 2012); *Aspergillus* isolates were cultured in SDA at 35 °C for 72 h (Faria-Ramos et al., 2014). *Escherichia coli* ATCC 25922 was also included in this study for control of antibacterial drugs.

Antimicrobial drugs and susceptibility testing

Liposomal amphotericin B (provided by Gilead Sciences, Inc, San Dimas, CA, USA), rifampicin (Sanofi Aventis, Anagni, Italy), azithromycin (Farmoz, Sintra, Portugal), clarithromycin (Alcala Farma, Madrid, Spain), colistin sulfate salt (C4461, Sigma-Aldrich, Munich, Germany), tetracycline (T3258, Sigma-Aldrich), and linezolid (LZD; Pfizer, New York, USA), were prepared according to the manufacturer's guidelines, in order to obtain stock solutions of 2 mg/L. The minimal inhibitory concentration (MIC) of L-AMB was determined according to M27-A3 protocol and M27-S4 supplement for *Candida* sp. and M38-A2 protocol of Clinical and Laboratory Standards Institute for *Aspergillus* sp. (CLSI, 2008a,b and 2012). The MIC of each antibacterial drugs was also determined; the tested antibacterial concentrations ranged between 0.25-128 mg/L. In order to evaluate the effect between L-AMB and the different antibacterial agents, the MIC to L-AMB was determined in the presence of 0.5-, 1-, 2- and 4-fold peak plasma concentrations of each antibacterial drug. Peak plasma levels described in the literature are: RIF 12 mg/L (Van Ingen et al., 2011), AZM 4 mg/L (Sevillano et al., 2006), CLR 2 mg/L (Kees et al., 1995), CST 3 mg/L (Michalopoulos and Falagas, 2006), TET 2 mg/L (Agwuh and MacGowan, 2006), and LZD 12 mg/L (Dryden, 2011). Briefly, the drugs were diluted 2-fold in culture medium

in order to obtain a 1:4 dilution. Fifty microliters of each L-AMB concentration (ranging from 0.03 to 16 mg/L) were combined with 50 µL of each of the concentration of the distinct antibacterial agents (described above). The plates were incubated at 35° C and the L-AMB MIC values determined after 24 and 48 h; MIC was the lowest concentration that prevented any discernible growth. Since clinical breakpoints were not yet established for L-AMB, the *Candida* isolates were classified in wild type (wt) whenever the MIC was ≤ 2 mg/L and non-wild type (nwt) when the MIC was > 2 mg/L according to the epidemiological cutoff values (ECVs) proposed by Pfaller and Diekema (Pfaller and Diekema, 2012). For *A. fumigatus*, the clinical breakpoint (CBP) for L-AMB is 1 mg/L (Elefanti et al., 2014). *C. albicans* ATCC 90028 and *A. fumigatus* ATCC MYA-3626 type strains were used as controls, as recommended by CLSI protocol (CLSI, 2008a,b and 2012). *E. coli* ATCC 25922 was used as a bacterial quality control to assure that antibacterial dilutions were correct. MIC determination assays were performed in triplicate.

Evaluation of cell viability

The evaluation of cell viability was performed with *C. albicans* 596, as a representative example, for the most synergic association, L-AMB/CST. The concentrations tested were selected according the susceptibility results. Yeast suspensions (10^6 yeast cells/mL) were exposed to L-AMB 0.125 mg/L alone and in combination with CST 3 mg/L, for 24 h at 35°C, 150 rpm. Another suspension was prepared and treated with single CST 3 mg/L. At specific time points, 1, 3, 6 and 24 h, aliquots were collected and tested for viability. Cell viability was determined in triplicate by counting colony forming units (CFU). Briefly, the number of viable cells for each treatment was determined by plating 100 µL of serial dilutions on SDA agar medium and incubating at 35°C for 24 h; the number of CFUs was determined and compared with control plates (not exposed to drugs or exposed to each antimicrobial drug, L-AMB and CST); before being plated, cells were washed once and resuspended in fresh medium in order to prevent antifungal carryover.

Functional characterization of drug interaction

The distinct cellular physiological status resulting from the interaction between L-AMB and CST were assessed by flow cytometry in a time-course assay, using *C. albicans* 596 as a

representative example. A cell suspension (10^6 yeast cells/mL) was used in all assays described below. Yeast cells were incubated with single L-AMB 0.125 mg/L and in association with CST 3 mg/L during 24 h. In order to evaluate whether there was a CST concentration-dependent effect, the following treatment conditions were also tested: L-AMB 0.125 mg/L with i) CST 1.5 mg/L and ii) CST 6 mg/L. The cellular status induced by single CST treatment was evaluated as control. At specific time points, 1, 3, 6 and 24 h, aliquots were collected and evaluated. All cytometric evaluations were performed in a FACSCalibur cytometer (BD Biosciences, Sydney, Australia) standard model, equipped with 3 photomultipliers, standard filters, a 15-mW 488-nm Argon laser, and using CellQuest Pro software (version 4.0.2). All the assays were performed in triplicate.

The effect of L-AMB 0.125 mg/L alone and in association with CST 3 mg/L was evaluated regarding: i) membrane potential, ii) membrane integrity, iii) metabolic activity, and iv) endogenous reactive oxygen species (ROS) production.

i) Evaluation of membrane potential

The cell membrane potential was assessed by staining the cells with Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol ($DiBAC_4(3)$, Sigma-Aldrich), as described by Teixeira-Santos et al. (Teixeira-Santos et al., 2012). The fluorescence intensity (FI) at FL1 (fluorescent detector; 530 nm) was registered and a staining index (SI) was defined as the ratio between the FI of treated cells and the FI of non-treated cells.

ii) Evaluation of membrane integrity

Cell membrane integrity impairment was evaluated using propidium iodide (PI, Sigma-Aldrich) staining. After antimicrobial treatment, yeast cells were stained with 1 mg/L of PI for 30 min at 35°C, in the dark (Pina-Vaz et al., 2005). The FI was measured at FL3 (fluorescent detector; 630 nm). The amount of injured cells in each sample was defined as the percentage of PI-positive cells.

iii) Evaluation of metabolic activity

Metabolic changes were evaluated using 5-Carboxyfluorescein diacetate (5-CFDA, Sigma-Aldrich), at 10 µM final concentration. Antimicrobial treated cells were stained with 5-CFDA and incubated for 45 min, at 35 °C, at 150 rpm, in the dark (Liao et al., 2003). The mean intensity of fluorescence (MIF) was registered at FL1 (530 nm).

iv) Evaluation of endogenous ROS production

Reactive oxygen species production was evaluated as previously described (Yan et al., 2009). In brief, yeast cells were incubated with 20 mg/L of 2',7'-dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich) for 30 min at 35°C, at 150 rpm. Cells were washed once (2,655 x *g* for 5 min at room temperature; 5417R, Eppendorf) and resuspended in phosphate-buffered saline (PBS, Sigma-Aldrich); afterwards, cells were treated with the antimicrobials as described in "Functional characterization of drug interactions" section. FI was determined at FL1 (530 nm). ROS production was calculated by subtracting the FI value displayed by cells treated with antimicrobials from that of cells treated with both antimicrobials and DCFH-DA.

Simultaneously, 10 µL of the treated cell suspension were placed in a microscope slide for further analysis under fluorescence microscopy, in a Carl Zeiss Axiovert inverted microscope, using laser wavelength of 488 nm.

Molecular dynamics studies

The Automated Topology Builder (ATB) webserver was used for the AMB ($C_{47}H_{73}NO_{17}$) and CST ($C_{52}H_{98}N_{16}O_{13}$) molecular dynamics (MD) topology parametrization, corresponding to the new residues name IDs D3JY and 09SS, respectively, as a GROMACS G54A7FF United-Atom force field (Malde et al., 2011).

The ATB derived force-field was tested in a series of independent MD simulations in an explicit water box. Then, AMB and CST molecules were placed in the same simulation box, together with 6230 SPCE water molecules, at more than 17 Å apart from each other, in order to avoid any computational bias. The entire MD protocol, including energy minimization, equilibration, and production steps, were performed using GROMACS 4.6.1 simulation package

(Hess et al., 2008). MD simulations were performed at the in-house Fermi GPU high performance computing workstation. The global charge of the system was zero and simulations run under periodic boundary conditions in an isothermal-isobaric (NPT) ensemble at 300K and 1.0 bar, during 20 ns, as described in previous studies (Branco et al., 2012).

Statistical analysis

Results are detailed as mean value and the respective standard deviations. Paired-sample Student's *t* test was used to compare the effect of L-AMB and CST alone and in association.

All statistical analysis was performed using the SPSS software (v. 23.0).

RESULTS

Susceptibility to L-AMB alone and in association with antibacterial drugs

Single RIF, AZM, CLR, CST, TET, and LZD did not inhibit the growth of all the fungal species studied, even at the maximum tested concentrations (4-fold peak plasma concentrations). The fungal strains showed variable susceptibility profiles to L-AMB, being all *Candida* strains wild-type (MIC value ≤ 1 mg/L), except *C. krusei* with a MIC value of 8 mg/L. Regarding *A. fumigatus* MIC values of 2 and 4 mg/L were registered (Table 1).

Table 1 – Minimal inhibitory concentration (MIC) of liposomal amphotericin B alone and in association with several antibacterial drugs at 4-fold peak plasma concentrations.

Fungal isolates	MIC value of L-AMB (mg/L)						
		L-AMB in association with					
	L-AMB	RIF	AZM	CLR	CST	TET	LZD
C. albicans ATCC 90028	1	0.25	0.25	0.25	0.125	0.25	1
C. albicans 596	1	0.125	0.25	0.25	0.125	0.25	1
C. albicans 38	0.5	0.06	0.25	0.25	0.06	0.25	0.5
C. glabrata 590	0.25	0.125	0.125	0.125	0.06	0.125	0.25
C. krusei 120	8	2	4	8	2	2	8
A. fumigatus ATCC MYA-3626	1	0.25	0.5	0.25	0.06	0.125	1
A. fumigatus 676	4	1	1	1	0.125	0.25	4
A. fumigatus 88	2	0.5	1	0.5	0.125	0.125	2

L-AMB, liposomal amphotericin B; RIF, Rifampicin; AZM, Azithromycin; CLR, Clarithromycin; CST, Colistin; TET, Tetracycline; LZD, Linezolid.

Minimal inhibitory concentration of L-AMB was reduced 2-to 4-fold in the presence of peak plasma concentrations of RIF, AZM, CLR and TET. The maximum effect of these antibacterial agents was obtained at 4-fold plasma concentrations with a reduction of L-AMB MIC of 2-to 8-fold, in the case of *Candida* (except CLR regarding *C. krusei* 120) and 2-to 16-fold for *A. fumigatus* (Table 1). CST at 0.5-fold peak plasma concentration (1.5 mg/L) resulted in a reduction of L-AMB MIC of 2-to 4-fold for all fungal isolates tested. At the peak plasma concentration (3 mg/L), CST reduced L-AMB MIC in 4-to 8-fold for *Candida* spp. and 16-to 32-fold for *A. fumigatus.* Interestingly, CST at 2- and 4-fold peak plasma concentration (6 and 12 mg/L, respectively)

resulted in the same effect upon L-AMB MIC as CST 3 mg/L. Linezolid, even at 4-fold peak plasma concentration did not associate with any L-AMB MIC reduction, regarding all tested fungal strains.

Colistin was selected for further studies since it was the drug that exhibited the highest synergistic effects with L-AMB.

Impact of L-AMB/CST association upon:

i) Fungal cell viability

Figure 1A represents *C. albicans* 596 viability at subinhibitory concentration of L-AMB (0.125 mg/L), alone and in association with CST 3 mg/L. Treatment with L-AMB 0.125 mg/L significantly decreased yeast cell viability up to 3 h of exposure ($p = 0.039$); however, after 3 h of treatment, cells recovered the ability to replicate and viability increased up to 24 h. At this time point, a significant difference was registered between cells treated with L-AMB and non-treated cells ($p = 0.011$). In the case of cells exposed to CST, significant growth reduction was only registered after 24 h of incubation ($p = 0.009$). Whenever cells were treated with L-AMB in association with CST there was a significant reduction of CFU counts (10^7 to 10^5 cells/mL) soon after 3 h of incubation ($p = 0.037$). This CFU reduction was consistently observed along the time up to 24 h ($p = 0.004$).

ii) Cell membrane permeability

L-AMB acts on fungal cells by binding to the sterol component of the cell membrane, first leading to alterations in cell permeability (Gray et al., 2012). In order to evaluate whether antibacterial agents improve the effect of L-AMB on cell membrane permeability, the membrane potential and cell membrane integrity were assessed with the fluorescent dyes $DiBAC_4(3)$ and PI, respectively. $DiBAC_4(3)$ enters only in depolarized cells, where it binds reversibly to intracellular components, resulting in an increased fluorescent signal. The results obtained regarding membrane depolarization of *C. albicans* 596 are depicted in Figure 1B; after 3 h of treatment, the SI of cells exposed to L-AMB 0.125 mg/L was 2.65 ($p = 0.173$); at 6 h, the SI increased to 3.88 ($p = 0.043$), remaining constant up to 24 h ($p = 0.042$). Whenever yeast cells were exposed to CST, the SI was about 1.00 up to 6 h of treatment ($p = 0.082$), increasing to 2.36 at 24 h ($p = 0.052$). Regarding the association of L-AMB 0.125 mg/L with CST 3 mg/L, soon after 1 h of treatment the

SI was 3.79, increasing up to 14.38 following 24 h. Significant differences were observed between the association L-AMB/CST and single L-AMB at 1 h (p = 0.025), 3 h (p = 0.028), 6 h (p = 0.011), and 24 h (p = 0.011). L-AMB 0.125 mg/L in association with CST 1.5 mg/L induced an increase of SI up to 24 h (SI = 6.69) (p = 0.028). This effect was more pronounced when L-AMB 0.125 mg/L was combined with CST 3 mg/L, as previously described. However, when L-AMB 0.125 mg/L was combined with CST 6 mg/L no significant differences were found, compared to the association L-AMB/CST (3 mg/L) (data not shown). Such results show the key role of L-AMB/CST association on the increase of cell membrane depolarization.

Conversely, PI is a cell viability marker, which enters cells only when membrane has been seriously injured. The results regarding membrane lesion of *C. albicans* 596 are represented in Figure 1C. In cells treated with L-AMB 0.125 mg/L, the % of PI(+) cells after 1 h of exposure was about 10.00%, increasing slightly up to 24 h (% of PI(+) cells = 14.93); significant differences were found at 1 h (p = 0.019), 3 h (p = 0.036), 6 h (p = 0.008), and 24 h (p = 0.036), compared to control (non-treated cells). Cells treated with CST 3 mg/L showed PI-uptake only after 24 h of incubation, with a % of PI(+) cells equal to 5.93 (p = 0.046). Concerning the L-AMB/CST association, soon after 1 h of exposure, PI stains 21.82% of the treated cells, and after 6 h of incubation, this value reaches 39.47%; at 24 h, the percentage of cells with membrane lesion was about 45.01%. Significant differences were observed between the L-AMB/CST association and L-AMB treatment at 1 h (p = 0.011), 3 h (p = 0.008) 6 h (p = 0.002), and 24 h (p = 0.040). Thus, data suggests that L-AMB/CST association promotes the membrane lesion effect of L-AMB, in a time-dependent fashion.

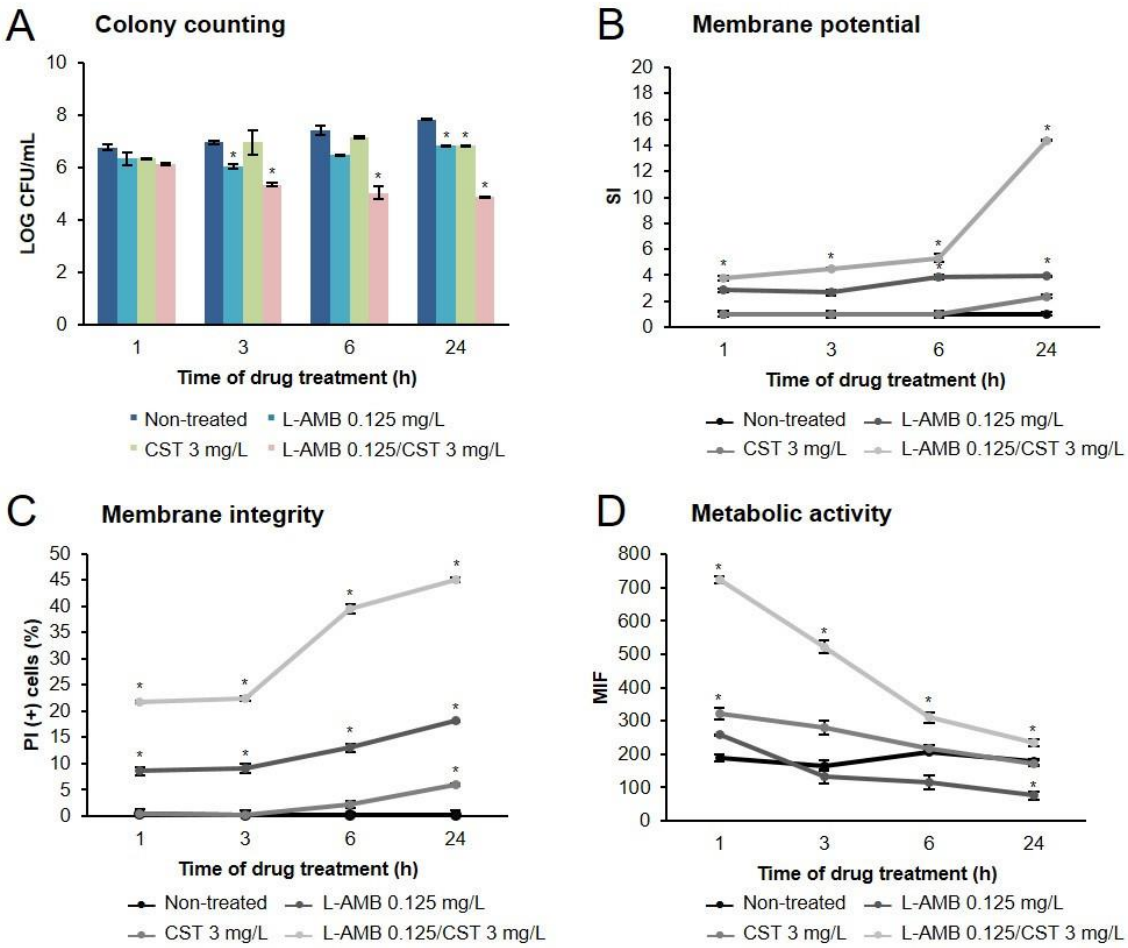

Figure 1 - Effect of liposomal amphotericin B in combination with colistin upon *C. albicans* (strain 596) physiological parameters. (A) Viability assessment by colony forming units (CFU) enumeration of *C. albicans* cells exposed to (■) L-AMB, (■) CST, and (■) L-AMB in association with CST. The CFU counts of cells non-treated are represented as (■). (B) Cell membrane potential was evaluated using $DiBAC_4(3)$ staining. (C) Cell membrane integrity was evaluated using propidium iodide (PI) staining. (D) Cell metabolic activity was evaluated using 5-CFDA staining. Data at respective time points corresponds to mean ± standard deviations. **p* values < 0.05, significant differences between the treatments with L-AMB alone and CST alone *vs.* the control (non-treated cells); and between the association L-AMB/CST and L-AMB alone.

iii) Metabolic activity

To assess the metabolic effects of L-AMB/CST upon *C. albicans* 596, the cells were stained with 5-CFDA; 5-CFDA is a cell-permeant esterase substrate that measures enzymatic activity (Breeuwer et al., 1995; Boender et al., 2011). Only the cells metabolically active will display a high level of fluorescence. The results obtained for MIF are detailed in Figure 1D. Mean

intensity of fluorescence displayed by viable cells (not exposed to the drugs) remained constant up to 24 h; at this time point, the MIF was 176.99. Cells treated with L-AMB did not display significant differences in MIF up to 3 h (p = 0.198). After 6 h, MIF decreased over time up to 75.53 (p = 0.049) at 24 h of exposure. When yeast cells were treated only with CST 3 mg/L, the MIF increased after 1 h (322.59) (p = 0.029), indicating that the cells were metabolically active. However, after 3 h of treatment, the MIF decreased up to a value of 172.11 (p = 0.220), at 24 h, similar to the MIF of cells not exposed to the drugs. Cells exposed to the association L-AMB/CST initially displayed a very high MIF (724.30); however, it decreased until 24 h of treatment (234.91). Significant differences were registered between the metabolic activity of cells exposed to the drug association and to L-AMB alone at 1 h (p = 0.001), 3 h (p = 0.021) 6 h (p = 0.014), and 24 h (p = 0.025).

iv) Endogenous ROS production

Reactive oxygen species production was assessed by DCFH-DA staining. DCFH-DA is oxidized to highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF) by ROS. ROS production by cells exposed to L-AMB 0.125 mg/L was constant up to 6 h of incubation (≈ 7.00%) (p = 0.005); at 24 h, the number of fluorescent cells increased up to 18% (p = 0.001) (Figure 2A). Exposure to CST 3 mg/L resulted in reduced ROS formation in *C. albicans* 596 cells, reaching a value of 7.00% (p = 0.010), following a 24 h of incubation. Images obtained by fluorescence microscopy (Figure 2B) showed vesicle formation in the presence of CST, after 24 h of incubation. Cells exposed to L-AMB/CST displayed a growing ROS production pattern; 7.00% following 6 h (p = 0.056); 25.53% following 24 h of incubation (p = 0.022) (Figure 2A). These results reveal that L-AMB/CST leads to an increase of intracellular accumulation of ROS in relation to single L-AMB after 24 h.

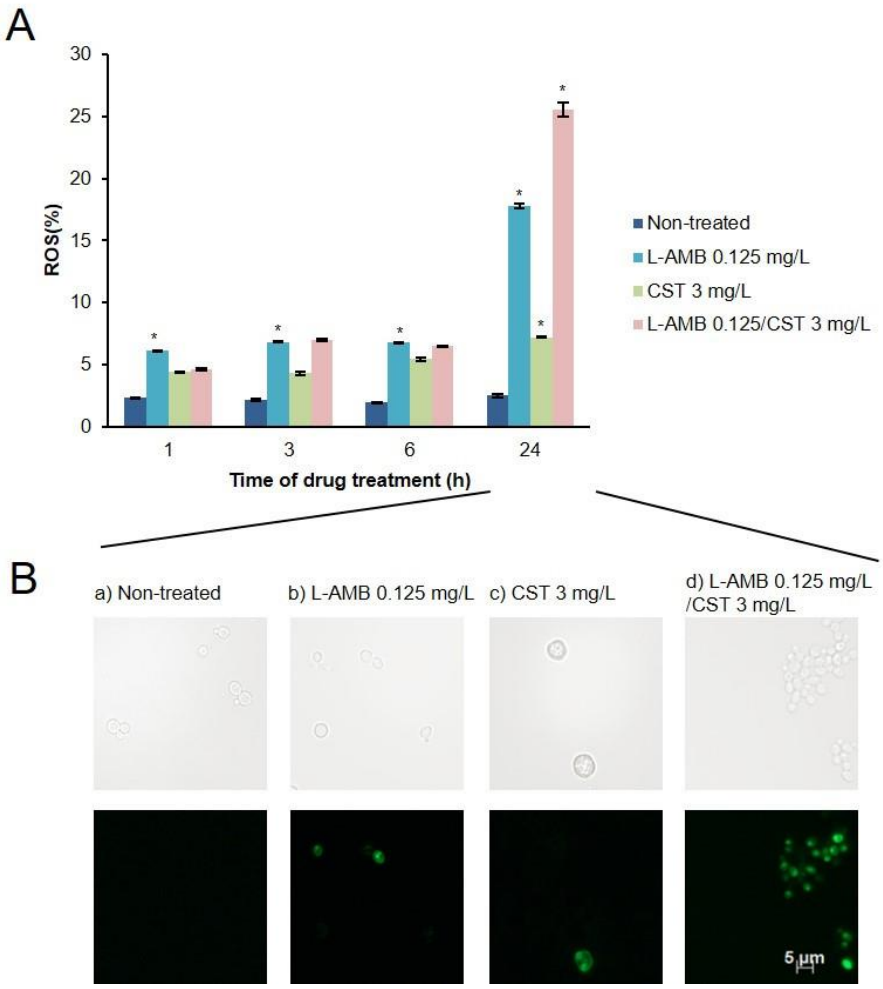

Figure 2 - Effect of liposomal amphotericin B (L-AMB) in association with colistin upon endogenous ROS production. (A) Endogenous reactive oxygen species (ROS) production determined by DCFH-DA staining, assessed by flow cytometry. (B) Fluorescence microscopy imaging showing ROS-positive cells after treatment with b) L-AMB and c) CST alone and d) in association after 24 h of exposure; a) control, non-treated cells. *p values < 0.05, significant differences between the treatments with L-AMB alone and CST alone *vs.* the control (non-treated cells); and between the association L-AMB/CST and L-AMB alone.

Self-association propensity assessed by atomic molecular dynamics simulations

It is known the propensity of AMB to form dimeric or even higher complex tetrameric structures once in water medium, which interfere with the electrophysiology of living cells through transmembrane ion channel formation (Starzyk et al., 2014). Herein, we describe the propensity of AMB and CST in water also to self-assembly based on molecular dynamics simulations. The results demonstrate that the two molecules tend spontaneously form a natural complex in solution, starting from a perfect unbound, solvated form, separated by more than 17 Å. Once the

two molecules meet each other after the first 4 ns of simulation, they are steadily attracted into a complex formation that stands stable for the rest of the simulation time. This complex is characterized by polar interactions, namely hydrogen bond interactions between the C_5- and C_9-OH groups of AMB and amide groups of CST, as depicted in Figure 4A and B. These two H-bond pairs (AMB-O_{58}:CST-N_{18} and AMB-O_{66}:CST-O_9) converged for a minimum distance of 2.7 Å and 2.5 Å, respectively. It seems that the complex is quite stable on the simulation window, supporting an analogous mechanism to the one predicted and validated experimentally for the dimerization of AMB alone. However, the main dimer-stabilizing contacts of the AMB:CST system seems to be the polar interactions and not the van der Waals forces contribution, as described for the hydrophobic nature of dimerization process of AMB molecules, which still might be essential for speeding up transmembrane intercalation of the complex and consequent disruption of the cell membrane through pore formation, resulting into a perfect and synergetic Trojan mechanism for the antimicrobial internalization (Starzyk et al., 2014).

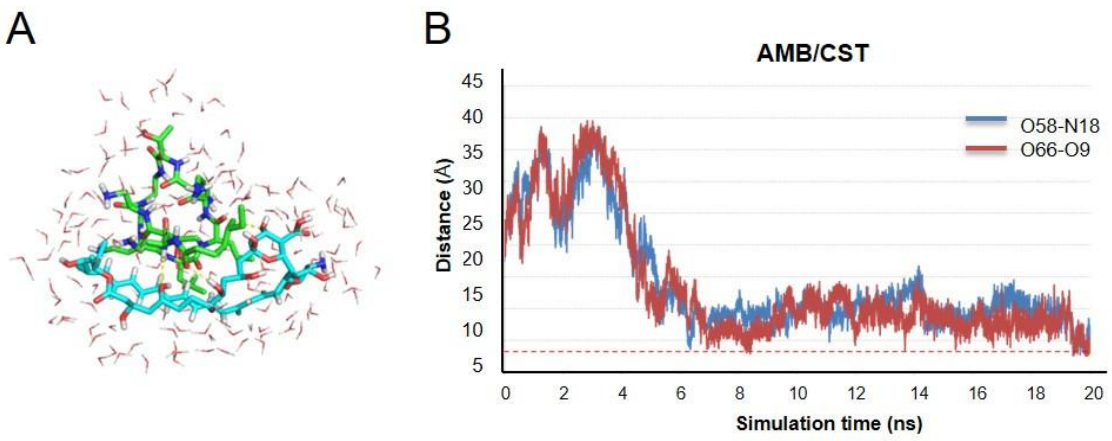

Figure 3 - Self-assembly of amphotericin B (AMB) and colistin (CST) (represented in sticks) in water medium (represented in lines) through molecular dynamics (MD) simulation studies. (A) Complex conformation snapshot after 20 ns of simulation, including water molecules within 4 Å of the complex. (B) Time evolution of the h-bond distance between two molecules during the complex formation.

DISCUSSION

This study demonstrates that all the tested antibacterial agents except linezolid interact synergistically with liposomal amphotericin B against several pathogenic fungi, namely *C. albicans*, *C. glabrata*, *C. krusei*, and *A. fumigatus* (Table1). These antibacterial agents alone did not exhibit antifungal activity, thus suggesting that such compounds do not intrinsically target the fungal cells. The agents tested belong to distinct antibacterial classes with different mechanisms of action. Rifampicin inhibits the DNA-dependent RNA polymerase (RNAP), leading to the suppression of RNA synthesis and cell death (Campbell et al., 2001). The basic architecture of bacterial RNAP and yeast RNAP presents several structural differences (Minakhin et al., 2001), explaining why RIF by itself does not display antifungal activity. Azithromycin and CLR are macrolide antibiotics that bind to 50S ribosomal subunits of bacteria, blocking the protein synthesis and inhibiting cell growth (Kanoh and Rubin, 2010; Parnham et al., 2014); LZD also binds to the 50S subunit of the prokaryotic ribosome, blocking the assembly of a functional initiation complex for protein synthesis (Livermore, 2003); TET inhibits protein translation in bacteria by binding to the 30S ribosomal subunit (Chopra and Roberts, 2001). Although the basic mechanism of protein synthesis in eukaryotes is similar to that in bacteria, some noteworthy differences were described between the structure of eukaryotic and prokaryotic ribosomes (Berg et al., 2002). Colistin belongs to the family of polymyxin antibiotics, which target bacterial cell membrane. CST displaces Mg^{2+} and Ca^{2+} ions, which stabilize the negatively charged lipopolysaccharide, disrupting the membrane integrity in gram-negative bacteria (Falagas and Kasiakou, 2005). The antifungal activity of polymyxin antibiotics against several fungal species has been previously reported (Schwartz et al., 1972); however, at the hereby tested concentrations, CST did not inhibit fungal growth. Colistin plasma concentrations usually range from 0 to 15 mg/L, with free CST levels ranging from 0.5 to 3 mg/L (Akers et al., 2015). It is important to emphasize that about 50% of CST molecules bind to plasma proteins (Falagas and Kasiakou, 2005).

Considering all the antibiotic drug combinations evaluated, the one that resulted in the strongest synergic effect was L-AMB/CST. Additionally, the synergistic combination of CST with other antifungal agents, namely echinocandins class, has been previously described (Zeidler et

al., 2013). Therefore, we explored the underlying mechanism of synergism of L-AMB/CST interaction.

Amphotericin B is a lifesaving antifungal drug used to treat deep-seated fungal infections, exhibiting broad-spectrum fungicidal activity. This activity is based on its interactions with fungal cell membranes (Starzyk et al., 2014). AMB primarily binds to ergosterol, inserts into the cytoplasmic membrane, and forms pore-like structures; the result is osmotic instability, loss of membrane integrity, metabolic disruption, and ultimately cell death (Gray et al., 2012). Our results showed a significant growth reduction of fungal cells treated with L-AMB/CST during 24 h of exposure (10^8 to 10^5 cells/mL), with a simultaneous increase of cell membrane permeability, as documented by $DiBAC_4(3)$ (SI was 14.38) and PI staining (% of PI(+) cells was 45.01%) (Figures 1B and C). Altogether, these results strongly indicate that the association of CST significantly improves the fungicidal activity of L-AMB.

It is known that yeast cells exposed to L-AMB pressure reprogram their metabolism in response to an environmental stress (Zhang et al., 2002; Belenky et al., 2013; Teixeira-Santos et al., 2015). The combination of L-AMB and CST triggered an increase of metabolic activity in treated cells, soon after 1 h of exposure (3.8 fold higher than single L-AMB treatment; Figure 1D), suggesting an early strong stress response induced by membrane permeabilization. Along 24 h of exposure, the metabolic activity decreased overtime, nevertheless, still exhibiting a MIF 3.1 fold higher than single L-AMB treatment (Figure 1D). This decrease can be explained by membrane pore formation, which may cause the accelerated loss of fluorescence, or by a reduced metabolic activity (Breeuwer et al., 1995; Teixeira-Santos et al., 2015). Interestingly, the higher cell metabolic activity induced by the association of L-AMB/CST versus single L-AMB along 24 h period can be related with a higher endogenous ROS production in cells exposed to this association (Figure 2), which will result in oxidative damage and possibly is involved in induced programmed cell death (Phillips et al., 2003; Al-Dhaheri and Douglas, 2010). Curiously, single CST (3 mg/L) has a significant effect on ROS production by itself. Accordingly, cells exposed to CST display a different phenotype compared to cells exposed to the other treatment conditions, i.e. the formation of vesicles (apoptotic bodies), which is a characteristic event of apoptosis (Fink and Cookson, 2005). Although the functional studies that support the mechanism of action of L-

AMB/CST association have been conducted only in *C. albicans*, according to the MIC determination assays, this mechanism seems to be transversal to the different species studied.

All of our results point to a considerable improvement of L-AMB antifungal effect, at subinhibitory concentrations, whenever associated to CST 3 mg/L. However, how do AMB and CST molecules interact? The computational molecular dynamics results demonstrate that the two molecules spontaneously form a natural complex in solution, characterized by a strong bond 1:1. Thus, AMB and CST molecules act together on fungal cells. In support of such a finding was the fact that the maximum synergistic effect was detected in the presence of peak plasma concentration of CST (3 mg/L); its increase to 4-fold peak plasma concentration did not increase the synergistic effect, as documented by MIC determination and membrane potential evaluation assays. Moreover, a recent study described that the interactions of AMB with biomembranes are managed by the molecular organization of AMB (Starzyk et al., 2014). AMB self-associates to dimeric structures; AMB dimers can further assemble into tetramers, which induce the formation of transmembrane ion channels, impairing the electrophysiological homeostasis of a living cell (Starzyk et al., 2014; Davis et al., 2015). Considering all these findings, it is possible that CST binds to AMB molecules and accelerates the assembly of AMB tetramers, thus inducing pore formation on fungal cell membranes, also triggering a strong cell stress response, which is typical of AMB action.

Our results are extremely promising since AMB is an important therapeutic alternative for the treatment of IFIs, particularly when infection persists, despite treatment with other drugs, and the clinical response to AMB is reduced in about 40% of treated patients (Mora-Duarte et al., 2002; Ito and Hooshmand-Rad, 2005; Park et al. 2006). Simultaneously, this synergic association may be a clue for drug discovery.

Studies are being conducted in order to characterize the functional groups of the AMB/CST complex that interact with the fungal membrane aiming to design a more active antifungal compound.

Chapter VI

Overall Discussion

More than fifty years after its availability for clinical use, AMB remains a very important therapeutic option in case of deep seated mycosis, particularly when the infection persists despite treatment with azoles or echinocandin drugs. While the development of resistance is an extremely rare event, the clinical response to AMB often does not match the predicted response by the *in vitro* susceptibility profile (Ullmann et al., 2006; Moen et al., 2009). The present work provides data that suggests an explanation for the clinical failure of AMB treatment, and also propose some antimicrobial compounds as enhancers of AMB activity. Amphotericin B therapy may fails, not because fungi may acquire resistance as it happens with other antifungals (Shapiro et al., 2011); instead, treatment failure could be linked to other factors, including plasma AMB concentrations and the inability of the drug to penetrate certain niches of the patient's body, allowing infection to persist (Paterson et al., 2003; Vincent et al., 2013). Based on this assumption, the yeast response to liposomal-AMB exposure at simulated plasma concentrations and a constant concentration was clarified in a comprehensive phenotypic and functional assay. Indeed, the results demonstrated that the effectiveness of AMB depends on the concentration and time of exposure. The yeast exposure to L-AMB plasma concentrations promotes a series of mechanisms at a distinct levels, like replicative ability, membrane integrity, metabolic activity, endogenous ROS production, and DNA damage, which allow homeostatic regulation and recovery from drug stress. While membrane depolarization and loss of replication competency is an early event (occurring within the first 6 h) in a phase of decline caused by exposure to high concentrations (5 to 20 mg/L), recovery can occur at low drug concentrations, along the remaining 18 h. Yeast cells initially exposed to high L-AMB concentrations suffer membrane depolarization followed by pore formation. The membrane pores induced by L-AMB action promote ion exchange between the intracellular and extracellular environment (Baginski et al., 1997; Milhaud et al., 2002; Cohen, 2014). Therefore, such an action triggers the activation of signaling pathways responsible for sensing and responding to the osmotic stress (Cohen, 2014). According to the AMB mechanism of action recently proposed, AMB firstly extracts ergosterol from lipid membranes (Anderson et al., 2014); as result, the pathways involved in the control of the lipid composition and ergosterol content of the cell membrane are activated, justifying the slightly high cellular metabolic activity in the first hour of exposure to high AMB concentrations. Amphotericin B has been found to downregulate genes involved in the biosynthesis of ergosterol, fatty acid elongases, and

synthetases as well as ceramide, which are enzymes and lipid precursors required for the biosynthesis of sphingolipids (Zhang et al., 2002; Cohen, 2014). Sterols and sphingolipids are important components of lipid rafts, the membrane microdomains that perform important roles in the targeting and activity of signaling proteins in eukaryote cells. Ergosterol is also an important element in fungal cell physiology, being associate to cell division, endocytosis, cell signaling, membrane compartmentalization, and functional regulation of membrane proteins (Mollinedo, 2012; Cohen, 2014). Owing to the essential role of ergosterol in fungal membranes, its depletion leads to fungal cell death. This fact may explain the loss of replication competency during the firsts 6 h wherein the cells are exposed to high L-AMB concentrations. However, when L-AMB concentrations start to decay, the fungal cells can recover from AMB effect: cell membrane potential is restored during the remaining 18 h that the cells are exposed to levels of drug close to or below the MIC. The intracellular equilibrium of ion concentration as a result of environmental adaptation is restored. As a consequence, the fungal cells return to a functional physiological status, reestablishing the components and full biological activity of their membranes. The initial loss of replication competency is restored and the cells maintain constant their metabolic activity up to 24 h. Other authors have also confirmed this finding; yeast cells exposed to AMB have a capacity for “resuscitation”, although they are unable to replicate (Liao et al., 1999). This result may be explained by the increase, and then the decrease of AMB-L plasma concentrations that rapidly evoke compensatory responses in yeast cells. Residual reactive oxygen species (ROS) production also happened in yeast cells exposed to L-AMB at plasma concentrations after 6 h of exposure. The production of ROS is associated with oxidative damage, and possibly is involved in induced cell death. Indeed, the production of ROS induced by AMB has been described as a universal and important mechanism of action that is correlated with fungicidal effect, and might explain the low rate of resistance to AMB (Mesa-Arango et al., 2014). Notably, about 20-30% of cells treated with L-AMB plasma concentrations suffered apoptosis. This results indicate that yeast cells exposed to L-AMB at plasma concentrations, initially compensate by upregulating their physiological responses to minimize the drug-induced stress; when the L-AMB concentrations fall, the cells recover from L-AMB action, allowing the persistence of the fungal infection and possibly resulting in a poor clinical outcome. In contrast, yeast cells exposed to a constant concentration of L-AMB (3 mg/L) during 24 h, despite initially developing compensatory

mechanisms to counteract the drug-induced stress, as the drug concentration is kept constant, the cells will succumb to the drug effect. Cells constantly exposed to L-AMB 3 mg/L lose viability, with clear evidence of cell membrane depolarization and lesion. Soon after 1 h of exposure, the cell metabolic activity increased as a response to environmental stress (Belenky et al., 2013); afterwards, it dropped significantly up to 24 h. The endogenous production of ROS was also registered in cells exposed to L-AMB 3 mg/L; at 24 h, the amount of accumulated ROS was much higher than in cells exposed to plasma concentrations. These molecules are toxic to the cells and have been described as the mains mediators of programmed cell death (Madeo et al., 1999). As result, in cells exposed constantly to 3 mg/L, apoptosis was registered in about 60% of yeast cells, twice the proportion of cells exposed to L-AMB plasma concentrations. Our data suggested that constant L-AMB concentration induces programmed cell death and necrosis in exposed cells, being more effective.

Another aim of our study was to determine whether yeast recovery mechanism to L-AMB effect was *Candida* species specific or was a transversal mechanism, common to other yeasts, with and without clinical relevance. This study comprised a wide range of yeasts (pathogenic and nonpathogenic) with different phylogenetic relationships and distinct antifungal susceptibility profiles. We confirmed that in all species studied (*Candida albicans, C. glabrata, C. parapsilosis, C. krusei, C. tropicalis, C. dubliniensis, C. lusitaniae, Cryptococcus neoformans, Saccharomyces cerevisiae, Debaromyces hansenii, Kluveromyces lactis,* and *Zygosaccharomyces rouxii*), yeast cells exposed to initially high L-AMB plasma concentrations (5 to 20 mg/L) suffered membrane depolarization up to 6 h; however, when the L-AMB concentrations fell, along the remaining 18 h, the cell membrane repolarized and the yeasts recovered from L-AMB action. Interestingly, the mechanism of yeast recovery seems to be unrelated to the MIC values corresponding to the strains studied. In all clinical isolates, independently of the corresponding L-AMB MIC value, these findings were registered. Thus, the recovery mechanism to L-AMB, at low plasma concentrations, might be responsible for a reduced susceptibility to L-AMB and the poor correlation between *in vitro* results and clinical outcome. Moreover, the results obtained suggest that this mechanism might impair the *in vivo* fungicidal activity of L-AMB. Despite these findings may provide important insights about *in vivo* L-AMB antifungal activity, its hypothetical clinical implications should be interpreted with particular caution, since yeast infections are mainly located at tissues and not in

the plasma (Smith et al., 2007). Noteworthy, previous results involving organ biopsies revealed that in some tissues like the kidney and liver, drug levels main remain high overtime. However, such findings are not consistent when considering other organs such as the lung or the central nervous system (Vogelsinger et al., 2006; Smith et al., 2007). In addition, since the diagnosis of invasive fungal infections is often delayed, the fungal burden within the tissues can be considerable at the time of initiation of treatment, requiring a large amount of antifungal drug to be delivered to the tissues to effectively reduce the fungal burden (Jones et al., 2003; Smith et al., 2007). Moreover, patients with IFIs are often administered multiple medications concomitantly with L-AMB, usually repeated daily, which may interfere with L-AMB molecules. During the prolonged periods following L-AMB administration, when drug levels fall to much lower concentrations, the possibility that fungal organisms may recover from the antifungal effect is to be remembered. The present type of investigation may be useful in helping to develop appropriate clinical L-AMB therapeutic regimens. The therapeutic monitoring of AMB should be performed for a better understanding of AMB exposure-response relationship, since the pharmacokinetics and distribution of AMB are sometimes poor, allowing some fungi to hide at certain niches where drug exposure is limited (Ashbee et al., 2013, Vincent et al., 2013). This practice might increase the probability of a successful outcome. However, in clinical routine the therapeutic drug monitoring is not performed (Ashbee et al., 2013).

One possible alternative to improve the clinical response to L-AMB is to focus on combined antifungal therapy. Late diagnosis and poor response to antifungal therapy frequently encourages the use of empirical antifungal combination as salvage therapy, even without scientific base (Baddley and Pappas, 2005). Studies about the *in vitro* effect of antifungal combinations involving the most frequent pathogenic yeasts are scarce and some provide contradictory results (Cuenca-Estrella, 2004). Notably, the available methodologies for evaluation of *in vitro* antifungal associations are very cumbersome, time consuming, and difficult to interpret. We developed an original protocol based on flow cytometry for *in vitro* assessment of associations between amphotericin B and anidulafungin, and anidulafungin and azoles, against *Candida* species. Anidulafungin belongs to the echinocandin class, and inhibits 1,3-β-D-glucan synthase, an enzyme that is necessary for synthesis of an essential component of the fungal cell wall (Gil-Alonso et al., 2015). AMB and azoles are membrane active drugs; the first extracts ergosterol

from the fungal membrane and produces pores, while the latter inhibits the ergosterol synthesis (Anderson et al., 2014; Ghannoum and Rice, 1999). Based on the literature, the echinocandin possibly facilitates the access of AMB and azoles to its target site, thus resulting in a synergistic effect (Baddley and Pappas, 2005 and Karlowsky et al., 2006; Kiraz et al., 2009). Despite the association of two antifungal drugs with distinct mechanisms of action might represent a promising therapeutic alternative for the treatment of IFIs, it cannot be assumed that it will improve the outcome in comparison to monotherapy. According with Kiraz et al., the association between caspofungin and amphotericin B against *C. glabrata* had an indifferent effect in 54-58% of tested isolates, and was antagonistic against 2% of the isolates (Kiraz et al., 2009). Another study has reported that the association between anidulafungin and fluconazole was indifferent in about 67% of *Candida* isolates tested (Karlowsky et al., 2006). These results highlighted the importance of critically evaluate the effect of combination therapy.

Recent reports have demonstrated the advantages of flow cytometry for the evaluation of antimicrobial susceptibility profile in yeasts (Ramani et al., 1997; Pina-Vaz et al., 2001a, 2001b and 2010; Rudensky et al., 2005). Through the use of suitable fluorescent dyes, this approach allows to identify different physiological states, being able to discriminate viable cells from nonviable in a short time (less than 2 h). For assessment of antifungal drug combinations, we selected two fluorescent dyes, $DiBAC_4(3)$, and FUN-1. $DiBAC_4(3)$ is a membrane potential marker that enters depolarized cells, where it binds to intracellular proteins or membranes and exhibits enhanced fluorescence; a higher depolarization results in a higher influx of the dye, and consequently, an increase in cell fluorescence (Baxter et al., 2002). FUN-1 is a metabolic activity dye that passively diffuses through yeast cell membranes; in metabolically active cells, FUN-1 is converted into intravacuolar structures that exhibit orange/red fluorescence, while in metabolically inactive cells it remains intact in the cytoplasm, displaying green fluorescence (Pina-Vaz et al., 2005). According to the data published, FUN-1 is not able to reveal the effect of AMB against yeast cells (Pina-Vaz et al., 2001a); in turn, the precocious effect of azoles can only be demonstrated using FUN-1 (Pina-Vaz et al., 2005). Regarding AND, the obtained results indicate that its activity may be studied using $DiBAC_4(3)$ or FUN-1. Thus, the study of AND/AMB association was performed using $DiBAC_4(3)$, while AND/azoles association was performed with FUN-1. This novel protocol revealed a high accuracy and an excellent agreement with the

checkerboard method, thus allowing the evaluation of different antifungal combinations in less than 2h. Nevertheless, it has the disadvantage that it can only be applied for yeasts, although Ramani et al., already had developed a protocol for antifungal susceptibility testing in *Aspergillus fumigatus* by flow cytometry (Ramani et al., 2003).

The association between AND and AMB resulted mostly in synergism (46%) or no interaction (49%), as previously described (Rosato et al., 2012; Valentin et al., 2016); antagonism was only detected in *C. albicans* isolates (5%). The combination of other echinocandins, i.e. caspofungin or micafungin, with AMB was also described as potentially synergic against *Candida* isolates (Kiraz et al., 2009; Nishi et al., 2009). Regarding the association of AND with azoles, a synergistic effect was detected in 36% of isolates, while no interaction was found in 49% of isolates. The synergistic interaction between anidulafungin and azoles against *Candida*, *Fusarium* and *Aspergillus* species has also been described in other studies (Philip et al., 2005; Rosato et al., 2012; Siopi et al*.,* 2016). The only case of antagonism was found in a *C. parapsilosis* strain. For *C. glabrata* isolates, both associations seem to be promising, since there were no cases of antagonism, and the cases of no interaction were few (29%). The results obtained suggest that drug interaction potentially is species and strain dependent, which reinforces the importance of the novel flow cytometric protocol hereby described. In addition, the synergic association of antifungal drugs seems to increase the action of each other, possibly allowing a decrease of individual dosage thus, reducing side effects for patients (Mukherjee et al., 2005; Karlowsky et al., 2006).

Likewise, the literature has described the synergic interaction between amphotericin B and antibacterial agents, namely those that inhibit RNA or protein synthesis, such as rifampicin, azithromycin, clarithromycin, and tetracycline, against *Candida* and *Aspergillus* species (Azevedo et al., 2015). This kind of association may represent an important therapeutic alternative for the treatment of IFIs, overcoming the AMB limited efficacy and toxicity. However, there are few studies addressing this topic, and there is somewhat limited knowledge about the underlying mechanisms of synergism. In the present work, it was evaluated the association between L-AMB and several antibacterial drugs against *Candida* spp. and *Aspergillus fumigatus*. The association of RIF, AZM, CLR, CST, and TET with L-AMB was synergistic against several pathogenic fungi, encompassing *C. albicans*, *C. glabrata*, *C. krusei*, and *A. fumigatus.* Since these antibacterial

compounds alone were not active against the fungal isolates, apparently, they do not have an intrinsic target in fungal cells, and the synergism occurs by an unknown mechanism. Most of these antibacterial agents, act at the level of bacterial polymerases (RNAP) or ribosome subunits (Campbell et al., 2001; Chopra and Roberts, 2001; Kanoh and Rubin, 2010; Parnham et al., 2014). Indeed, the structure of the prokaryotic targets displays noteworthy differences in comparison with their homologs in eukaryotic organisms (Minakhin et al., 2001; Berg et al., 2002). Another possible explanation for the lack of activity upon fungal cells, is related with the difficulty that antibacterial agents might have in accessing a possible target in fungal cells. The fungal cell damaged due to AMB action, may allow entrance of antibacterial agents, inhibiting RNA/protein synthesis, and resulting in a synergistic effect (Azevedo et al., 2015). Regarding colistin, its antifungal activity has been previously reported, although at the tested concentrations, CST did not inhibit fungal growth (Schwartz et al., 1972). According to our results, despite all antibacterial agents having improved the L-AMB fungicidal action at sub-inhibitory concentrations, the CST was the drug that exhibited the strongest synergistic effect. Thus, the association L-AMB/CST was scrutinized and the underlying mechanism described at a functional and molecular level. Colistin is a polymyxin antibiotic, which targets bacterial cell membrane (Falagas and Kasiakou, 2005). The synergistic effect between CST and antifungal drugs, namely those belonging to echinocandin class, has been described (Zeidler et al., 2013). The functional studies shown that L-AMB in association with CST induced a significant cell growth reduction compared with single L-AMB treatment. At the same time, cells exposed to L-AMB/CST suffered an increase of membrane permeability throughout 24 h. In the first hour of treatment, cells shown a high metabolic activity. As previously mentioned, the drug-induced stress triggers an increase of cell metabolic activity in the first hours of exposure. Signaling pathways are activated in order to maintain cellular structure and function (Zhang et al., 2002; Cohen, 2014). Afterwards, the metabolic activity of cells exposed to this association decreased overtime. The initial high metabolic activity of cells exposed to this association can be related with an intense endogenous ROS production, resulting in oxidative damage for cells, which can lead to induced programmed cell death (Mesa-Arango et al., 2014). The cells exposed to single L-AMB treatment suffered a similar effect, however, less pronounced than in cells exposed to L-AMB/CST association. All these findings are consistent with the mechanism of action of AMB, and with the results described

above (Anderson et al., 2014). That is, the association of CST with L-AMB enhances the fungicidal activity of the latter, even at subinhibitory concentrations. Interestingly, our computational molecular dynamics studies have demonstrated that CST and L-AMB form a natural complex in solution, acting together on fungal cells. It is possible that the formation of this complex catalysis the assemble of AMB structure required for its fungicidal action (Gagós and Arczewska, 2010; Starzyk et al., 2014).

Altogether, the presented results might represent a step forward in the management of invasive fungal infections treatment, for which, frequently liposomal amphotericin B is the only remaining therapeutic option.

Chapter VII

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This dissertation envisaged to explore the fungal cell mechanisms that allow the recovery from amphotericin B treatment, and to investigate strategies to improve the fungicidal activity of this drug. In this section, the scientific contribution of this dissertation will be discussed in conjunction with topics for future research.

CONCLUDING REMARKS

- The results presented in Chapter III demonstrated that the fungal response to L-AMB exposure clearly relates to the drug concentration. Accordingly, the yeast cell can respond in two ways, namely by: (i) expression of compensatory mechanisms as a response to drug-induced stress, and then, survival, whenever are initially exposed to high concentrations of L-AMB, and then to much lower concentrations for long periods of time; (ii) induction of programmed cell death and necrosis, whenever the cells are exposed to a constant high L-AMB concentration along the time. Therefore, the often-insufficient L-AMB concentration at the site of infection may compromise the L-AMB fungicidal action, allowing the increase of fungal burden, which ultimately may result in a poor clinical outcome.

- In chapter III we also concluded that the mechanism of recovery from L-AMB action was common among pathogenic and non-pathogenic yeasts, and was a phenomenon independent of the MIC corresponding to the distinct fungal strains.

- In chapter IV we detailed a novel method for the *in vitro* evaluation of antifungal associations, since this is an empirical and frequent practice, often encouraged by clinical therapeutic failure. This new methodology showed high sensitivity and specificity, and a good correlation with the traditional checkerboard method, providing results in less than 2 h.

- The association of antifungal drugs with different mechanisms of action might represent a possible therapeutic option for the treatment of IFIs. In chapter IV, the association of anidulafungin with amphotericin B or azoles resulted in a synergistic effect in a vast amount of yeast isolates studied.

- In Chapter V we demonstrated that the association of L-AMB with colistin improved the fungicidal activity of the first, and elucidated about the underlying mechanism of synergism, showing that these two drugs act together as a stable complex. Molecular dynamics study was an important tool for unveiling the mechanism of synergism.

- Flow cytometric assays were crucial for the development of the studies described in this dissertation. In chapters III e V this approach allowed to explore the mechanism of action of single L-AMB and in association with colistin. In chapter IV, FC was used to develop a new and reliable method to evaluate the *in vitro* antifungals association.

FUTURE PERSPECTIVES

In the end of this dissertation, some questions remained unanswered and a few new ones came up.

We consider of relevance to corroborate the results obtained *in vitro* concerning the mechanism of yeast cells recovery at L-AMB plasma concentrations, and test the cidal effect of this drug at a constant fixed concentration, in an *in vivo* study. The confirmation of these results using an animal model, could lead to the design of new therapeutic regiments for the treatment of invasive fungal infections. Simultaneously, we would like to develop a novel tool for the therapeutic monitoring of L-AMB possibly based on flow cytometry, since its adoption in routine may increase the probability of a successful outcome. Notably, all the available methodologies are cumbersome, and therefore, impractical in the clinical routine.

Regarding the association of L-AMB with colistin, we would like to characterize the molecular interaction of the complex AMB:CST with the fungal membrane, and afterwards, to design a more active antifungal compound. The study of this interaction should involve molecular dynamics simulations (MDS), and be confirmed using knock-out strains for the best hits identified with MDS.

In addition, we intend to explore further the association of L-AMB with other antibacterial agents. At the moment, the association between L-AMB and tigecycline is being characterized at a phenotypic and a functional level.

Chapter VIII

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Chapter IX

PUBLICATIONS

Publication I

(Chapter IV)

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Novel Method for Evaluating *In Vitro* Activity of Anidulafungin in Combination with Amphotericin B or Azoles

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A combination of drugs possessing different targets has been used as salvage therapy, although without scientific support. *In vitro* studies validating such combinations are scarce, and the methodology is very laborious and time-consuming. This study proposes a flow cytometric (FC) protocol as an alternative to evaluate the effect of the combination of anidulafungin (AND) with amphotericin B (AMB) and azoles (fluconazole and voriconazole), tested upon 39 and 36 *Candida* strains, respectively. The concentration assayed in the combination was 0.5× MIC of each drug. The membrane potential marker $DiBAC_4(3)$ [Bis-(1,3-dibutylbarbituric acid) trimethine oxonol] was used for AND-AMB, and the metabolic marker FUN-1 was used for AND-azoles. Drug interaction was determined by calculating a staining index (SI): the sum of the percentage of depolarized cells (DC) after treatment with drug combinations divided by the DC of the drug alone, and the sum of the mean intensity of fluorescence (MIF) displayed by cells treated with drug combinations divided by the MIF of the drug alone for FUN-1. An SI of <1 means antagonism, an SI between 1 and 4 means no interaction, and an SI of >4 means synergism. The combination of AND and AMB by FC and checkerboard was synergistic for 46 and 43% of isolates and antagonistic for 5 and 8%, respectively. For the combination of AND and azoles, it was synergistic for 36% and antagonistic for 3% by FC and synergistic for 44% and antagonistic for 3% by checkerboard. When the FC method was compared to the gold standard checkerboard method, the agreement was 0.91 (95% confidence interval [95% CI] of 0.88 to 0.94), sensitivity was 0.88 (95% CI of 0.73 to 0.95), and specificity was 0.95 (95% CI of 0.84 to 1). Thus, FC is a rapid and reliable method (<2 h) to assess the effect of antifungal combinations.

Candida species represent an important cause of nosocomial infections with high morbidity and mortality rates (28, 33). Over the past few years, epidemiological changes have been registered: the incidence of *C. albicans* has been reduced followed by a growing incidence of non-*albicans* species (9, 21, 34). Late diagnosis and high mortality rates frequently foment the use of empirical antifungal combinations as salvage therapy without a sound scientific basis (1). The availability of new antifungal drugs with novel targets of action has enlivened the interest in combination therapy. Likewise, it is not possible to assume that the simultaneous administration of two or more drugs with distinct mechanisms of action would improve the clinical outcome compared to monotherapy (3). It is unknown whether a combination might reduce the effectiveness of each drug or increase the potential for drug interactions or even toxicity, keeping in mind that this carries a significantly increased cost to the health care system without previously proven clinical benefits (7). Thus, it is important and timely to critically evaluate the role of combination therapy.

The methods available for studying drug combinations are few and cumbersome and often provide contradictory results. The most commonly utilized are the checkerboard method (based on a mathematical model) and the time-kill assay, both impossible to implement in the routine of clinical laboratories because they are very laborious (15, 35). Therefore, the Etest was proposed as an alternative; however, it also has serious limitations, including the cost, despite its good correlation with the classical method. The Etest is difficult to interpret when dealing with the azoles, due to the inconsistent growth patterns, and it takes at least 24 h to provide results since it is based on microbial growth (15). Critical patients need a rapid response.

Flow cytometry (FC) is a valuable tool for studying antifungal susceptibility, since it can be used to detect different physiological cell stages by using the appropriate fluorescent markers (5, 8). FC susceptibility testing to azoles, amphotericin B (AMB), and echinocandins has already been described (23–25, 27, 30). The goal of this study was to develop an FC protocol to characterize the effects of the combinations of AMB or azoles with the echinocandin anidulafungin (AND) upon *Candida* spp.

MATERIALS AND METHODS

Fungal strains. Thirty-nine *Candida* strains were tested regarding the association between AND and AMB: 14 *Candida albicans*, 8 *Candida glabrata*, 9 *Candida parapsilosis*, 4 *Candida tropicalis*, 1 *Candida guilliermondii*, 2 *Candida krusei*, and 1 *Candida lusitaniae*. For the association between AND and azoles (fluconazole [FLU] or voriconazole [VOR]), 36 strains were tested: 16 *C. albicans*, 9 *C. glabrata*, 7 *C. parapsilosis*, 7 *C. tropicalis*, and 1 *C. krusei*. Clinical isolates of *Candida* spp. with a previously characterized antifungal susceptibility phenotype were selected from the collection of the Department of Microbiology, Faculty of Medicine of Porto, Portugal, in order to study all possibilities: susceptible or nonsusceptible to both drugs or susceptible to one and nonsusceptible to the other. *C. albicans* ATCC 90028 was used as the reference strain. Before

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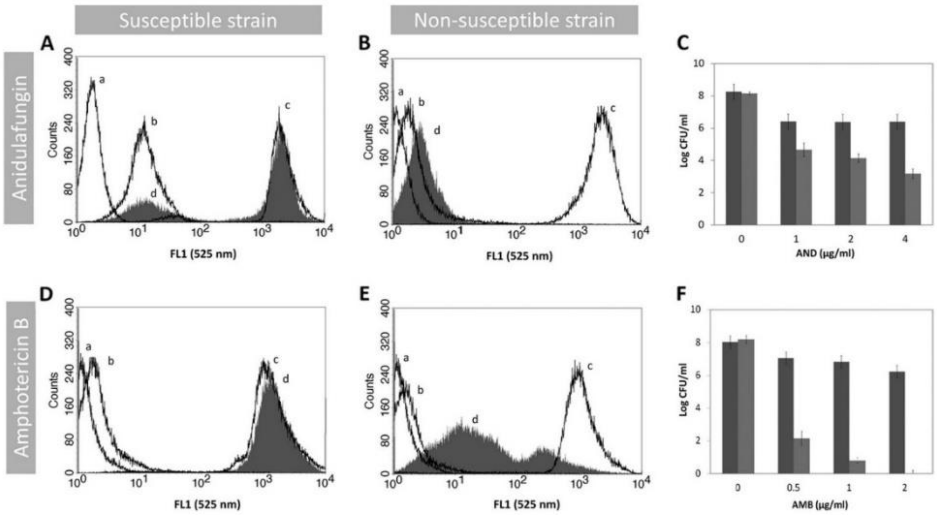

FIG 1 *In vitro* antifungal activities of anidulafungin and amphotericin B. Distribution of fluorescence intensity of the *C. albicans* 0207 AND-susceptible strain (A), *C. parapsilosis* 0136 AND-nonsusceptible strain (B), *C. albicans* O207 AMB-susceptible strain (D), and *C. lusitaniae* D51 AMB-nonsusceptible strain (E). In each histogram, the autofluorescence is represented by line a, line b represents the fluorescence of untreated cells stained with $DiBAC_4(3)$, line c is the fluorescence of cells treated with 70% ethanol and stained with $DiBAC_4(3)$ (positive control), and line d is the fluorescence of cells treated with 1 μg/ml of antifungal drugs during 1 h and stained with $DiBAC_4(3)$. (C, F) Determination of the number of CFU (CFU/ml) of cell suspensions treated with different antifungal concentrations under conditions identical to those of the flow cytometric assay. The nonsusceptible strain is represented by the dark-gray bars and the susceptible strain by the light-gray bars.

the initiation of each experiment, the yeasts were subcultured twice on Sabouraud agar (Liofilchem, Teramo, Italy) to ensure both the viability and that the culture was pure.

Drugs and chemicals. Stock solutions of AMB (Sigma-Aldrich, Taufkirchen, Germany), FLU (Sigma), and VOR and AND (Pfizer, New York) were prepared as recommended by the CLSI protocol M27-A3 (6) and stored at −80°C. Fluorescent dyes 2-chloro-4-(2,3-dihydro-3-methyl-[benzo-1,3-thiazol-2-yl]-methylidene)-1 (FUN-1) and Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] (both acquired from Molecular Probes, Leiden, Netherlands) were prepared in phosphate-buffered saline (PBS) (Sigma) and kept at −20°C.

Checkerboard microdilution studies. Checkerboard assays were performed for AND plus AMB and for AND plus azoles (FLU or VOR). MIC values of each antifungal and for the associations were determined after 24 h of incubation. The concentrations tested for each antifungal ranged between 0.06 and 32 μg/ml for FLU, 0.015 and 8 μg/ml for VOR and AMB, and 0.06 and 2 μg/ml for AND. Endpoints were determined according to values defined by the M27-A3 protocol (6). The fractional inhibitory concentration index (FICI), which is defined as the sum of the MIC of each drug when used in combination divided by the MIC of the drug when used alone, was calculated to determine the interaction; a FICI of ≤0.5 represents synergy, >0.5 to 4 represents no interaction, and >4 represents antagonism (10, 18).

Flow cytometry studies. For FC assays, the strains were subcultured in Sabouraud broth and incubated with agitation at 35°C until the exponential growth phase in order to obtain a homogenous population and thus to correlate the perturbations in cellular parameters observed by FC with the drug action and independently of growth phase. Then, 0.5 McFarland standard density yeast suspensions were prepared in PBS, corresponding to 10^6 yeast cells/ml. The cell suspensions were incubated at 35°C with subinhibitory concentrations (0.5× MIC value) of each antifungal alone and in combination as described above. In order to standardize the FC protocol for all strains, the 0.5× MIC of each drug was chosen since the breakpoints to AND and FLU are being reviewed and their values are species dependent (20, 22), because a new CLSI protocol is not already available, and because antagonistic and synergistic classifications usually rely on deviations from additivity (36). According to the Loewe additivity definition, 0.5× MIC of drug A combined with 0.5× MIC of drug B is equivalent to 1 MIC of drug A or 1 MIC of drug B in an additive drug pair. Even so, for strains inhibited at high MIC values and that do not present antagonism with the combination of 0.5× MIC, a new test was carried out using the breakpoint of the drug (e.g., 8 μg/ml for FLU and 4 μg/ml for AND) in order to evaluate its clinical significance.

Following 1 h of incubation, the cells were washed and incubated for 15 min in the dark at room temperature with 0.5 μg/ml of $DiBAC_4(3)$, a lipophilic anion able to diffuse across depolarized membranes, in the case of the association of AND plus AMB and with 0.5 μg/ml of FUN-1, a metabolic marker, in the case of association of AND plus azoles. The intensity of fluorescence of 30,000 cells was registered at FL1 (530 nm) for $DiBAC_4(3)$ and FL2 (575 nm) for FUN-1. The samples were analyzed in a FACSCalibur cytometer (BD Biosciences, Sydney, Australia) standard model equipped with 3 photomultiplayers (PMTs), standard filters, and a 15-mW 488-nm Argon laser and using CellQuest Pro software (version 4.0.2). Instrument controls followed the standard procedures described by the manufacturer. All trials were performed in triplicate. The evaluation of *in vitro* drug interactions by FC was determined according to the staining index (SI), which is similar to the FICI described above. The SI was calculated as the sum of the percentage of depolarized cells (DC) after treatment with drug combinations divided by the DC of the drug alone for $DiBAC_4(3)$ and the sum of mean intensity of fluorescence displayed by cells treated with drug combinations divided by the fluorescence of the drug alone for FUN-1. Hence, SI = (DC AND + AMB/DC AND) + (DC AND + AMB/DC AMB) for AND-AMB association and SI = (MIF AND + azole/MIF AND) + (MIF AND + azole/MIF azole) (MIF, ratio between mean intensity of fluorescence of treated cells and viable cells) for AND-azoles association. Taking into account the standard classification

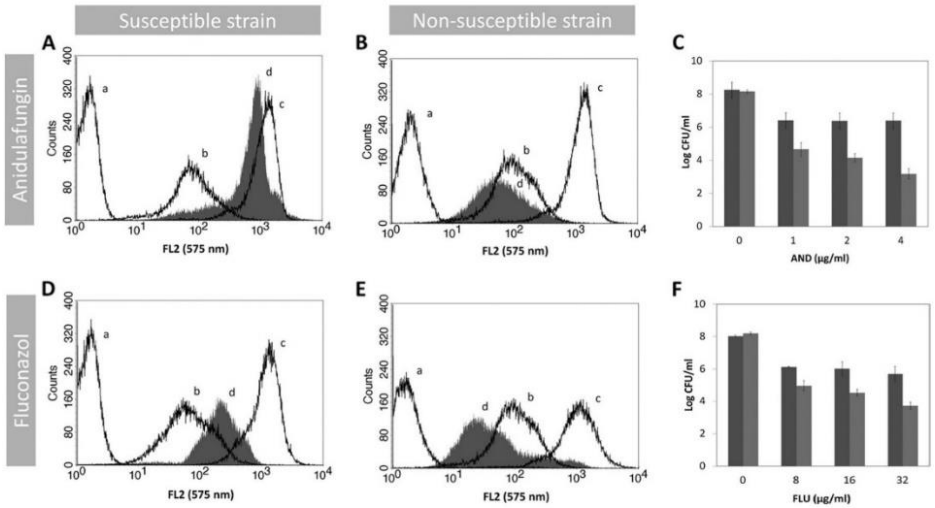

FIG 2 *In vitro* antifungal activities of anidulafungin and fluconazole. Distribution of fluorescence intensity of *C. albicans* 0207 AND-susceptible strain (A), *C. parapsilosis* 0136 AND-nonsusceptible strain (B), *C. albicans* O223 FLU-susceptible strain (D), and *C. albicans* O216 FLU-nonsusceptible strain (E). In each histogram, the autofluorescence is represented by line a, line b represents the fluorescence of untreated cells stained with FUN-1, line c is the fluorescence of cells treated with 70% ethanol and stained with FUN-1 (positive control), and line d is the fluorescence of cells treated with antifungal drugs (1 μg/ml of ANI and 16 μg/ml of FLU) during 1 h and stained with FUN-1. (C, F) Determination of the number of CFU (CFU/ml) of cell suspensions treated with different antifungal concentrations under conditions identical to those of the flow cytometric assay. The nonsusceptible strain is represented by the dark-gray bars and the susceptible strain by the light-gray bars.

of the checkerboard results, an association provided by FC was defined as antagonism for SI of <1, no interaction for SI between 1 and 4, and synergy for an SI of >4.

Determination of viable cells. The number of viable cells in each FC assay was determined by plating 100 μl of serial dilutions on Sabouraud agar medium and incubating at 35°C for 24 h. Afterwards, the number of CFU was determined. No carryover antifungal effect was detected. All assays were performed in triplicate.

Statistical analysis. To evaluate the agreement between checkerboard and FC studies, the proportion of agreement (PA) and the value of Kappa (K) were calculated (31). In order to check the diagnostic validity of FC to detect the effect of AND with AMB and azoles, having the checkerboard method as the reference, sensitivity and specificity were calculated (with confidence intervals at 95%). For calculation of all measures, the SPSS program (version 19.0) was used.

RESULTS

A protocol for evaluation of AND and AMB susceptibility by FC using $DiBAC_4(3)$ as a marker was optimized. Our method is indeed able to discriminate for both drugs the susceptible and nonsusceptible *Candida* strains. A typical example of FC analysis for antifungal susceptibility testing is represented in Fig. 1. Accordingly, for each strain, the autofluorescence of the cell population in analysis is measured. This value is always represented on the first decade of the log scale of intensity of fluorescence, while the ethanol-treated cells (dead cells, the positive control) showed a high increase in the green fluorescence intensity (FL1 [530 nm]) as expected. The viable nontreated cells stained with $DiBAC_4(3)$ had a slight increase in the fluorescence (2-fold) in comparison with the viable nontreated and nonstained cells (autofluorescence). Treatment of susceptible strains with AND produced a dose-dependent increase for the fourth decade of intensity of fluorescence of cells, which was not observed in the nonsusceptible strains (Fig. 1A, B). The results obtained after 2 h of incubation with the antifungal were similar to data after 1 h (data not shown). This increase in fluorescence intensity corresponds to a decrease in the number of CFU (Fig. 1A to C). Likewise, treatment of susceptible strains with 1 μg/ml of AMB also induced an increase in fluorescence intensity similar to that of the positive control after 1 h of incubation. In contrast, for strains having higher MIC values, only a slight increase in fluorescence intensity was observed after incubation with AND or AMB, highlighting the fact that plasma membrane depolarization is dependent on antifungal action and thus dependent on the antifungal susceptibility profile (Fig. 1D, E). The CFU values determined in similar conditions agree very well with the FC data (PA = 0.92) (Fig. 1C, F). Since azole treatment did not result in any increase in the intensity of fluorescence after $DiBAC_4(3)$ staining, even after 2 h of incubation, neither in susceptible nor in nonsusceptible strains (data not shown), a different fluorescent probe was chosen. FUN-1, a metabolic marker, has already been demonstrated by our group to be an excellent probe for azole susceptibility testing of *Candida* spp. (25). Thus, we developed an FC protocol to evaluate FLU, VOR, and AND susceptibility by FC using FUN-1 as a marker (Fig. 2). An increase in the intensity of fluorescence was registered only for susceptible strains after 1 h of incubation. Conversely, this increase was not found for nonsusceptible strains as expected (Fig. 2B, E). The CFU values were consistent with FC data (PA = 0.86) (Fig. 2C, F).

Regarding the antifungal association studies, the FC assays for antifungal associations were performed with subinhibitory concentrations (0.5× MIC values) of each drug either alone or in association using the previous optimized conditions (Fig. 1 and

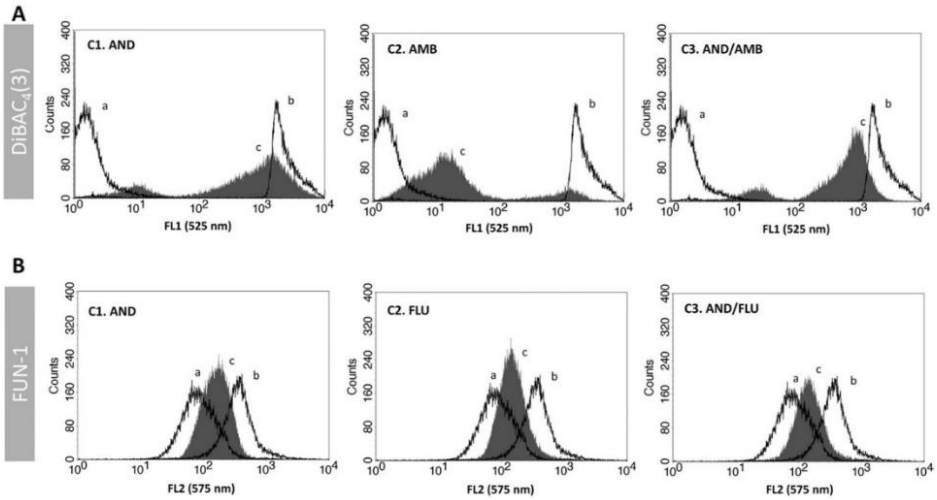

FIG 3 Evaluation of antifungal combination effect using flow cytometry. (A) Flow cytometric analysis of the combination effect between anidulafungin and amphotericin B on the *C. albicans* 0215 strain, an example of synergistic association. Line a, fluorescence of untreated cells stained with $DiBAC_4(3)$; line b, fluorescence of cells treated with 70% ethanol and stained with $DiBAC_4(3)$; line c, fluorescence of cells treated with antifungal drugs and stained with $DiBAC_4(3)$; C1, cells treated with a subinhibitory concentration of AND (0.5× MIC); C2, cells treated with a subinhibitory concentration of AMB (0.5× MIC); and C3, cells treated with a subinhibitory concentrations of both antifungal drugs in association (AND 0.5× MIC + AMB 0.5× MIC). (B) Flow cytometric analysis of the combination effect between anidulafungin and fluconazole on the *C. albicans* OL196 strain, an example of indifferent association. Line a, fluorescence of untreated cells stained with FUN-1; line b, fluorescence of cells treated with 70% ethanol and stained with FUN-1; line c, fluorescence of cells treated with antifungal drugs and stained with FUN-1; C1, cells treated with a subinhibitory concentration of AND (0.5× MIC); C2, cells treated with a subinhibitory concentration of FLU (0.5× MIC); and C3, cells treated with subinhibitory concentrations of both antifungal drugs in association (AND 0.5× MIC + FLU 0.5× MIC).

2). These values were used to be able to standardize since the MIC varies for each strain. $DiBAC_4(3)$ was used for staining cells treated with AND-AMB and FUN-1 for staining cells treated with AND-FLU or AND-VOR. A typical synergistic interaction of AND-AMB evaluated by FC is represented in Fig. 3A. The lack of interaction between AND and FLU association is represented in Fig. 3B. Taking into account the FC data, the association between AND and AMB was synergistic in 46% of cases (18 of 39); there was no interaction in 49% of isolates (19 of 39), and the association was antagonistic in 5% (2 of 39) (Table 1). The association between AND and FLU was synergistic in 36% of cases (13 of 36), there was no interaction in 61% (22 of 36), and the association was antagonistic in 3% (1 of 36) (Table 2). The association between AND and AMB evaluated by the checkerboard microdilution method was synergistic in 43% of strains tested (17 of 39), there was no interaction in 49% (19 of 39), and the association was antagonistic in 8% (3 of 39). Antagonistic interaction was detected only for *C. albicans*. For *C. glabrata* isolates, this association was quite promising since there was no evidence of an antagonistic effect and "no interaction" was not a frequent event (Table 1). The results of AND and FLU association were similar to that for AND and VOR. Synergism was observed in 44% of strains (16 of 36), no interaction in 53% (19 of 36), and an antagonistic effect in 3% (1 of 36). These antagonistic events were detected for only one isolate of *C. parapsilosis*. Concerning *C. glabrata*, once again no antagonism was observed between AND and the azoles, and no interaction was also a rare event, it being detected only in two strains. For strains that are susceptible-dose dependent or resistant to FLU and those which are nonsusceptible to AND, not showing antagonism with the combination of 0.5× MIC, the results of the assays performed using the breakpoint concentration were similar to those obtained with 0.5× MIC (data not shown).

Our FC assay showed three more cases of "no interaction" than the checkerboard method, and there was one more case of antagonism in the checkerboard method related to one *C. albicans* strain (O195) in comparison with the FC method. The Kappa value obtained between both methods was 0.83 (95% CI of 0.79 to 0.87), it being 0.82 (95% CI of 0.76 to 0.88) for AND-AMB association and 0.84 (95% CI of 0.78 to 0.90) for the AND-azoles association. The proportion of agreement calculated was 0.91 (95% CI of 0.88 to 0.94), namely 0.90 (95% CI of 0.85 to 0.95) for the AND-AMB association and 0.92 (95% CI of 0.87 to 0.97) for the AND-azoles association. Regarding sensitivity and specificity of the FC method, considering the checkerboard assay as the reference methodology, sensitivity was 0.88 (95% CI of 0.73 to 0.95) for detection of synergic effects, and specificity was 0.95 (95% CI of 0.84 to 1). In order to detect an antagonistic interaction, FC sensitivity was 0.75 (95% CI of 0.3 to 0.95), and its specificity was 1.

DISCUSSION

The use of drugs with different mechanisms of action in association may play a key role in treatment of invasive fungal infections (3, 7). In the past few years, this combined antifungal therapy has received increased attention. Antifungal interaction involving *Cryptococcus* has been studied; however, reports on the effect of

TABLE 1 *In vitro* interaction of anidulafungin and amphotericin B by the checkerboard and flow cytometry methods against 39 *Candida* species[a]

Strain	Checkerboard MIC (μg/ml)			FICI (interpretation)	Flow cytometry DC (%)			SI (interpretation)
	AND	AMB	AND/AMB		AND	AMB	AND-AMB	
C. albicans O236	0.25	0.25	0.125/0.03	0.62 (NI)	45.04	13.53	22.42	2.15 (NI)
C. albicans O223	0.25	0.125	0.06/0.015	0.36 (S)	71.23	14.58	69.08	5.71 (S)
C. albicans O216	0.125	0.25	0.06/0.015	0.54 (NI)	49.68	8.24	25.17	3.56 (NI)
C. albicans O189	0.25	0.125	0.06/0.015	0.36 (S)	26.33	1.78	24.77	14.86 (S)
C. albicans OL196	0.03	0.25	0.06/0.015	2.06 (NI)	83.45	27.89	33.56	1.61 (NI)
C. albicans O207	0.015	0.125	0.06/0.015	4.12 (A)	48.96	10.45	8.53	0.99 (A)
C. albicans O245	0.015	0.25	0.06/0.015	4.06 (A)	48.13	37.23	20.21	0.99 (A)
C. albicans O237	0.25	0.25	0.06/0.015	0.30 (S)	42.59	1.57	44.53	29.41 (S)
C. albicans O183	0.015	0.06	0.015/0.015	1.25 (NI)	22.69	10.41	13.20	1.85 (NI)
C. albicans O222	1	0.125	0.06/0.015	0.18 (S)	37.40	8.78	32.65	4.59 (S)
C. albicans O215	0.25	0.125	0.06/0.015	0.36 (S)	68.42	13.05	65.04	5.93 (S)
C. albicans O190	0.25	2	0.125/0.015	0.51 (NI)	19.34	11.08	17.57	2.49 (NI)
C. albicans O195	0.015	0.125	0.06/0.015	4.12 (A)	53.12	12.98	17.89	1.72 (NI)
C. albicans ATCC	0.03	0.06	0.015/0.015	0.75 (NI)	61.34	37.12	53.11	2.30 (NI)
C. glabrata OL158	4	0.25	1/0.03	0.37 (S)	79.44	19.36	75.85	4.87 (S)
C. glabrata O206	0.03	0.25	0.06/0.015	2.06 (NI)	35.48	70.21	50.81	2.16 (NI)
C. glabrata O188	0.50	0.125	0.06/0.015	0.24 (S)	82.83	3.95	58.39	15.49 (S)
C. glabrata OL163	0.25	0.25	0.06/0.015	0.30 (S)	73.94	15.09	57.93	4.62 (S)
C. glabrata OL149	0.25	0.25	0.125/0.03	0.62 (NI)	75.39	36.95	58.23	2.35 (NI)
C. glabrata O175	0.06	0.125	0.015/0.015	0.37 (S)	66.85	10.44	63.11	6.99 (S)
C. glabrata O180	0.03	0.25	0.015/0.015	0.56 (NI)	78.27	3.01	73.13	25.23 (S)
C. glabrata O181	4	0.06	0.015/0.03	0.50 (S)	59.89	5.66	37.48	7.25 (S)
C. guilliermondii 33	1	0.125	0.25/0.03	0.49 (S)	23.28	2.46	21.11	9.49 (S)
C. krusei OL16	0.25	0.25	0.06/0.015	0.30 (S)	29.72	4.81	27.58	6.66 (S)
C. krusei O234	0.125	0.125	0.06/0.015	0.60 (NI)	14.92	2.11	10.53	5.70 (S)
C. lusitaniae D51	0.5	2	0.25/0.25	0.63 (NI)	79.93	49.79	69.93	2.28 (NI)
C. parapsilosis OL143	2	0.25	1/0.03	0.62 (NI)	72.14	23.71	55.46	3.11 (NI)
C. parapsilosis O246	1	0.25	0.5/0.06	0.74 (NI)	67.93	48.06	58.03	2.06 (NI)
C. parapsilosis ATO17	4	0.125	1/0.06	0.73 (NI)	2.94	0.34	0.78	2.56 (NI)
C. parapsilosis OL144	2	0.25	0.06/0.03	0.15 (S)	21.15	0.63	16.10	26.32 (S)
C. parapsilosis O204	4	0.125	2/0.06	0.98 (NI)	82.33	31.18	80.53	3.56 (NI)
C. parapsilosis O136	2	0.125	1/0.03	0.74 (NI)	15.58	14.50	16.98	2.26 (NI)
C. parapsilosis Cpo41	8	0.125	0.06/0.06	0.49 (S)	8.87	5.98	15.06	4.22 (S)
C. parapsilosis O158	2	0.125	0.5/0.03	0.49 (S)	45.08	4.92	21.61	4.87 (S)
C. parapsilosis O56	4	0.125	2/0.03	0.74 (NI)	21.72	11.53	18.67	2.48 (NI)
C. tropicalis OL202	0.06	0.25	0.015/0.015	0.31 (S)	90.22	3.45	89.79	27.02 (S)
C. tropicalis OL205	0.25	0.25	0.06/0.015	0.30 (S)	70.98	23.04	53.85	3.10 (NI)
C. tropicalis OL193	0.03	0.5	0.06/0.015	2.03 (NI)	42.01	11.21	11.01	1.24 (NI)
C. tropicalis 1304	1	0.06	0.5/0.03	1 (NI)	11.54	1.98	3.96	2.34 (NI)

[a] S, synergism; A, antagonism; NI, no interaction; FICI, fractional inhibitory concentration index; DC, percentage of depolarized cells; SI, staining index. DC percentage is based on the use of 0.5× MIC of each drug. FICI = (MIC AND/AMB)/MIC AND + (MIC AMB/AND)/MIC AMB. SI = (DC AND + AMB)/DC AND + (DC AND + AMB)/DC AMB.

antifungal combinations involving the most frequent *Candida* spp. are less common. Most of these studies have demonstrated synergism, whereas others have reported no interaction and occasionally antagonism (3, 11, 13, 29, 32).

Echinocandins are a novel class of antifungals which have the cell wall as their target. The literature addressing the relationship between such drugs and membrane-active drugs such as polyenes or azoles against *Candida* is still somewhat limited (12, 13, 15). Importantly, drugs with different targets of action could reinforce each other, allowing a decrease in doses and thus reducing side effects for patients (12, 16). AND acts by inhibiting the 1,3-β-D-glucan synthesis, the major component of the fungal cell wall (26). AMB and azoles are membrane-active drugs; the former acts by making holes in the membrane and the latter by inhibiting its synthesis. Echinocandins probably enhance the effect of membrane-active drugs by increasing their access to the target (1, 12). Nevertheless, it cannot be assumed that the use of two or more effective drugs with distinct mechanisms of action would produce an improved outcome compared to the results seen with a single compound (3, 7).

Like in most previously published studies, the association between azoles or AMB with AND resulted, for the majority of the strains, in a synergy or no interaction (13, 17, 19). In fact, in our study, antagonism for AND-AMB association was observed only for three strains of *C. albicans* (O207, O245, O195) and one for the AND-FLU association in a strain of *C. parapsilosis* (Cpo41). Each drug alone had very low MIC values for these strains, but they increased following the association, although still remaining low. With regard to echinocandins, most authors found no antagonism between micafungin and azoles or AMB (4, 17, 32). Barchiesi et al. did not find advantages in associating caspofungin and the polyene, with the exception of *C. parapsilosis*, but the study in-

TABLE 2 *In vitro* interaction of anidulafungin and fluconazole by the checkerboard and flow cytometry methods against 36 *Candida* species[a]

Strain	Checkerboard MIC (μg/ml)			FICI (interpretation)	Flow cytometry MIF			SI (interpretation)
	AND	FLU	AND/FLU		AND	FLU	AND-FLU	
C. albicans O189	0.25	32	0.06/0.06	0.24 (S)	1.10	1.26	2.60	4.44 (S)
C. albicans O190	0.25	64	0.06/0.015	0.24 (S)	0.48	0.48	1.24	5.13 (S)
C. albicans O195	0.015	4	0.015/0.06	1.02 (NI)	2.70	1.62	2.34	2.31 (NI)
C. albicans O205	0.06	2	0.06/0.015	1.01 (NI)	1.68	0.93	0.82	1.36 (NI)
C. albicans O207	0.015	4	0.015/0.06	1.02 (NI)	2.30	1.74	1.55	1.57 (NI)
C. albicans O216	0.25	64	0.06/0.06	0.24 (S)	4.77	3.16	7.72	4.06 (S)
C. albicans O223	0.25	1	0.06/0.06	0.30 (S)	1.75	1.39	4.37	5.65 (S)
C. albicans O236	0.25	16	0.06/0.06	0.24 (S)	1.10	1.03	2.17	4.08 (S)
C. albicans O237	0.25	64	0.06/0.06	0.24 (S)	2.67	1.29	3.50	4.03 (S)
C. albicans O245	0.015	64	0.015/0.06	1 (NI)	1.41	1.27	1.34	2.00 (NI)
C. albicans OL122	0.125	0.5	0.125/0.015	1.03 (NI)	8.00	4.79	5.69	1.90 (NI)
C. albicans OL160	0.03	16	0.015/0.125	0.51 (NI)	0.38	0.38	0.59	3.09 (NI)
C. albicans OL171	0.015	0.5	0.015/0.015	1.03 (NI)	0.48	0.49	0.48	2.00 (NI)
C. albicans OL172	0.03	16	0.015/0.125	0.51 (NI)	1.26	0.91	1.68	3.19 (NI)
C. albicans OL196	0.015	64	0.015/0.06	1 (NI)	1.28	1.41	1.28	1.91 (NI)
C. albicans ATCC	0.03	0.125	0.015/0.015	0.62 (NI)	1.69	1.40	1.79	2.33 (NI)
C. glabrata O158	4	4	0.5/1	0.38 (S)	7.93	1.01	8.90	9.93 (S)
C. glabrata O181	4	4	0.25/1	0.31 (S)	2.48	0.51	2.83	6.72 (S)
C. glabrata O188	0.5	8	0.06/0.06	0.13 (S)	3.77	2.81	7.15	4.44 (S)
C. glabrata O180	0.5	16	0.125/0.06	0.25 (S)	0.46	1.02	1.30	4.09 (S)
C. glabrata O206	0.03	16	0.015/0.06	0.51 (NI)	1.88	1.80	2.04	2.22 (NI)
C. glabrata OL149	0.25	16	0.06/0.06	0.24 (S)	2.08	1.22	3.24	4.20 (S)
C. glabrata OL158	4	8	0.06/4	0.52 (NI)	1.27	1.01	1.14	2.03 (NI)
C. glabrata OL163	0.25	16	0.06/0.06	0.24 (S)	1.78	1.57	1.96	2.35 (NI)
C. glabrata OL164	0.25	8	0.06/0.015	0.24 (S)	0.96	0.62	1.61	4.29 (S)
C. krusei OL16	0.25	64	0.125/0.06	0.51(NI)	1.10	1.06	1.15	2.13 (NI)
C. parapsilosis Cpo41	8	0.125	0.06/0.5	4.01 (A)	1.34	1.11	0.59	0.97 (A)
C. parapsilosis O136	4	2	0.25/2	1.06 (NI)	1.64	0.91	0.92	1.57 (NI)
C. parapsilosis O246	2	0.5	0.25/0.25	0.625 (NI)	1.53	1.72	1.66	2.05 (NI)
C. parapsilosis O56	4	0.5	0.5/0.25	0.625 (NI)	2.33	4.06	3.07	2.07 (NI)
C. parapsilosis OL143	2	2	0.25/0.5	0.38 (S)	3.37	4.37	4.96	2.61 (NI)
C. parapsilosis ATO16	4	0.25	0.5/0.06	0.37 (S)	3.10	3.53	4.31	2.61 (NI)
C. parapsilosis OL144	2	2	0.5/1	0.75 (NI)	1.95	1.18	1.94	2.65 (NI)
C. tropicalis OL193	0.015	2	0.015/0.06	1.03 (NI)	1.12	1.68	1.53	2.27 (NI)
C. tropicalis OL202	0.015	2	0.015/0.06	1.03 (NI)	0.47	0.75	0.46	1.60 (NI)
C. tropicalis OL295	0.25	2	0.06/0.06	0.27 (S)	1.43	0.98	2.57	4.42 (S)

[a] S, synergism; A, antagonism; NI, no interaction; FICI, fractional inhibitory concentration index; MIF, mean intensity of fluorescence; SI, staining index. MIF was determined using 0.5× MIC of each drug. FICI = (MIC AND/FLU)/MIC AND + (MIC FLU/AND)/MIC FLU. SI = (MIF AND + FLU)/MIF AND + (MIF AND + FLU)/MIF FLU.

cluded a much more limited number of strains (2). In some *Candida* strains, echinocandins are highly active, and so the fungicidal activity may be difficult to improve after combination (17). Moreover, we have shown that the drug interaction potential is species and strain dependent, which enhances the importance of the novel protocol described here for the first time.

The mathematic model used for the checkerboard method, which allows a quantification analysis after calculating the FICI, has been the most commonly used procedure to characterize the activity of antimicrobial combinations in clinical laboratories. Other methods, such as time-kill assays and Etest, have been used (13); however, despite their good correlation with the checkerboard assay, all of them take at least another 24 h to provide results. The terminology used to assign the results into interpretative categories is often a subject of debate and confusion, with difficult resolution (10). Synergism and antagonism have clear and intuitive meanings, although "no interaction" is a somewhat subjective category without a clear clinical relevance (10).

At the moment, few but relevant reports have helped to demonstrate the value of cytometric assays as excellent yet underexplored tools in clinical microbiology (8, 23, 30). Flow cytometry is a powerful high-throughput technology which allows the characterization of several thousands of cells per second, distinguishing between different physiological states. Differentiations between viable, intermediate, and nonviable cells are possible using fluorescent dyes. Using these tools, fast, reliable data could be obtained with great benefit for the patient. Echinocandin antifungal activity was studied using $DiBAC_4(3)$ or FUN-1, although for azoles only FUN-1 could be used after a short incubation time. Regarding AMB, previous studies reported the impossibility of studying its activity with FUN-1 (25). Thus, the study of the AND-AMB association was performed using $DiBAC_4(3)$ and that of the AND-azoles association was performed using FUN-1 (Fig. 1 to 3). $DiBAC_4(3)$ is a membrane potential marker that can enter depolarized cells, where it binds to intracellular proteins or membranes and exhibits enhanced fluorescence; and FUN-1 is a metabolic activity staining that passively diffuses through yeast cell walls. In metabolically inactive cells, FUN-1 remains in the cytoplasm, dis-

playing a green fluorescence, while in active cells it is processed, which results in the formation of distinct vacuolar structures that exhibit a red fluorescence, accompanied by a reduction in the green cytoplasmic fluorescence (14, 24). For both markers, an increase in fluorescence intensity [FL1 (530 nm) for $DiBAC_4(3)$ and FL2 (575 nm) for FUN-1] agrees with the reduction of CFU counts, meaning a reduction of cell viability.

The FC protocols described show an excellent agreement with the checkerboard method and high sensitivity and specificity, thus allowing the study of different combinations of antifungal drugs in less than 2 h.

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Publication II

(Chapter III)

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New Insights Regarding Yeast Survival following Exposure to Liposomal Amphotericin B

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***In vitro* resistance to amphotericin B is an extremely rare event among pathogenic yeasts. However, *in vivo* response is sometimes reduced, resulting in an unfavorable outcome. Such adverse outcomes might be related to subfungicidal plasma concentrations. We aimed to clarify the mechanisms of liposomal amphotericin B (AMB-L; AmBisome)-induced lesions and the mechanisms responsible for yeast cell recovery following exposure at plasma concentrations. The physiological statuses developing following exposure to AMB-L at simulated plasma concentrations (20 to 0.1 mg/liter) and at a constant concentration (3 mg/liter) were assessed in a 24-h time course assay. Time-kill experiments also were carried out under the same AMB-L treatment conditions. Our results suggest that yeast cells develop compensatory responses related to membrane polarization, metabolic activity, and reactive oxygen species (ROS) production after exposure to high plasma concentrations (20 to 5 mg/liter) during the first 6 h; in the remaining 18 h, when exposed to lower concentrations, cells reveal almost full recovery with no evidence of fungicidal activity. In contrast, whenever cells are exposed to a constant concentration above the MIC, despite initially exhibiting compensatory stress responses, soon afterwards they exhibit membrane depolarization, a decrease of metabolic activity, increasing ROS production, and lastly, programmed cell death and necrosis, resulting in succumbing to AMB-L fungicidal effects. This study may represent a step forward in the support of AMB-L use for clinical treatment of invasive fungal infections, since it demonstrates the importance of maintaining levels of AMB-L above the MIC in plasma and tissues to ensure it produces its fungicidal effects.**

The understanding of how fungal organisms respond to antimicrobial therapy is a relevant question both in terms of evolutionary biology and for treatment of invasive fungal infections. During recent decades, fungi have emerged as major human pathogens; *Candida albicans* represents the fourth most common agent of all hospital-acquired infections (1).

Despite over 50 years of use as monotherapy, amphotericin B (AMB) still represents an important therapeutic alternative for the treatment of systemic fungal infections, particularly when infection persists despite treatment with alternative drugs (2). Amphotericin B belongs to the polyene drug class and exhibits a broad-spectrum fungicidal activity. For decades, the prevailing mechanism of action has been that AMB primarily binds to ergosterol, inserts into the cytoplasmic membrane, and forms pore-like structures; the result is osmotic instability, loss of membrane integrity, metabolic disruption, and ultimately cell death (3, 4). Recently, Anderson et al. proposed a new mechanism of action of AMB. Accordingly, amphotericin exists primarily in extramembranous aggregates that kill yeast cells by extracting ergosterol from the plasma membrane. Consequently, membrane ergosterol depletion will interfere not only with cell membrane integrity but also with other cellular processes which highly depend on membrane ergosterol (5).

The development of genetic resistance to AMB among *Candida* species remains extremely rare, in contrast to what is observed with other drugs in the triazole or echinocandin classes (6, 7). However, in spite of the observed high *in vitro* susceptibility (0.125 to 1 mg/liter), the *in vivo* response to AMB is somewhat reduced in about 40% of treated patients (8–10). The exact reasons for this lower-than-expected response still remain unclear. Some authors do not associate response failure with target modification, as has been observed with other antifungal drugs (11), but with inappropriate concentrations of AMB at the infection site (12). In accordance with this hypothesis, cells exposed to amphotericin B may exhibit different physiological conditions which are related to drug concentration and exposure time (12). In addition, studies of yeast apoptosis have revealed the occurrence of a programmed cell death or a cellular necrotic response depending on the AMB concentration (13, 14).

Considering the clinical relevance of liposomal amphotericin B (AMB-L; AmBisome) and based on plasma levels described previously (15), this study aims to explore yeast survival mechanisms in response to AMB-L in order to identify different physiological conditions following exposure to decreasing concentrations in a time course assay. In addition, we intend to determine whether

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TABLE 1 Yeast strains used in this study[a]

ID	Species	Source	MIC (mg/liter)	% of depolarized cells		
				3 h	6 h	24 h
ATCC 90028	*Candida albicans*	American Type Culture Collection	0.5	61.21	77.42	26.66
596	*Candida albicans*	Clinical isolate	0.5	33.62	40.16	21.72
590	*Candida glabrata*	Clinical isolate	1	28.19	37.51	20.04
597	*Candida parapsilosis*	Clinical isolate	0.5	34.62	45.98	9.57
120	*Candida krusei*	Clinical isolate	8	31.08	43.29	19.81
514	*Candida tropicalis*	Clinical isolate	1	48.65	77.92	21.54
479	*Candida dubliniensis*	Clinical isolate	0.06	21.64	36.73	25.57
520	*Candida lusitaneae*	Clinical isolate	0.25	30.18	33.21	22.94
D1	*Cryptococcus neoformans*	Clinical isolate	0.5	28.14	35.79	19.31
BY4741	*Saccharomyces cerevisiae*	American Type Culture Collection[b]	0.5	20.13	45.83	19.67
PYCC 6480	*Debaryomyces hansenii*	Portuguese Yeast Culture Collection[b]	1	43.78	89.90	30.47
PYCC 4166	*Kluyveromyces lactis*	Portuguese Yeast Culture Collection[b]	0.06	27.89	38.89	5.63
CBS 732	*Zygosaccharomyces rouxii*	CBS-KNAW Fungal Biodiversity Center[b]	0.06	14.85	34.71	12.38

[a] Liposomal amphotericin B susceptibility test results and membrane potential of yeast cells treated with AMB-L plasma concentrations after 3, 6, and 24 h, expressed as the percentage of depolarized cells.
[b] Kindly provided by Catarina Prista, Instituto Superior de Agronomia, Lisbon, Portugal.

cell survival following exposure to AMB-L is a common phenomenon among different yeast species, some of which are relevant clinical pathogens.

Our results provide novel insights regarding mechanisms by which yeast cells can escape AMB-L fungicidal action depending upon the time of exposure and the concentration of the drug.

MATERIALS AND METHODS

Yeast strains and growth conditions. A broad range of pathogenic and nonpathogenic yeasts were grown in yeast extract-peptone-dextrose (YPD) liquid medium (1% yeast extract, 2% peptone, 2% dextrose; Formedium, Norfolk, United Kingdom) at 35°C with shaking (150 rpm) until the exponential growth phase (details are presented in Table 1) (16). All of the strains were subjected to antifungal susceptibility testing, cell viability assays, and membrane potential evaluation. For membrane integrity, metabolic activity, reactive oxygen species (ROS) production, and apoptotic assays, only *Saccharomyces cerevisiae* BY4741 was used as a model organism. Prior to experiments, yeasts were subcultured twice in YPD agar to ensure the purity of cultures.

Liposomal amphotericin B susceptibility. The MIC of AMB-L (provided by Gilead Sciences, Inc., San Dimas, California) was determined according to the M27-A3 protocol and M27-S3 supplement of the Clinical and Laboratory Standards Institute (CLSI) (17, 18). MIC values were determined after 24 and 48 h of incubation with AMB-L; the MIC was the lowest concentration that prevented any discernible growth. Due to a lack of established clinical breakpoints for AMB-L, the *Candida* isolates were considered wild type (wt) whenever the MIC was ≤2 mg/liter and non-wild type (nwt) when the MIC was >2 mg/liter according to the epidemiological cutoff values (ECVs) proposed by Pfaller and Diekema (19). For non-*Candida* genera, only the MIC value is displayed, since amphotericin B ECVs and clinical breakpoints remain undefined (19). The *C. albicans* ATCC 90028 reference type strain was used as recommended by the CLSI protocol.

Evaluation of cell viability. Yeast suspensions (10^6 yeast cells/ml) were exposed to AMB-L in accordance with the plasma levels established by Walsh et al. (15). Briefly, cell suspensions were treated with 10 mg/liter AMB-L for 30 min; afterwards, cells were harvested by centrifugation at 1,610 × *g* for 10 min at room temperature (Universal 320R; Hettich). The same cells then were resuspended in fresh culture medium and exposed sequentially to serial concentrations of AMB-L: 20 mg/liter (for 30 min), 10 mg/liter (for 2 h), 5 mg/liter (for 3 h), 1 mg/liter (for 6 h), and 0.1 mg/liter (until 24 h) (detailed in Fig. 1A). Another cell suspension was treated with 3 mg/liter AMB-L for 24 h. During that period at 1, 3, 6, and 12 h of treatment, those cells were harvested (Universal 320R; Hettich) and resuspended in fresh medium containing 3 mg/liter AMB-L, similar to the protocol developed for the cells exposed to various dosages of AMB-L (plasma conditions). Following 1, 3, 6, and 24 h from the start of incubation, the number of viable cells was determined by plating on YPD agar and incubating at 35°C for 24 h; the number of CFU was determined and compared to that of the plate control (not exposed to AMB-L). Before being plated, cells were washed and resuspended in fresh medium in order to prevent antifungal carryover.

Functional characterization of liposomal amphotericin B-induced action. The physiological status of yeast cells which developed following AMB-L exposure to decreasing concentrations or a constant concentration of AMB-L in a time course assay were assessed by flow cytometry. A cell suspension corresponding to 10^6 yeast cells/ml was used in all assays described below. Yeast cells were incubated with various concentrations of AMB-L, in accordance with the scheme previously proposed (Fig. 1A). At 1, 3, 6, and 24 h, aliquots were collected and tested; all cytometric evaluations were performed in a standard FACSCalibur cytometer (BD Biosciences, Sydney, Australia) equipped with 3 photomultipliers (PMTs), standard filters, and a 15-mW, 488-nm argon laser using CellQuest Pro software (version 4.0.2). All trials were performed in triplicate.

Assessment of membrane integrity. Cell membrane integrity was assessed with propidium iodide (PI; Sigma-Aldrich, Munich, Germany) staining. After antifungal treatment, yeast cells were stained with 1 mg/liter of PI for 30 min at 35°C at 150 rpm in the dark (20). The fluorescence intensity (FI) was measured at FL3 (630 nm). The amount of injured cells in each sample was defined as the percentage of PI-positive cells.

Assessment of membrane potential. The effect of AMB-L exposure on cell membrane potential was evaluated using bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$; Sigma-Aldrich, Munich, Germany] as described by Teixeira-Santos et al. (21). After AMB-L treatment, cells were incubated for 15 min in the dark at 35°C at 150 rpm with 0.5 mg/liter of $DiBAC_4(3)$. The FI was registered at FL1 (530 nm).

Assessment of metabolic activity. Metabolic changes induced by AMB-L in *S. cerevisiae* were evaluated using 5-carboxyfluorescein diacetate (5-CFDA; Sigma-Aldrich, Munich, Germany) at a 10 μM final concentration. Antifungal-treated cell suspensions were stained with 5-CFDA and incubated for 45 min at 35°C at 150 rpm in the dark (12). The mean intensity of fluorescence (MIF) was registered at FL1 (530 nm).

Assessment of endogenous ROS production. ROS production was assessed as previously reported (22). In brief, the cell suspension was incubated with 20 mg/liter of 2,7-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich, Munich, Germany) for 30 min at 35°C at 150 rpm.

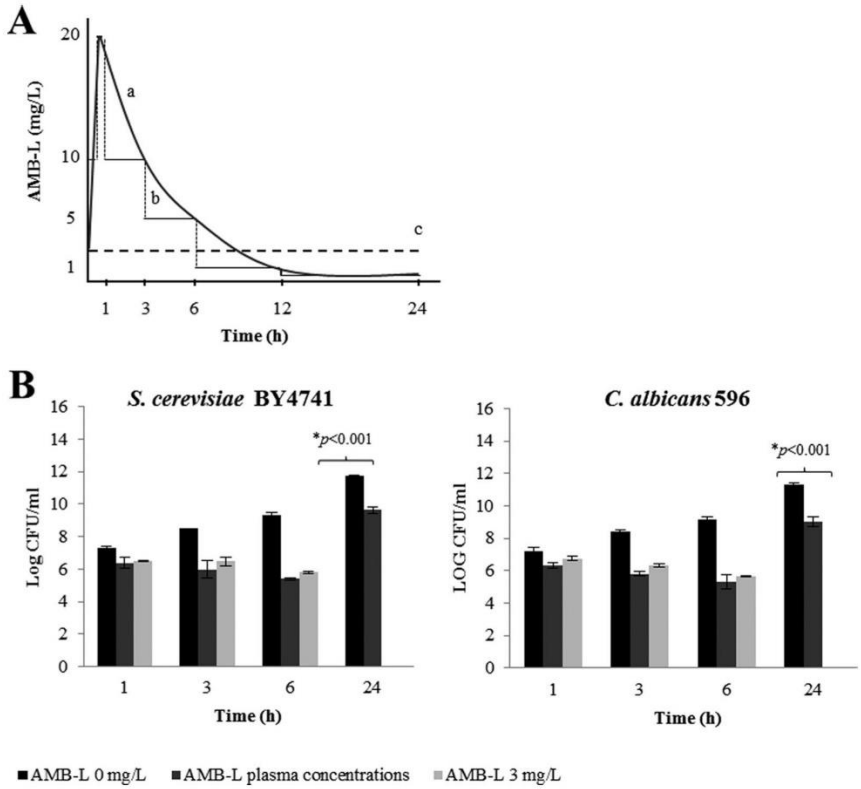

FIG 1 Effect of liposomal amphotericin B on *S. cerevisiae* BY4741 and *C. albicans* 596. (A) Study design scheme. (a) Plasma concentration-time curve obtained after a first infusion of AMB-L at 3 mg/liter. (b) The scheme proposed in accordance with AMB-L plasma levels described by Walsh et al (15). (c) Dashed line represents the treatment with a constant concentration of 3 mg/liter AMB-L during 24 h. (B) Viability assessment by CFU enumeration of *S. cerevisiae* BY4741 and *C. albicans* 596 cells exposed to treatment conditions b and c. Data at respective time points are given as means ± standard deviations. An asterisk indicates significant differences between the two treatment conditions.

Yeast cells were washed (2,655 × *g* for 5 min at room temperature; 5417R; Eppendorf) and resuspended in phosphate-buffered saline (PBS; Sigma-Aldrich, Munich, Germany); afterwards, cells were treated as described above. As a control, cells were treated with 0.4 mM H_2O_2 (PanReac, Castellar del Valles, Spain). The FI was determined at FL1 (530 nm). ROS production was calculated by subtracting the FI value of cells treated with simple antifungal agent from that of cells treated with both antifungal agent and DCFH-DA.

TUNEL assay. DNA strand splitting was demonstrated by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) with the *in situ* cell death detection kit, fluorescein, from Roche (Mannheim, Germany). Yeast cells were fixed with 3.7% (vol/vol) formaldehyde (Applichem, Darmstadt, Germany) for 30 min at room temperature and washed thrice with PBS (2,655 × *g* for 5 min at room temperature; 5417R; Eppendorf). Afterwards, cell walls were digested with 24 mg/liter of lyticase (Sigma-Aldrich, Munich, Germany) at 37°C for 60 min. Cells were rinsed with PBS, incubated in permeabilization solution (0.1% [vol/vol] Triton X-100 and 0.1% [wt/wt] sodium citrate) for 2 min on ice, and rinsed twice with PBS (5417R; Eppendorf). For a positive control, cells were treated with DNase I enzyme (Roche, Mannheim, Germany) for 10 min and then washed twice in PBS (5417R; Eppendorf). Yeast cells subsequently were incubated with 15 μl of TUNEL reaction mixture for 60 min at 37°C and then washed twice in PBS (5417R; Eppendorf). Finally, cell suspensions were submitted to flow cytometric analysis, and the percentage of apoptotic cells was determined at FL1 (530 nm).

Simultaneously, 10 μl of the treated cell suspension was placed in a microscope slide, and the protocol for 4′,6-diamidino-2-phenylindole (DAPI; Roche, Mannheim, Germany) nuclear staining was carried out as previously described, using 1 mg/liter of DAPI (23), for further analysis under fluorescence microscopy with a Carl Zeiss Axiovert inverted microscope and laser wavelengths of 405 nm (DAPI) and 488 nm (TUNEL).

Statistical analysis. Results are presented as mean values and the respective standard deviations. A comparison of results was performed using paired-sample Student's *t* test. $P < 0.05$ was considered significant. For calculations of all measures, the SPSS program (version 19.0) was used.

RESULTS

Liposomal amphotericin B effect on cell viability. Our primary goal in this study was to determine the effect of therapeutic plasma concentrations of AMB-L upon cell viability. We focused on AMB-L effects within the first 24 h of exposure, a time period corresponding to the first drug infusion. The effect of AMB-L on yeast cells was evaluated by colony-forming unit count following incubation with decreasing concentrations of AMB-L (Fig. 1B). As

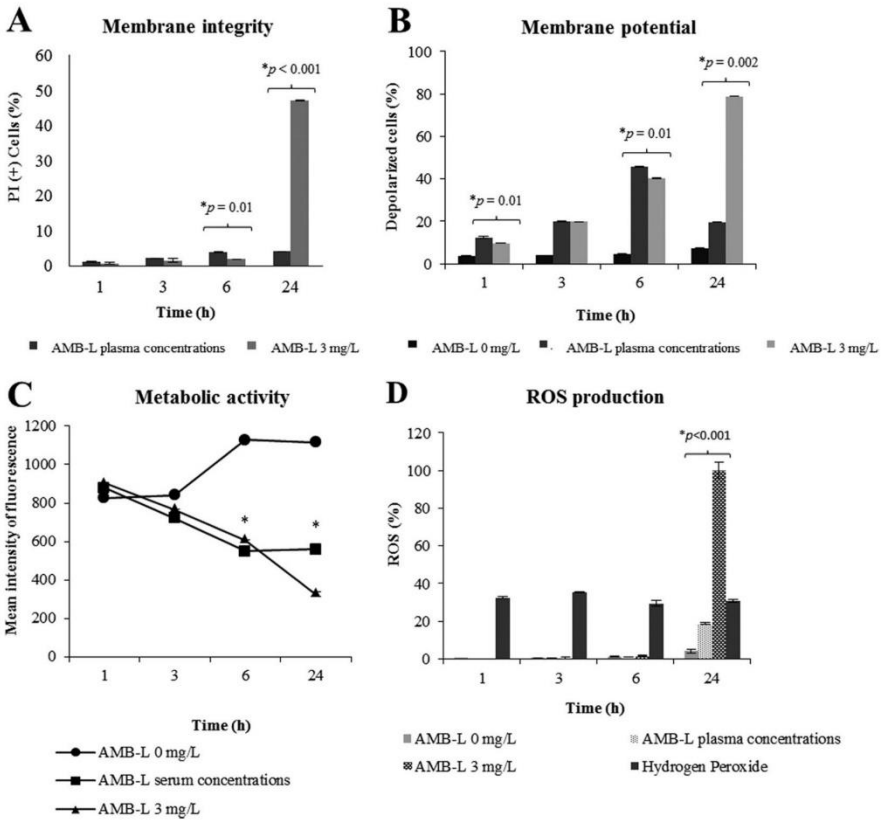

FIG 2 Effect of liposomal amphotericin B on *S. cerevisiae* BY4741 physiological parameters. (A) Cell membrane integrity was assessed with propidium iodide (PI). (B) Cell membrane potential was evaluated using $DiBAC_4(3)$. (C) Metabolic activity was determined by 5-CFDA staining. (D) Endogenous reactive oxygen species (ROS) production as determined by DCFH-DA staining. An asterisk indicates significant differences ($P < 0.05$) between the two treatment conditions.

detailed in Fig. 1B, a slight reduction of viability was observed for up to 6 h of exposure; however, after 6 h of exposure to decreasing concentrations, cells recovered their replication ability, with this effect being most marked after 24 h of exposure (10^6 to 10^9 cells/ml). Conversely, in the case of cells exposed to 3 mg/liter AMB-L for 24 h, a growth reduction was consistently registered throughout the 24-h period, and the cells were unable to replicate. At 24 h, a significant difference between both experimental conditions was registered ($P < 0.001$). Overall, the killing kinetics were similar among all species studied (Table 1). *S. cerevisiae* BY4741 and *C. albicans* 596 are representative examples (Fig. 1B). When comparing such results with the assessment of cell viability with PI (Fig. 2A), we observed some discrepancies. While after 1, 3, and 6 h of incubation with AMB-L under both drug treatment conditions there was a reduction of colony-forming units per milliliter, the cells were not permeable to PI. Only after 24 h was any PI uptake observed, but it was seen in only about 50% of the cells treated with 3 mg/liter AMB-L.

Liposomal amphotericin B-induced alterations on membrane potential. To assess the effect of AMB-L on the cytoplasmic membrane potential of yeast cells, we used $DiBAC_4(3)$ staining. $DiBAC_4(3)$ enters only depolarized cells, where it binds reversibly to intracellular components, resulting in an increased fluorescent signal. The results obtained regarding membrane depolarization of *S. cerevisiae* BY4741 are depicted in Fig. 2B. Viable nontreated cells stained with $DiBAC_4(3)$ exhibited a small percentage of depolarized cells (≈5%), which remained constant over time. Cells treated with AMB-L plasma concentrations displayed an increase of depolarized cells (DC) up to 6 h of incubation; the percentage of DC at 1, 3, and 6 h of incubation was 13.01%, 20.13%, and 45.83%, respectively. Interestingly, after 24 h of incubation, membrane yeast cells seemed to repolarize, corresponding to a DC final value of 19.67%. Conversely, treatment of yeast cells with 3 mg/liter AMB-L resulted in a time-dependent increase of depolarized cells. Figure 2B shows that following 24 h of incubation, cells treated with 3 mg/liter AMB-L displayed higher membrane depolarization than cells treated with plasma concentrations; the percentage of depolarized cells at this time point was 80.45%.

To evaluate whether this mechanism of cell salvage is conserved in the presence of decreasing concentrations of AMB-L during a 24-h period among distinct yeasts, a broad range of yeast species with different phylogenetic relationships (Table 1) were

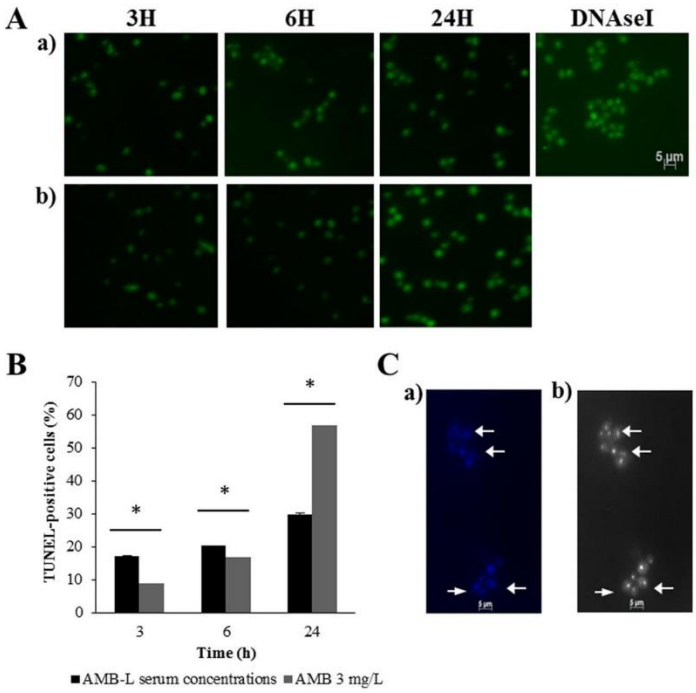

FIG 3 TUNEL staining of *S. cerevisiae* BY4741 cells exposed to liposomal amphotericin B. (A) Fluorescence microscopy imaging showing TUNEL-positive cells after treatment with AMB-L plasma concentrations (a) and with 3 mg/liter (b) after 3 h, 6 h, and 24 h. (B) Percentage of cells exhibiting damaged DNA (i.e., cells positive by TUNEL) after treatment with AMB-L, as assessed by flow cytometry. An asterisk indicates significant differences ($P < 0.05$) between the two treatment conditions. (C) Nuclear fragmentation as shown by DAPI staining. Fluorescence microscopy imaging with (a) and without (b) a DAPI filter. *S. cerevisiae* cells exposed to AMB-L exhibit an irregular shape and fragmented DNA, two findings in accordance with DNA damage during apoptosis.

assessed by flow cytometry regarding the respective cell membrane potential. The species studied showed different susceptibility profiles to AMB-L with all wild-type *Candida* strains (MIC, ≤2 mg/liter), except *C. krusei*, with a MIC of 8 mg/liter. Non-*Candida* yeast genera also showed MIC values of <2 mg/liter. Regardless of the susceptibility profile, all species studied revealed depolarization of the cell membrane after 6 h of incubation with AMB-L plasma concentrations, followed by repolarization of the cell membrane by 24 h (Table 1). However, cells treated with 3 mg/liter AMB-L exhibited a time-dependent increase in membrane depolarization (data not shown).

Metabolic alterations triggered by AMB-L exposure. To assess metabolic effects of AMB-L on *S. cerevisiae* cells, we performed 5-CFDA staining. 5-CFDA is a cell-permeant esterase substrate; it measures both enzymatic activity and cell membrane integrity. The results obtained for MIF displayed by cells stained with 5-CFDA are detailed in Fig. 2C. The MIF displayed by viable cells not treated with AMB-L following 1 h of treatment was 820.05; it increased after 6 h of incubation (MIF, 1,127.35) and remained constant up to 24 h. After 1 h of incubation, yeast cells treated with plasma concentrations of AMB-L displayed a MIF slightly higher than that of viable cells (901.01). However, following 3 and 6 h, the MIF decreased to values of 721.33 and 549.78, respectively; afterwards, the MIF remained constant until 24 h of treatment. When yeast cells were treated with 3 mg/liter AMB-L over time, the MIF increased after 1 h of incubation (MIF, 923.54), indicating that the cells were metabolically active. However, after 3 h of incubation, the MIF decreased over time; at 24 h the MIF was 327.49. This event may be related to a lower enzyme activity or cell membrane injury.

Induction of endogenous ROS production by AMB-L. Reactive oxygen species production was assessed by DCFH-DA staining. DCFH-DA is oxidized to highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) by ROS. Exposure to AMB-L plasma concentrations resulted in reduced ROS formation in *S. cerevisiae* cells (≈18%) (Fig. 2D). In contrast, prolonged and constant exposure to 3 mg/liter AMB-L resulted in high formation of ROS ($P < 0.001$); after 6 h of incubation, a significant increase of the number of fluorescent cells (reaching 100%) was registered. This assay reveals that 3 mg/liter AMB-L leads to intracellular accumulation of ROS, which is associated with oxidative damage and possibly is involved in induced programmed cell death.

DNA damage and nuclear fragmentation. DNA fragmentation was measured by TUNEL assay. The percentage of *S. cerevisiae* cells that exhibited TUNEL-positive nuclei after exposure to plasma concentrations of AMB-L for 3 and 6 h was about 20% (Fig. 3). There were significant differences between cells exposed to AMB-L plasma concentrations and to 3 mg/liter AMB-L ($P < 0.001$); following exposure to AMB-L at a constant 3 mg/liter concentration, TUNEL-positive cells increased over time, indicating

apoptotic-like DNA fragmentation; after 24 h of incubation with 3 mg/liter of AMB-L, 60.8% of yeast cells exhibited TUNEL-positive nuclei, twice the proportion of TUNEL-positive cells following exposure to AMB-L plasma concentrations (Fig. 3A and B). During apoptosis, *S. cerevisiae* cells exposed to AMB-L exhibited evidence of nuclear fragmentation associated with DNA damage (Fig. 3C), including irregularly shaped DNA, as previously described (24).

DISCUSSION

Our study clearly demonstrated that exposure to AMB-L plasma concentrations induces compensatory responses at distinct levels, like replicative ability, membrane potential, metabolic activity, endogenous ROS production, and DNA damage. In the treatment regimen with plasma concentrations, yeast cells are exposed to high concentrations of AMB-L (5 to 20 mg/liter) over a short time period (6 h), which can trigger a stress response. Consequently, the yeast cells initially compensate by upregulating their physiological responses to minimize this stress; afterwards, over the remaining 18 h they are exposed to a much lower AMB-L concentration, which allows its recovery. This may explain why the yeast cells exposed to the simulated plasma concentrations are less AMB-L susceptible. Accordingly, no evidence of fungicidal activity was found, which may be responsible for fungal infection persistence in the blood of these patients. Conversely, following constant exposure to 3 mg/liter, cells develop compensatory mechanisms for survival initially; however, as the concentration is kept constant for 24 h, the cells succumb to the drug effects. In addition, in interpreting this data, one also has to consider the fact that most fungal infections localize in the tissues, such as the lungs, kidneys, liver, and spleen. In these tissues, concentrations of AMB-L reach well above the MIC and are maintained at these levels for at least 1 week and longer depending upon the tissues (25, 26).

An important challenge for cell physiology and microorganism survival is a successful, balanced growth when confronted with environmental imbalances. A variety of cellular processes and physiological changes have to be coordinated to allow yeast cells to reproduce, grow, and respond to environmental stresses (27). We demonstrated that yeast cells use distinct time-programmed mechanisms to respond to AMB-L-induced stress. When yeast cells were exposed initially to high concentrations of AMB-L (5 to 20 mg/liter) during the first 6 h, followed by much lower levels of drug, closer to their MIC (simulated plasma concentrations), the loss of replication competency seems to be a relatively early event that could be easily overcome, provided that the yeast then were exposed for longer periods of time to drug concentrations at or below their MICs (Fig. 1B). Previous studies have shown that some yeast cells exposed to amphotericin B demonstrate a capacity for resuscitation, although they are unable to replicate (12, 28). It is notable that increasing and then decreasing AMB-L concentrations rapidly evoke compensatory responses by yeast cells, including the recovery of replication ability. Interestingly, such cells appear to exhibit an intact cell membrane, as shown by PI staining (only about 5% of cells are PI positive after 24 h) (Fig. 2A). Conversely, yeast cells constantly exposed to 3 mg/liter AMB-L lose their viability, as confirmed by colony-forming unit determination, also with clear evidence of cell membrane injury (50% of cells PI positive at 24 h).

Regarding membrane potential, the phenomenon of cell salvage was found to be conserved among different yeasts (pathogenic and nonpathogenic) with different phylogenetic relationships and antifungal susceptibility profiles, suggesting that this recovery is related to insufficient AMB-L exposure. An essential aspect of environmental adaptation is the equilibrium of ion concentration, which determines cell membrane potential (29). It is well known that amphotericin B increases fungal cell membrane permeability to ions (30). Yeast cells initially exposed to high AMB-L concentrations exhibited evidence of plasma membrane depolarization soon after 1, 3, and 6 h of incubation, as shown by $DiBAC_4(3)$ staining; however, in such cells membrane potential was restored afterwards during the time that the cells were exposed to levels of drug close to or below their MICs (Fig. 2B). Our results suggest that with initial exposure to high AMB-L levels, there is a cellular response characterized by recovery of plasma membrane potential which is maintained when the cells then are incubated with low levels of AMB-L (at or below MICs). In contrast, yeast cells exposed to 3 mg/liter AMB-L exhibited a marked time-dependent impairment in membrane potential (Fig. 2B). These results suggest that yeast cell recovery after exposure to high levels of AMB-L is related to subsequent exposure to nonfungicidal concentrations of AMB-L.

Cell metabolic activity was significantly reduced by AMB-L plasma concentrations at 3 and 6 h of incubation, remaining constant for up to 24 h (Fig. 2C). In contrast, yeast cells continuously exposed to AMB-L at constant concentration (3 mg/liter) initially displayed a high metabolic activity after 1 h of incubation, suggesting a different metabolic stress response. This fact is in accordance with the hypothesis that yeast cells exposed to antifungal pressure reprogram their metabolism in response to an environmental stress (31). A previous study involving yeast genome-scale microarrays demonstrated that amino acid metabolism, phosphate metabolism, and carbohydrate metabolism genes are upregulated after treatment with 2.5 mg/liter of amphotericin B (4). After 3, 6, and 24 h of incubation with 3 mg/liter AMB-L, the fluorescence resulting from cFDA cleavage decreased (Fig. 2C); this finding can be explained by membrane pore formation, which may lead to the loss of fluorescence, or by an extremely reduced metabolic activity (32).

Previous studies have shown that amphotericin B induces *C. albicans* apoptosis (13, 14). According to our results, endogenous ROS production was induced following 6 h of AMB-L exposure (Fig. 2D); this finding also was corroborated by results for DNA damage (Fig. 3A and B). Notably, apoptosis was more evident when *S. cerevisiae* was exposed to 3 mg/liter AMB-L than when exposed to AMB-L plasma concentrations, as documented by both types of assays. Interestingly, AMB-L at plasma concentrations did not kill *S. cerevisiae* cells by necrosis; in fact, cells exposed to such concentrations displayed no evidence of plasma membrane damage, as shown by PI staining.

In conclusion, our results using *S. cerevisiae* as a model organism clearly demonstrate that yeast cells can respond in two ways to AMB-L: (i) expression of compensatory responses and survival when, in the plasma, the yeast are initially exposed to high concentrations of AMB-L and then much lower concentrations of the drug for extended periods of time; and (ii) induction of programmed cell death and/or necrosis, occurring at constant high concentrations. These findings provide important insights regarding AMB-L antifungal activity and ultimately may lead to the need to review therapeutic regimens.

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Publication III

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Unveiling the Synergistic Interaction Between Liposomal Amphotericin B and Colistin

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Patients with multiple comorbidities are often administered simultaneously or sequentially antifungals and antibacterial agents, without full knowledge of the consequences of drug interactions. Considering the clinical relevance of liposomal amphotericin B (L-AMB), the association between L-AMB and six antibacterial agents was evaluated against four clinical isolates and one type strain of *Candida* spp. and two clinical isolates and one type strain of *Aspergillus fumigatus*. In order to evaluate such combined effects, the minimal inhibitory concentration (MIC) of L-AMB was determined in the presence of 0.5-, 1-, 2-, and 4-fold peak plasma concentrations of each of the antibacterial drugs. Since the L-AMB/colistin (CST) association was the most synergic, viability assays were performed and the physiological status induced by this association was characterized. In addition, computational molecular dynamics studies were also performed in order to clarify the molecular interaction. The maximum synergistic effect with all antibacterial agents, except CST, was reached at fourfold the usual peak plasma concentrations, resulting in 2-to 8-fold L-AMB MIC reduction for *Candida* and 2-to 16-fold for *Aspergillus*. For CST, the greatest synergism was registered at peak plasma concentration (3 mg/L), with 4-to 8-fold L-AMB MIC reduction for *Candida* and 16-to 32-fold for *Aspergillus*. L-AMB at subinhibitory concentration (0.125 mg/L) combined with CST 3 mg/L resulted in: a decrease of fungal cell viability; an increase of cell membrane permeability; an increase of cellular metabolic activity soon after 1 h of exposure, which decreased until 24 h; and an increase of ROS production up to 24 h. From the molecular dynamics studies, AMB and CST molecules shown a propensity to form a stable molecular complex in solution, conferring a recognition and binding added value for membrane intercalation. Our results demonstrate that CST interacts synergistically with L-AMB, forming a stable complex, which promotes the fungicidal activity of L-AMB at low concentration.

Keywords: liposomal amphotericin B, colistin, synergistic effect, *Candida* species, *Aspergillus fumigatus*

INTRODUCTION

Fungi are recognized as major pathogens in critically ill patients. *Candida* and *Aspergillus* species are the most common agents of invasive fungal infections (IFIs), although other yeasts and filamentous fungi are becoming emerging pathogens (Gullo, 2009). In the clinical practice, patients at risk for IFIs often receive concomitantly or sequentially antifungal therapy and antibacterial agents, either for prophylactic or therapeutic purposes (Stergiopoulou et al., 2009). However, this procedure is often adopted without full knowledge of the consequences resulting from pharmacological drug interactions.

A recent literature review showed that the combination of amphotericin B (AMB) or fluconazole with antibacterial agents that impair RNA or protein synthesis, such as rifampicin (RIF), azithromycin (AZM), clarithromycin (CLR), and tetracycline (TET), enhances the *in vitro* activity of the antifungal agent against yeast and filamentous fungi (Azevedo et al., 2015). In addition, Zeidler et al. (2013) demonstrated that colistin (CST), an antibiotic that targets Gram-negative membranes, exhibits a synergistic effect with antifungal agents belonging to the echinocandin class against *Candida* spp. According to the authors, the echinocandin weakens the fungal cell wall facilitating the colistin action upon fungal membranes, and, consequently, this effect enhances the antifungal activity of the echinocandin (Zeidler et al., 2013). These results are particularly promising since the available antifungal panoply is narrow, encompassing only a few classes of agents, and the discovery of new drugs is a slow and exhaustive process (Roemer and Boone, 2013). Moreover, some antifungals demonstrate limited efficacy, high toxicity, and are prone to the development of antifungal resistance. Therefore, the association of compounds that enhance the efficacy of antifungal drugs may contribute to a more effective reduction of fungal burden and minimize the development of resistance (Zeidler et al., 2013).

Although the association of antifungal and antibacterial agents may have beneficial implications in clinical terms, little is known about the pharmacological drug interactions and the underlying mechanisms of synergism. While there are few studies available addressing this topic, it is important to stress that antagonistic or indifferent effects may also be found (Petrou and Rogers, 1988; Stergiopoulou et al., 2009; Venturini et al., 2011). Thus, an extensive study aiming to evaluate the mechanisms involved in the association between antifungal and antibacterial agents upon fungal growth inhibition is mandatory.

We hereby propose to evaluate the association between liposomal amphotericin B (L-AMB) and several antibacterial drugs against *Candida* spp. and *Aspergillus fumigatus*, as well as to unveil the mechanisms underlying such drug interactions. In the case of synergistic interaction, we have investigated using molecular dynamics (MD) simulations whether (i) the antibacterial agent has an intrinsic activity upon fungal cells; or (ii) the antibacterial agent acts as a facilitator of AMB activity. MDs simulations have been widely applied to study the interaction of AMB molecules with the fungal membranes, since the mechanism of action of AMB is not well characterize (Baginski et al., 1997; Sternal et al., 2004; Czub and Baginski, 2006; Czub et al., 2007; Neumann et al., 2009; Cohen, 2010; Foglia et al., 2014). Thus, this approach may be essential to unveil the mechanism of synergism.

Our results demonstrate which antibacterial agents improve L-AMB efficacy and elucidate about the hypothetical underlying mechanism.

MATERIALS AND METHODS

Strains and Growth Conditions

Four *Candida* clinical isolates and one type strain (C. *albicans* 596, *C. albicans* 38, *C. glabrata* 590, *C. krusei* 120, and *C. albicans* ATCC 90028) and two clinical isolates and one type strain of *A. fumigatus* (*A. fumigatus* 76, *A. fumigatus* 88, and *A. fumigatus* ATCC MYA-3626) were used in this study. Clinical isolates of *Candida* spp. and *A. fumigatus* were obtained from patients admitted at Centro Hospitalar São João, Porto, Portugal. Isolates are included in the Collection of fungal clinical isolates deposited at Department of Microbiology, Faculty of Medicine of Porto, Portugal. *Candida* isolates were grown in Sabouraud dextrose agar (SDA; Formedium, Norfolk, United Kingdom) at 35°C for 24 h (Teixeira-Santos et al., 2012); *Aspergillus* isolates were cultured in SDA at 35°C for 72 h (Faria-Ramos et al., 2014). *Escherichia coli* ATCC 25922 was also included in this study for control of antibacterial drugs.

Antimicrobial Drugs and Susceptibility Testing

Liposomal amphotericin B (provided by Gilead Sciences, Inc, San Dimas, CA, USA), rifampicin (Sanofi Aventis, Anagni, Italy), azithromycin (Farmoz, Sintra, Portugal), clarithromycin (Alcala Farma, Madrid, Spain), colistin sulfate salt (C4461, Sigma–Aldrich, Munich, Germany), tetracycline (T3258, Sigma–Aldrich), and linezolid (LZD; Pfizer, New York, NY, USA), were prepared according to the manufacturer's guidelines, in order to obtain stock solutions of 2 mg/L. The minimal inhibitory concentration (MIC) of L-AMB was determined according to M27-A3 protocol and M27-S4 supplement for *Candida* sp. and M38-A2 protocol of Clinical and Laboratory Standards Institute for *Aspergillus* sp. (CLSI, 2008a,b, 2012). The MIC of each antibacterial drugs was also determined; the tested antibacterial concentrations ranged between 0.25 and 128 mg/L. In order to evaluate the effect between L-AMB and the different antibacterial agents, the MIC to L-AMB was determined in the presence of 0.5-, 1-, 2-, and 4-fold peak plasma concentrations of each antibacterial drug. Peak plasma levels described in the literature are: RIF 12 mg/L (Van Ingen et al., 2011), AZM 4 mg/L (Sevillano et al., 2006), CLR 2 mg/L (Kees et al., 1995), CST 3 mg/L (Michalopoulos and Falagas, 2011), TET 2 mg/L (Agwuh and MacGowan, 2006), and LZD 12 mg/L (Dryden, 2011). Briefly, the drugs were diluted twofold in culture medium in order to obtain a 1:4 dilution. Fifty microliters of each L-AMB concentration (ranging from 0.03 to 16 mg/L) were combined with 50 μL of each of the concentration of the distinct antibacterial agents (described above). The plates were incubated at 35°C and the L-AMB MIC values determined after 24 and 48 h; MIC was

the lowest concentration that prevented any discernible growth. Since clinical breakpoints were not yet established for L-AMB, the *Candida* isolates were classified in wild type (wt) whenever the MIC was ≤2 mg/L and non-wild type (nwt) when the MIC was >2 mg/L according to the epidemiological cutoff values (ECVs) proposed by Pfaller and Diekema (2012). For *A. fumigatus*, only the MIC value is displayed, since AMB ECVs and clinical breakpoints remain undefined (Pfaller and Diekema, 2012). *C. albicans* ATCC 90028 and *A. fumigatus* ATCC MYA-3626 type strains were used as controls, as recommended by CLSI protocol (CLSI, 2008a,b, 2012). *E. coli* ATCC 25922 was used as a bacterial quality control to assure that antibacterial dilutions were correct. MIC determination assays were performed in triplicate.

Evaluation of Cell Viability

The evaluation of cell viability was performed with *C. albicans* 596, as a representative example, for the most synergic association, L-AMB/CST. The concentrations tested were selected according the susceptibility results. Yeast suspensions (10^6 yeast cells/mL) were exposed to L-AMB 0.125 mg/L alone and in combination with CST 3 mg/L, for 24 h at 35°C, 150 rpm. Another suspension was prepared and treated with single CST 3 mg/L. At specific time points, 1, 3, 6, and 24 h, aliquots were collected and tested for viability. Cell viability was determined in triplicate by counting colony forming units (CFU). Briefly, the number of viable cells for each treatment was determined by plating 100 μL of serial dilutions on SDA agar medium and incubating at 35°C for 24 h; the number of CFUs was determined and compared with control plates (not exposed to drugs or exposed to each antimicrobial drug, L-AMB and CST); before being plated, cells were washed once and resuspended in fresh medium in order to prevent antifungal carryover.

Functional Characterization of Drug Interaction

The distinct cellular physiological status resulting from the interaction between L-AMB and CST were assessed by flow cytometry in a time-course assay, using *C. albicans* 596 as a representative example. A cell suspension (10^6 yeast cells/mL) was used in all assays described below. Yeast cells were incubated with single L-AMB 0.125 mg/L and in association with CST 3 mg/L during 24 h. In order to evaluate whether there was a CST concentration-dependent effect, the following treatment conditions were also tested: L-AMB 0.125 mg/L with (i) CST 1.5 mg/L and (ii) CST 6 mg/L. The cellular status induced by single CST treatment was evaluated as control. At specific time points, 1, 3, 6, and 24 h, aliquots were collected and evaluated. All cytometric evaluations were performed in a FACSCalibur cytometer (BD Biosciences, Sydney, NSW, Australia) standard model, equipped with three photomultipliers, standard filters, a 15-mW 488-nm Argon laser, and using CellQuest Pro software (version 4.0.2). All the assays were performed in triplicate.

The effect of L-AMB 0.125 mg/L alone and in association with CST 3 mg/L was evaluated regarding: (i) membrane potential, (ii) membrane integrity, (iii) metabolic activity, and (iv) endogenous reactive oxygen species (ROS) production.

Evaluation of Membrane Potential

The cell membrane potential was assessed by staining the cells with Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol ($DiBAC_4(3)$, Sigma–Aldrich), as described by Teixeira-Santos et al. (2012). The fluorescence intensity (FI) at FL1 (fluorescent detector; 530 nm) was registered and a staining index (SI) was defined as the ratio between the FI of treated cells and the FI of non-treated cells.

Evaluation of Membrane Integrity

Cell membrane integrity impairment was evaluated using propidium iodide (PI, Sigma–Aldrich) staining. After antimicrobial treatment, yeast cells were stained with 1 mg/L of PI for 30 min at 35°C, in the dark (Pina-Vaz et al., 2005). The FI was measured at FL3 (fluorescent detector; 630 nm). The amount of injured cells in each sample was defined as the percentage of PI-positive cells.

Evaluation of Metabolic Activity

Metabolic changes were evaluated using 5-Carboxyfluorescein diacetate (5-CFDA, Sigma–Aldrich), at 10 μM final concentration. Antimicrobial treated cells were stained with 5-CFDA and incubated for 45 min, at 35°C, at 150 rpm, in the dark (Liao et al., 2003). The mean intensity of fluorescence (MIF) was registered at FL1 (530 nm).

Evaluation of Endogenous ROS Production

Reactive oxygen species production was evaluated as previously described (Yan et al., 2009). In brief, yeast cells were incubated with 20 mg/L of 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Sigma–Aldrich) for 30 min at 35°C, at 150 rpm. Cells were washed once (2,655 × *g* for 5 min at room temperature; 5417R, Eppendorf) and resuspended in phosphate-buffered saline (PBS, Sigma–Aldrich); afterward, cells were treated with the antimicrobials as described in "Functional characterization of drug interactions" section. FI was determined at FL1 (530 nm). ROS production was calculated by subtracting the FI value displayed by cells treated with antimicrobials from that of cells treated with both antimicrobials and DCFH-DA.

Simultaneously, 10 μL of the treated cell suspension were placed in a microscope slide for further analysis under fluorescence microscopy, in a Carl Zeiss Axiovert inverted microscope, using laser wavelength of 488 nm.

Molecular Dynamics Studies

The Automated Topology Builder (ATB) web server was used for the AMB ($C_{47}H_{73}NO_{17}$) and CST ($C_{52}H_{98}N_{16}O_{13}$) MDs topology parametrization, corresponding to the new residues name IDs D3JY and 09SS, respectively, as a GROMACS G54A7FF United-Atom force field (Malde et al., 2011).

The ATB derived force-field was tested in a series of independent MD simulations in an explicit water box. Then, AMB and CST molecules were placed in the same simulation box, together with 6230 SPCE water molecules, at more than

17 Å apart from each other, in order to avoid any computational bias. The entire MD protocol, including energy minimization, equilibration, and production steps, were performed using GROMACS 4.6.1 simulation package (Hess et al., 2008). MD simulations were performed at the in-house Fermi GPU high performance computing workstation. The global charge of the system was zero and simulations run under periodic boundary conditions in an isothermal-isobaric (NPT) ensemble at 300 K and 1.0 bar, during 20 ns, as described in previous studies (Branco et al., 2012).

Statistical Analysis

Results are detailed as mean value and the respective standard deviation. Paired-sample Student's *t*-test was used to compare the effect of L-AMB and CST alone and in association.

All statistical analysis was performed using the SPSS software (v. 23.0).

RESULTS

Susceptibility to L-AMB alone and in Association with Antibacterial Drugs

Single RIF, AZM, CLR, CST, TET, and LZD did not inhibit the growth of all the fungal species studied, even at the maximum tested concentrations (fourfold peak plasma concentrations). The fungal strains showed variable susceptibility profiles to L-AMB, being all *Candida* strains wt (MIC value $\leq$ 1 mg/L), except *C. krusei* with a MIC value of 8 mg/L. Regarding *A. fumigatus* MIC values of 2 and 4 mg/L were registered (**Table 1**).

Minimal inhibitory concentration of L-AMB was reduced 2- to 4-fold in the presence of peak plasma concentrations of RIF, AZM, CLR, and TET. The maximum effect of these antibacterial agents was obtained at fourfold plasma concentrations with a reduction of L-AMB MIC of 2-to 8-fold, in the case of *Candida* (except CLR regarding *C. krusei* 120) and 2-to 16-fold for *A. fumigatus* (**Table 1**). CST at 0.5-fold peak plasma concentration (1.5 mg/L) resulted in a reduction of L-AMB MIC of 2-to 4-fold for all fungal isolates tested. At the peak plasma concentration (3 mg/L), CST reduced L-AMB MIC in 4-to 8-fold for *Candida* spp. and 16-to 32-fold for *A. fumigatus*. Interestingly, CST at 2- and 4-fold peak plasma concentration (6 and 12 mg/L, respectively) resulted in the same effect upon L-AMB MIC as CST 3 mg/L. Linezolid, even at fourfold peak plasma concentration did not associate with any L-AMB MIC reduction, regarding all tested fungal strains.

Colistin was selected for further studies since it was the drug that exhibited the highest synergistic effects with L-AMB.

Impact of L-AMB/CST Association upon:

Fungal Cell Viability

Figure 1A represents *C. albicans* 596 viability at subinhibitory concentration of L-AMB (0.125 mg/L), alone and in association with CST 3 mg/L. Treatment with L-AMB 0.125 mg/L significantly decreased yeast cell viability up to 3 h of exposure ($p = 0.039$); however, after 3 h of treatment, cells recovered the ability to replicate and viability increased up to 24 h. At this time point, a significant difference was registered between cells treated with L-AMB and non-treated cells ($p = 0.011$). In the case of cells exposed to CST, significant growth reduction was only registered after 24 h of incubation ($p = 0.009$). Whenever cells were treated with L-AMB in association with CST there was a significant reduction of CFU counts (10^7 to 10^5 cells/mL) soon after 3 h of incubation ($p = 0.037$). This CFU reduction was consistently observed along the time up to 24 h ($p = 0.004$).

Cell Membrane Permeability

Liposomal amphotericin B acts on fungal cells by binding to the sterol component of the cell membrane, first leading to alterations in cell permeability (Gray et al., 2012). In order to evaluate whether antibacterial agents improve the effect of L-AMB on cell membrane permeability, the membrane potential and cell membrane integrity were assessed with the fluorescent dyes $DiBAC_4(3)$ and PI, respectively. $DiBAC_4(3)$ enters only in depolarized cells, where it binds reversibly to intracellular components, resulting in an increased fluorescent

TABLE 1 | Minimal inhibitory concentration (MIC) of liposomal amphotericin B alone and in association with several antibacterial drugs at fourfold peak plasma concentrations.

Fungal isolates	MIC value of L-AMB (mg/L)						
		L-AMB in association with					
	L-AMB	RIF	AZM	CLR	CST	TET	LZD
C. albicans ATCC 90028	1	0.25	0.25	0.25	0.125	0.25	1
C. albicans 596	1	0.125	0.25	0.25	0.125	0.25	1
C. albicans 38	0.5	0.06	0.25	0.25	0.06	0.25	0.5
C. glabrata 590	0.25	0.125	0.125	0.125	0.06	0.125	0.25
C. krusei 120	8	2	4	8	2	2	8
A. fumigatus ATCC MYA-3626	1	0.25	0.5	0.25	0.06	0.125	1
A. fumigatus 676	4	1	1	1	0.125	0.25	4
A. fumigatus 88	2	0.5	1	0.5	0.125	0.125	2

L-AMB, liposomal amphotericin B; RIF, Rifampicin; AZM, Azithromycin; CLR, Clarithromycin; CST, Colistin; TET, Tetracycline; LZD, Linezolid.

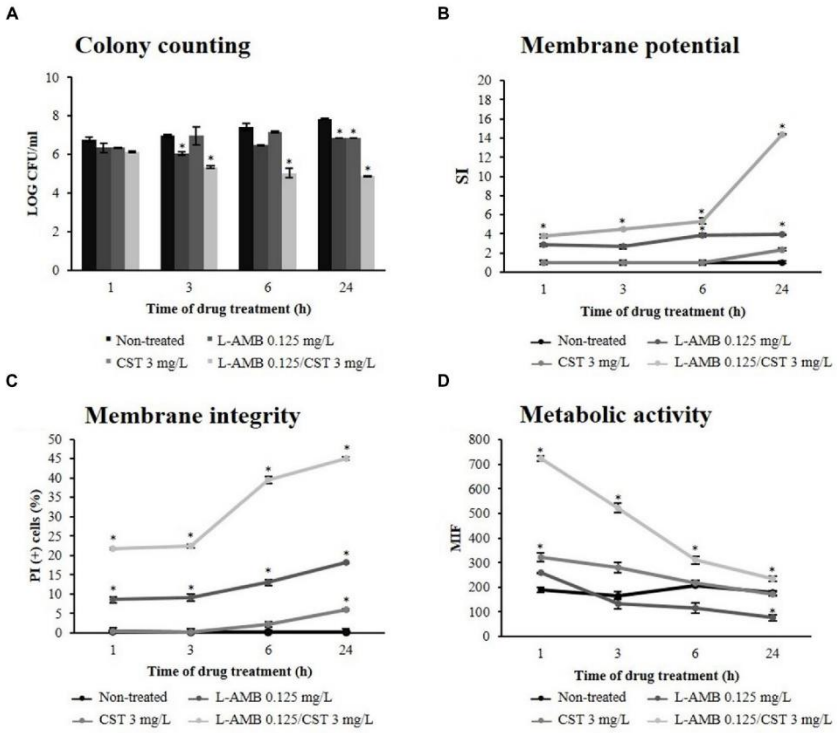

FIGURE 1 | Effect of liposomal amphotericin B (L-AMB) in combination with colistin upon *C. albicans* (strain 596) physiological parameters. **(A)** Viability assessment by colony forming units (CFU) enumeration of *C. albicans* cells exposed to (■) L-AMB, (■) CST, and (■) L-AMB in association with CST. The CFU counts of cells non-treated are represented as (■). **(B)** Cell membrane potential was evaluated using $DiBAC_4(3)$ staining. **(C)** Cell membrane integrity was evaluated using propidium iodide (PI) staining. **(D)** Cell metabolic activity was evaluated using 5-CFDA staining. Data at respective time points corresponds to mean ± standard deviation. *p-values < 0.05, significant differences between the treatments with L-AMB alone and CST alone vs. the control (non-treated cells); and between the association L-AMB/CST and L-AMB alone.

signal. The results obtained regarding membrane depolarization of *C. albicans* 596 are depicted in **Figure 1B**; after 3 h of treatment, the SI of cells exposed to L-AMB 0.125 mg/L was 2.65 ($p = 0.173$); at 6 h, the SI increased to 3.88 ($p = 0.043$), remaining constant up to 24 h ($p = 0.042$). Whenever yeast cells were exposed to CST, the SI was about 1.00 up to 6 h of treatment ($p = 0.082$), increasing to 2.36 at 24 h ($p = 0.052$). Regarding the association of L-AMB 0.125 mg/L with CST 3 mg/L, soon after 1 h of treatment the SI was 3.79, increasing up to 14.38 following 24 h. Significant differences were observed between the association L-AMB/CST and single L-AMB at 1 h ($p = 0.025$), 3 h ($p = 0.028$), 6 h ($p = 0.011$), and 24 h ($p = 0.011$). L-AMB 0.125 mg/L in association with CST 1.5 mg/L induced an increase of SI up to 24 h (SI = 6.69; $p = 0.028$). This effect was more pronounced when L-AMB 0.125 mg/L was combined with CST 3 mg/L, as previously described. However, when L-AMB 0.125 mg/L was combined with CST 6 mg/L no significant differences were found, compared to the association L-AMB/CST (3 mg/L; data not shown).

Such results show the key role of L-AMB/CST association on the increase of cell membrane depolarization.

Conversely, PI is a cell viability marker, which enters cells only when membrane has been seriously injured. The results regarding membrane lesion of *C. albicans* 596 are represented in **Figure 1C**. In cells treated with L-AMB 0.125 mg/L, the % of PI(+) cells after 1 h of exposure was about 10.00%, increasing slightly up to 24 h (% of PI(+) cells = 14.93); significant differences were found at 1 h ($p = 0.019$), 3 h ($p = 0.036$), 6 h ($p = 0.008$), and 24 h ($p = 0.036$), compared to control (non-treated cells). Cells treated with COL 3 mg/L showed PI-uptake only after 24 h of incubation, with a % of PI(+) cells equal to 5.93 ($p = 0.046$). Concerning the L-AMB/CST association, soon after 1 h of exposure, PI stains 21.82% of the treated cells, and after 6 h of incubation, this value reaches 39.47%; at 24 h, the percentage of cells with membrane lesion was about 45.01%. Significant differences were observed between the L-AMB/CST association and L-AMB treatment at 1 h ($p = 0.011$), 3 h ($p = 0.008$), 6 h ($p = 0.002$), and 24 h ($p = 0.040$). Thus, data suggests that L-AMB/CST association

promotes the membrane lesion effect of L-AMB, in a time-dependent fashion.

Metabolic Activity

To assess the metabolic effects of L-AMB/CST upon *C. albicans* 596, the cells were stained with 5-CFDA; 5-CFDA is a cell-permeant esterase substrate that measures enzymatic activity (Breeuwer et al., 1995; Boender et al., 2011). Only the cells metabolically active will display a high level of fluorescence. The results obtained for MIF are detailed in **Figure 1D**. MIF displayed by viable cells (not exposed to the drugs) remained constant up to 24 h; at this time point, the MIF was 176.99. Cells treated with L-AMB did not display significant differences in MIF up to 3 h ($p = 0.198$). After 6 h, MIF decreased over time up to 75.53 ($p = 0.049$) at 24 h of exposure. When yeast cells were treated only with CST 3 mg/L, the MIF increased after 1 h (322.59; $p = 0.029$), indicating that the cells were metabolically active. However, after 3 h of treatment, the MIF decreased up to a value of 172.11 ($p = 0.220$), at 24 h, similar to the MIF of cells not exposed to the drugs. Cells exposed to the association L-AMB/CST initially displayed a very high MIF (724.30); however, it decreased until 24 h of treatment (234.91). Significant differences were registered between the metabolic activity of cells exposed to the drug association and to L-AMB alone at 1 h ($p = 0.001$), 3 h ($p = 0.021$), 6 h ($p = 0.014$), and 24 h ($p = 0.025$).

Endogenous ROS Production

Reactive oxygen species production was assessed by DCFH-DA staining. DCFH-DA is oxidized to highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) by ROS. ROS production by cells exposed to L-AMB 0.125 mg/L was constant up to 6 h of incubation (≈7.00%; $p = 0.005$); at 24 h, the number of fluorescent cells increased up to 18% ($p = 0.001$) (**Figure 2A**). Exposure to CST 3 mg/L resulted in reduced ROS formation in *C. albicans* 596 cells, reaching a value of 7.00% ($p = 0.010$), following a 24 h of incubation. Images obtained by fluorescence microscopy (**Figure 2B**) showed vesicle formation in the presence of CST, after 24 h of incubation. Cells exposed to L-AMB/CST displayed a growing ROS production pattern; 7.00% following 6 h ($p = 0.056$); 25.53% following 24 h of incubation ($p = 0.022$) (**Figure 2A**). These results reveal that L-AMB/CST leads to an increase of intracellular accumulation of ROS in relation to single L-AMB after 24 h.

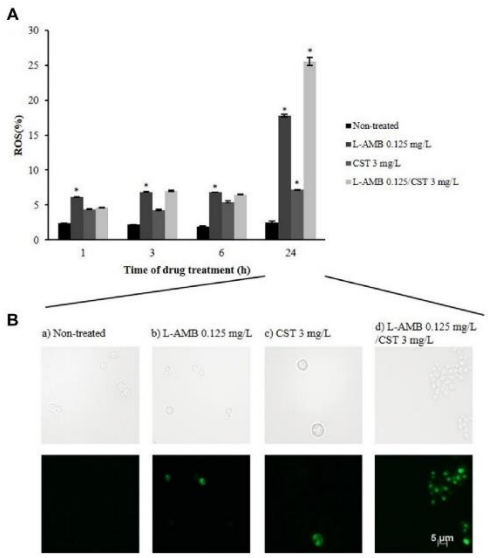

FIGURE 2 | Effect of L-AMB in association with colistin upon endogenous ROS production. (A) Endogenous reactive oxygen species (ROS) production determined by DCFH-DA staining, assessed by flow cytometry. **(B)** Fluorescence microscopy imaging showing ROS-positive cells after treatment with (b) L-AMB and (c) CST alone and (d) in association after 24 h of exposure; (a) control, non-treated cells. *p-values < 0.05, significant differences between the treatments with L-AMB alone and CST alone vs. the control (non-treated cells); and between the association L-AMB/CST and L-AMB alone.

Self-Association Propensity Assessed by Atomic Molecular Dynamics Simulations

It is known the propensity of AMB to form dimeric or even higher complex tetrameric structures once in water medium, which interfere with the electrophysiology of living cells through transmembrane ion channel formation (Starzyk et al., 2014). Herein, we describe the propensity of AMB and CST in water also to self-assembly based on MDs simulations. The results demonstrate that the two molecules tend spontaneously form a natural complex in solution, starting from a perfect unbound, solvated form, separated by more than 17 Å. Once the two molecules meet each other after the first 4 ns of simulation, they are steadily attracted into a complex formation that stands stable for the rest of the simulation time. This complex is characterized by polar interactions, namely hydrogen bond interactions between the C_5- and C_9-OH groups of AMB and amide groups of CST, as depicted in **Figures 3A,B**. These two H-bond pairs (AMB-O_{58}:CST-N_{18} and AMB-O_{66}:CST-O_9) converged for a minimum distance of 2.7 and 2.5 Å, respectively. It seems that the complex is quite stable on the simulation window, supporting an analogous mechanism to the one predicted and validated experimentally for the dimerization of AMB alone. However, the main dimer-stabilizing contacts of the AMB:CST system seems to be the polar interactions and not the van der Waals forces contribution, as described for the hydrophobic nature of dimerization process of AMB molecules, which still might be essential for speeding up transmembrane intercalation of the complex and consequent disruption of the cell membrane through pore formation, resulting into a perfect and synergetic Trojan mechanism for the antimicrobial internalization (Starzyk et al., 2014).

DISCUSSION

This study demonstrates that all the tested antibacterial agents except linezolid interact synergistically with L-AMB against

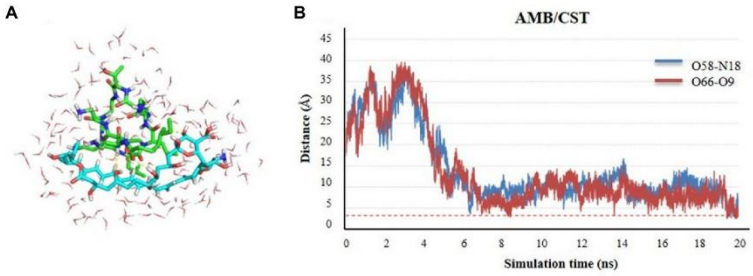

FIGURE 3 | Self-assembly of amphotericin B (AMB) and colistin (CST; represented in sticks) in water medium (represented in lines) through molecular dynamics (MD) simulation studies. (A) Complex conformation snapshot after 20 ns of simulation, including water molecules within 4 Å of the complex. **(B)** Time evolution of the h-bond distance between two molecules during the complex formation.

several pathogenic fungi, namely *C. albicans*, *C. glabrata*, *C. krusei*, and *A. fumigatus* (**Table 1**). These antibacterial agents alone did not exhibit antifungal activity, thus suggesting that such compounds do not intrinsically target the fungal cells. The agents tested belong to distinct antibacterial classes with different mechanisms of action. Rifampicin inhibits the DNA-dependent RNA polymerase (RNAP), leading to the suppression of RNA synthesis and cell death (Campbell et al., 2001). The basic architecture of bacterial RNAP and yeast RNAP presents several structural differences (Minakhin et al., 2001), explaining why RIF by itself does not display antifungal activity. Azithromycin and CLR are macrolide antibiotics that bind to 50S ribosomal subunits of bacteria, blocking the protein synthesis and inhibiting cell growth (Kanoh and Rubin, 2010; Parnham et al., 2014); LZD also binds to the 50S subunit of the prokaryotic ribosome, blocking the assembly of a functional initiation complex for protein synthesis (Livermore, 2003); TET inhibits protein translation in bacteria by binding to the 30S ribosomal subunit (Chopra and Roberts, 2001). Although the basic mechanism of protein synthesis in eukaryotes is similar to that in bacteria, some noteworthy differences were described between the structure of eukaryotic and prokaryotic ribosomes (Berg et al., 2002). Colistin belongs to the family of polymyxin antibiotics, which target bacterial cell membrane. CST displaces Mg^{2+} and Ca^{2+} ions, which stabilize the negatively charged lipopolysaccharide LPS, disrupting the membrane integrity in gram-negative bacteria (Falagas and Kasiakou, 2005). The antifungal activity of polymyxin antibiotics against several fungal species has been previously reported (Schwartz et al., 1972); however, at the hereby tested concentrations, CST did not inhibit fungal growth. Colistin plasma concentrations usually range from 0 to 15 mg/L, with free CST levels ranging from 0.5 to 3 mg/L (Akers et al., 2015). It is important to emphasize that about 50% of CST molecules bind to plasma proteins (Falagas and Kasiakou, 2005).

Considering all the antibiotic drug combinations evaluated, the one that resulted in the strongest synergic effect was L-AMB/CST. Additionally, the synergistic combination of CST with other antifungal agents, namely echinocandins class, has been previously described (Zeidler et al., 2013). Therefore, we explored the underlying mechanism of synergism of L-AMB/CST interaction.

Amphotericin B is a lifesaving antifungal drug used to treat deep-seated fungal infections, exhibiting broad-spectrum fungicidal activity. This activity is based on its interactions with fungal cell membranes (Starzyk et al., 2014). AMB primarily binds to ergosterol, inserts into the cytoplasmic membrane, and forms pore-like structures; the result is osmotic instability, loss of membrane integrity, metabolic disruption, and ultimately cell death (Gray et al., 2012). Our results showed a significant growth reduction of fungal cells treated with L-AMB/CST during 24 h of exposure (10^8 to 10^5 cells/mL), with a simultaneous increase of cell membrane permeability, as documented by $DiBAC_4(3)$ (SI was 14.38) and PI staining (% of PI(+) cells was 45.01%) (**Figures 1B,C**). Altogether, these results strongly indicate that the association of CST significantly improves the fungicidal activity of L-AMB.

It is known that yeast cells exposed to L-AMB pressure reprogram their metabolism in response to an environmental stress (Zhang et al., 2002; Belenky et al., 2013; Teixeira-Santos et al., 2015). The combination of L-AMB and CST triggered an increase of metabolic activity in treated cells, soon after 1 h of exposure (3.8-fold higher than single L-AMB treatment; **Figure 1D**), suggesting an early strong stress response induced by membrane permeabilization. Along 24 h of exposure, the metabolic activity decreased overtime, nevertheless, still exhibiting a MIF 3.1-fold higher than single L-AMB treatment (**Figure 1D**). This decrease can be explained by membrane pore formation, which may cause the accelerated loss of fluorescence, or by a reduced metabolic activity (Breeuwer et al., 1995; Teixeira-Santos et al., 2015). Interestingly, the higher cell metabolic activity induced by the association of L-AMB/CST versus single L-AMB along 24 h period can be related with a higher endogenous ROS production in cells exposed to this association (**Figure 2**), which will result in oxidative damage and possibly is involved in induced programmed cell death (Phillips et al., 2003; Al-Dhaheri and Douglas, 2010). Curiously, single CST (3 mg/L) has a significant effect on ROS production by itself. Accordingly, cells exposed to CST display a different phenotype compared to cells exposed to the other treatment conditions, i.e., the formation

of vesicles (apoptotic bodies), which is a characteristic event of apoptosis (Fink and Cookson, 2005). Although the functional studies that support the mechanism of action of L-AMB/CST association have been conducted only in *C. albicans*, according to the MIC determination assays, this mechanism seems to be transversal to the different species studied.

All of our results point to a considerable improvement of L-AMB antifungal effect, at subinhibitory concentrations, whenever associated to CST 3 mg/L. However, how do AMB and CST molecules interact? The computational molecular dynamics results demonstrate that the two molecules spontaneously form a natural complex in solution, characterized by a strong bond 1:1. Thus, AMB and CST molecules act together on fungal cells. In support of such a finding was the fact that the maximum synergistic effect was detected in the presence of peak plasma concentration of CST (3 mg/L); its increase to fourfold peak plasma concentration did not increase the synergistic effect, as documented by MIC determination and membrane potential evaluation assays. Moreover, a recent study described that the interactions of AMB with biomembranes are managed by the molecular organization of AMB (Starzyk et al., 2014). AMB self-associates to dimeric structures; AMB dimers can further assemble into tetramers, which induce the formation of transmembrane ion channels, impairing the electrophysiological homeostasis of a living cell (Starzyk et al., 2014; Davis et al., 2015). Considering all these findings, it is possible that CST binds to AMB molecules and accelerates the assembly of AMB tetramers, thus inducing pore formation on fungal cell membranes, also triggering a strong cell stress response, which is typical of AMB action.

Our results are extremely promising since AMB is an important therapeutic alternative for the treatment of IFIs, particularly when infection persists, despite treatment with other drugs, and the clinical response to AMB is reduced in about 40% of treated patients (Mora-Duarte et al., 2002; Ito and Hooshmand-Rad, 2005; Park et al., 2006). Simultaneously, this synergic association may be a clue for drug discovery.

Studies are being conducted in order to characterize the functional groups of the AMB/CST complex that interact with the fungal membrane aiming to design a more active antifungal compound.

AUTHOR CONTRIBUTIONS

RT-S, AR, and CP-V contributed with the experimental design and results interpretation of this study. RT-S, ER, MA carried out all experiments and RB perform the molecular dynamics studies. RT-S and CP-V wrote the manuscript and all authors performed a critical revision and approved the final version.

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

In vitro antifungal susceptibility study: MICs were determined using the E-test method® on RPMI agar medium.

Reading: at 24 h and 48 h.

Interpretation of MICS values: the categorization using new 24 h-CBPs was compared to the categorization obtained at 24 h and 48 h using previous BPs. The respective categorical agreements 24 h-CA and 48 h-CA were evaluated.

Results The 24 h-CA and 48 h-CA were 81%, 76.2%, 88.6%, 78% and 88.1%, 85.7%, 94.3%, 87.8% respectively for Poitiers, Reims, Rennes and Toulouse centres. Comparing interpretation using new 24 h-CBPs and previous 48 h-BPs, we obtained a different categorization for 18 of the 160 MIC (11.25%). Two late resistances at 48 h were not detected using the new 24 h-CBPs (SDD) for 2 pairs of *C. glabrata*/fluconazole (classed SDD). Three categorizations S (n = 1) or SDD (n = 2) at 48 h were ranged R using the new 24 h-CBPs, respectively *C. krusei*/caspofungin, *C. parapsilosis*/voriconazole and *C. parapsilosis*/fluconazole. The remaining 13 cases (8 strains) were categorized "S"using the previous BPs and "S"with the new 24hCBPs.

Conclusion The MIC determination at 24 h with application of the newly revised CBPs provide a good CA with categorization at 24 h and 48 h using previous BPs. The CA was better with 48 h-values in all centres, underlining that, in most cases, these new 24 h-CBPs take into account late apparition of an elevated MIC.

P028

Uncovering yeast recovery pathway to liposomal amphotericin B-induced stress

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Objectives Liposomal amphotericin B (L-AmB) is antifungal that targets to ergosterol, the principal sterol in the fungal cytoplasmic membrane. L-AmB exhibits broad fungicidal activity at very low concentrations. Nonetheless, in spite of the observed high in vitro susceptibility, in vivo response to L-AmB is often reduced. Pharmacokinetic studies indicate that serum levels decline rapidly during the first 2 h after the administration. In order to understand what happens to yeasts exposure to L-AmB serum concentrations, a kinetic study was performed in *Candida* spp., *Saccharomyces cerevisiae* and *Debaryomyces hansenii* by flow cytometry.

Methods Two *Candida* spp. clinical strains, one *S. cerevisiae* and one *D. hansenii*, all susceptible to liposomal amphotericin B were studied. Cells were incubated for 90 min with L-AmB 3 μg/ml, then submitted to three treatment conditions: 3, 0.3 and 0 μg/ml of L-AmB and incubated at 35°C, 180 rpm. After 90 min, 3 h, 6 h and 24 h of incubation cells were stained with $DiBAC_4$, (a membrane potential fluorescent probe) for 30 min in the dark, and analyzed in a FACSCalibur cytometer at FL1 (530 nm). Time-kill experiments were also performed to validate flow cytometry results.

Results The results showed that yeast cells are totally depolarized following exposure to L-AmB 3 μg/ml for 24 hours. Conversely, when exposed to the same concentration only for 90 min followed by 0 or 0.3 μg/ml of L-AmB in the remaining hours, the population re-polarizes following 3 hours of incubation only. Time-kill assay performed under the same conditions showed that cells exposed to 3 μg/ml of L-AmB (90 min, 3 h, 6 h or 24 h) are unable to replicate. However, in the presence of lower L-AmB concentrations, cells recovered the replication capacity. This phenomenon was observed in *Candida* spp., *S. cerevisiae* and *D. hansenii*.

Conclusions The expected L-AmB fungicidal effect is only observed when the concentration is high for long periods of time. The phenomenon of cell recovery at lower concentrations seems to be common on the studied yeasts, a conserved pathway should be present and can be related to a poor outcome.

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P029

In vitro susceptibility testing of *Candida albicans* by using Micronaut-Am method

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Objectives Microbiologists are looking for standardized AFST (antifungal susceptibility testing) to determine the minimal inhibitory concentration (MIC) without *trailing effect* and to monitor *Candida* resistance. The knowledge concerning antifungal resistance rate among *Candida* sp. is still lacking, thus a susceptibility testing of superficial yeast is of great importance to improve it and finally to better manage the treatment of opportunistic infections. The aim of the study was to determine the susceptibility of *Candida albicans* isolates using AFST systems.

Methods 170 *Candida albicans* strains were tested against six antifungals using MICRONAUT-AM system and doubt results were verified by E-test and reference procedure.

Results A total of *Candida albicans* isolates were susceptible to ketoconazole, fluconazole, voriconazole and 5- flucytosine according to CLSI while 4 (2%) were considered intermediate to fluconazole (MIC = 4 μg ml^{-1}) by EUCAST. Thirteen isolates (8%) were resistant to itraconazole (CLSI). Up to 48 isolates (28%) exhibited MIC of amphotericin B – 2 μg ml^{-1} but the MIC values were significantly reduced by E-test (0.094–0.38 μg ml^{-1}). *Trailing endpoints* for azoles were clearly inhibited.

Conclusion MICRONAUT-AM system seems to be a useful tool for *in vitro* yeast susceptibility testing to azoles while E-test is still the most reliable to determine MIC of amphotericin.

P030

Molecular identification and antifungal susceptibility of clinical *Scedosporium/Pseudallescheria* isolates from a Spanish teaching hospital

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Objectives The taxonomy of the *Scedosporium/Pseudallescheria* species complex has been extensively modified in recent years. However, there is still limited data about the rate at which the different species of this fungal group are recovered from patients. In this study we identified to the species level a collection of *Scedosporium/Pseudallescheria* isolates using molecular techniques. Additionally, for all available isolates we also determined their antifungal susceptibility.

Methods We studied 91 clinical isolates collected from 43 patients attended at our institution from 1996 to 2012, and morphologically identified as *Scedosporium* spp. Molecular identification of isolates was performed by partial sequencing the beta-tubulin encoding gene. Susceptibility to amphotericin B (AMB), isavuconazole (ISA), itraconazole (ITZ), posaconazole (POS), voriconazole (VCZ), anidulafungin (AND), caspofungin (CAS) and micafungin (MYC) was evaluated by the CLSI broth microdilution method.

Results The following species (isolates/cases) were identified: *Pseudallescheria boydii* (PB, 37.4%/37.2%), *Scedosporium apiospermum* (SA, 24.2%/32.6%), *S. prolificans* (SP, 37.4%/27.9%) and *P. ellipsoidea* (PE, 1%/2.3%). The results of antifungal susceptibility testing are shown in the table.