DEVELOPMENT OF NEW STRATEGIES FOR THE TREATMENT OF EMERGING OPPORTUNISTIC FUNGAL INFECTIONS

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Development of new strategies for the treatment of emerging opportunistic fungal infections

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Drs., Javier Capilla Luque, Katihuska Paredes Aguilar and Josep Guarro Artigas, researchers of the *Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili.*

STATE that the present study, entitled "Development of new strategies for the treatment of emerging opportunistic fungal infections", presented by Adela Martín Vicente for the award of the degree of Doctor, has been carried out under my supervision at the *Departament de Ciències Mèdiques Bàsiques*, and that it fulfills the requirements to obtain the International Doctorate mention.

Reus, September 2, 2016

Doctoral Thesis Supervisor/s

Javier Capilla Luque

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Josep Guarro Artigas

A Manuel Vicente Guillén

"Cuanto más grande es la dificultad,
más gloria hay en superarla."

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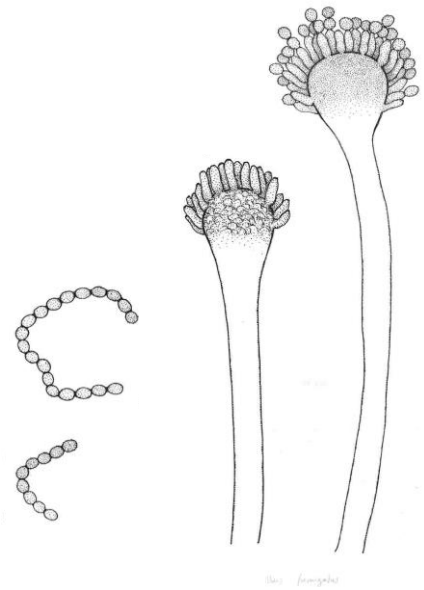


1. INTRODUCTION

1. Introduction

1.1 Medical mycology

Medical mycology is a relatively young sub-discipline of medicine, originally developed by dermatologists, pathologists, bacteriologists, microbiologists, or clinicians, rather than researchers or medical mycologists. It is, essentially, the study of infectious disease in human caused by fungi.

Possibly, the earliest description of a fungal infection is reported in the fourth collection of the sacred scriptures of Hinduism (Atharva Veda) (2000 – 1000 b.C.). It is attributable to mycetoma of the foot, which was differentiated by filarial elephantiasis and metamorphically defined as "padavalmika" that means "Madura foot" (foot-ant-hill). However, the term "mycetoma" was not introduced until 1861 by the anatomist, surgeon, and anatomical artist Henry Vandyke Carter (1831-1897). Although in the classic Rome and Greece the fact that microorganism are responsible of some diseases was unknown, some mycoses familiar to us today as thrush in infants and favus, were noted and medically described in the Greek and Roman classics, as appears in Hippocrates (5th century b.C.) and Aulus Cornelius Celsus (1st century a.C.) writings.

The fundamental merit of having demonstrated in an exemplary way that a microorganism may be the causative agent of an infectious disease in animals must be recognized to Agostino Bassi (1773-1856). Bassi, a civil servant and self-taught scientist published the results of his studies on the disease of the silkworm, the larva of the mulberry moth (*Bombyx mori*), which was destroying the silk industry in Italy and France (1835), demonstrating that the disease was caused by a fungus (subsequently named *Beauveria bassiana*, in his honour). In spite of his findings, the pathogenicity

of fungi to animals was not generally accepted until after the mid-nineteenth century, with the demonstrations of Louis Pasteur. Bassi also stated the idea that not only animal (insect), but also human infectious diseases were caused by other living organisms (1844). Through meticulous experiments, Bassi showed that the cause of death of the silkworm was an entomogenous fungus, and is rightly acknowledged to have been the first to refer the etiology of an animal disease to a microbial infection, being universally regarded as the "father of Medical Mycology". In 1839, Johann Lucas Schönlein (1793-1864) first observed and published that fungal elements were present in lesions of human favus, a widespread and virulent disease of the skin and scalp, which infested children and adults in Europe and caused by the fungus *Trychophyton schoenleinii*. The same year, the surgeon Bernhard Rudolf Konrad von Langenbeck (1810-1887) published the first case of candidiasis of the oesophageal mucosa of a patient who died of typhoid fever. He described in detail what is now recognized to be septate hyphae, branched pseudohyphae and blastoconidia.

The beginning of medical mycology as a distinct branch of medicine can be precisely dated as 1842 to 1844, the years during which David Gruby in Paris published a series of six short papers in which he showed that four types of ringworm and also thrush were mycotic in origin. It was in 1846 when Carl Ferdinand Eichstedt (1816-1892) identified the fungus *Microsporum furfur* as a causative agent of the contagious Pytiriasis versicolor. The next year, the first well-documented case of human pulmonary aspergillosis was published in the doctoral thesis of Theodor Sluyter (1817-1895).

The term "onychomycosis" was created in 1854 by Rudolf Virchow according to the observations of Georg Meissner, who first described *Tinea unguium,* based on the detection of a fungus in the nail material as the

etiologic agent one year before. Nine years later, the German pathologist Friedrich Albert von Zenker (1825-1898), first described a case of disseminated candidiasis as metastasis to the brain, commenting that the infection was probably bloodborne from the thrush lesions of the tongue and gullet.

At the end of the 19th century, protozoologists made important advances in mycopathology. In 1892, Alejandro Posadas first described the microorganism believed to be a protozoon responsible for "*mycosis fungoides*" and four years later the same organism was described in California and named *Coccidioides immitis* by Rixford & Gilchrist. Eight years later Ophuls & Moffitt recognized that the protozoon-like was a pathogenic phase of a fungus. In 1906, S. T. Darling described *Histoplasma capsulatum* but in this case it was 28 years before the fungal nature of the pathogen was demonstrated. After the First World War, advances in mycology were clearly observed, including taxonomy and characterization of sexual and asexual phases. Many pathogenic fungi were isolated from soil, animal dung or other substrata, concluding that many of the fungi causing mycoses are "opportunistic", that is, they establish themselves as pathogens only when a subject is by chance exposed to an exceptionally high concentration of their spores or when the subjects are immunocompromised.

1.2 Disseminated fungal infections

It has been suggested that there are around 5 million fungal species (Blackwell, 2011), of which only about 150 have been associated with human diseases. And of this subset only a few are regularly encountered in the clinical arena. In addition to the relative paucity of fungal species with pathogenic potential, life-threatening fungal diseases are more infrequent in immunologically intact human populations than other infectious diseases.

However, over the past two and a half decades, the incidence of both nosocomial and community-associated fungal infections has dramatically increased, mainly due to a growing population of immunocompromised patients whose mechanisms of host defense have been impaired by primary disease states (e.g. AIDS, cancer, hematologic malignancy). In addition, the mobile and aging population with an increased prevalence of chronic medical conditions, and the use of new and aggressive medical and surgical therapeutic strategies, including broad-spectrum antibiotics, cytotoxic chemotherapies, and organ transplantation have contributed to the increase in opportunistic fungal infections (Oliveira-Coelho *et al.*, 2015).

Infections are thought to be acquired by the inhalation of conidia or by the progression of previously localized cutaneous lesions, producing a wide spectrum of diseases depending on the anatomic location. Mycoses range from superficial infections involving the outer layer of the *stratum corneum* of the skin to disseminated infections involving the brain, heart, lungs, liver, spleen and kidneys.

Disseminated fungal infections are caused by primary pathogenic and opportunistic fungal pathogens. Primary pathogenic fungi are able to establish infection in a normal host, whereas opportunistic fungi require a compromised host. Primary pathogens usually gain access to the host via the respiratory tract, while opportunistic fungi can also invade via other routes such as the alimentary tract or intravascular devices (Walsh & Dixon, 1996). The primary systemic fungal pathogens include the dimorphic species *Coccidioides immitis, Histoplasma capsulatum, Blastomyces dermatitidis*, *Paracoccidioides brasiliensis* and *Sporothrix spp*. and the most important opportunistic fungi causing disseminated infections include *Candida* spp.,

Cryptococcus spp., *Aspergillus* spp., Mucorales, *Scedosporium* spp. and *Fusarium* spp.

1.2.1 Antifungal strategies used to treat disseminated fungal infections

The development of effective, well-tolerated antifungals has lagged behind the advances of antibacterial therapy. Bacteria are prokaryotic and hence offer numerous structural and metabolic targets that differ from those of the human host (Calvo & Martínez-Martínez, 2009). Fungi, in contrast, are eukaryotes, and consequently most agents toxic to fungi are also toxic to the host. Currently, the antifungal drugs available for the treatment of systemic mycoses are mostly directed against components of plasmatic membranes (polyenes and azoles) and cell wall (echinocandins) (Figure 1).

1.2.1.1 Polyenes

Polyenes are natural amphipathic organic molecules called macrolides. They are the oldest class of antifungals and are generally produced by *Streptomyces nodosus* (Nett & Andes, 2016). Polyenes directly bind to the ergosterol of fungal cell membranes, which leads to the formation of pores in the membrane and results in the loss of ionic balance, membrane integrity and cell death (Sanglard *et al.*, 2009) (Figure 1).

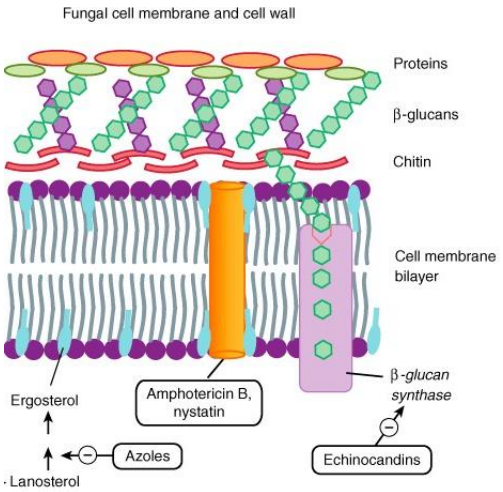


Figure 1. Cell targets of the different antifungal drugs.

The main polyenes are amphotericin B (Figure 2), natamycin and nystatin, the first of which is the only one that is effective against systemic invasive fungal infections. Amphotericin B deoxycholate was developed in the 1950s and marked a major therapeutic advance. It is one of the most potent antifungals, and is active against an array of yeast and filamentous fungal pathogens. It exhibits activity against *Cryptococcus* spp. and most *Candida* spp. (Diekema *et al.*, 2003; Pfaller *et al.*, 2005; Pfaller *et al.*, 2013) and also against *Aspergillus* spp., with the major exception of *Aspergillus terreus*, which is often resistant (Espinel-Ingroff *et al.*, 2011). In addition, amphotericin B formulations are active against dimorphic fungi, including *Histoplasma capsulatum, Blastomyces dermatitidis, Coccidioides* spp., *Paracoccidioides* spp. and *Sporothrix* spp. (Sabatelli *et al.*, 2006; Li *et al.*, 2000; Oliveira *et al.*, 2015; Borba-Santos *et al.*, 2014). Amphotericin B is active against many pathogenic organisms of the Mucorales group but *Scedosporium* spp. and *Fusarium* spp. are often resistant (Almyroudis *et al.*, 2007; Sabatelli *et al.*, 2006; Lackner *et al.*, 2012). In general, acquired resistance to amphotericin B is exceedingly uncommon even though it has been in clinical use for many decades.

Although polyenes are fungicidal in nature and have been used for a long time, they are of limited use because they have many side effects in humans. However, lipid formulations of amphotericin B are less toxic and are better for the treatment of fungal infections (Prasad *et al.*, 2016; Arikan 2002).

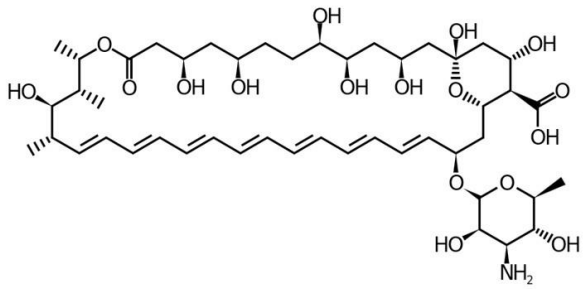


Figure 2. Structure of amphotericin B.

Amphotericin B is available as a deoxycholate formulation and as three lipid-based formulations: liposomal amphotericin B (L-AmB), amphotericin B lipid complex (ABLC) and amphotericin B colloidal dispersion (ABCD). Given the limited solubility of amphotericin B and its poor oral bioavailability, all formulations are parenteral. Although amphotericin B demonstrates potent antifungal activity, its use is often limited by significant common adverse effects such as renal toxicity, infusion reactions, electrolyte abnormalities, and hepatotoxicity (Bates *et al.*, 2001).

1.2.1.2 Azoles

In addition to the advent of lipid-based amphotericin B formulations, another major advance during the 1990s was the addition of azoles, which are tolerated significantly better than amphotericin B formulations. The antifungal azole drug class consists of imidazoles (clotrimazole, econazole, oxiconazole, ketoconazole, miconazole) and

triazoles (fluconazole, itraconazole, voriconazole, posaconazole, isavuconazole), the names of which reflect the number of nitrogen atoms in the azole ring. Imidazoles are mainly used for mucosal fungal infections while triazoles are administered for both systemic and mucosal infections (Prasad *et al.*, 2016).

These agents act primarily on ergosterol biosynthesis by targeting 14α-lanosterol demethylase encoded by the *ERG11* gene and inhibiting the cytochrome P450-dependent conversion of lanosterol to ergosterol (Figure 1). The resulting depletion of ergosterol interferes with the bulk functions of ergosterol as a membrane component but, more importantly, severe ergosterol depletion may also interfere with the "sparking" functions of ergosterol, affecting cell growth and proliferation (Sanglard *et al.*, 2009; Shapiro *et al.*, 2011). The blocking of 14α-demethylase results in the accumulation of toxic methylated sterols and membrane stress (Shapiro *et al.*, 2011).

First-generation triazoles (fluconazole and itraconazole) show excellent activity against *Candida* spp. The spectrum of itraconazole activity also includes filamentous and endemic fungi, such as histoplasmosis. However, they are less effective than amphotericin B for treating invasive filamentous fungal infections, such as aspergillosis and mucormycosis. Second-generation azole drugs (voriconazole, posaconazole and isavuconazole) (Figure 3) are broad-spectrum agents, which have additional activity against filamentous fungi while retaining anti-*Candida* activity. Voriconazole exhibits activity against *Scedosporium* spp. and *Fusarium* spp., and posaconazole is active against several Mucorales. The activity of the newest azole released in 2015 (isavuconazole) is similarly broad.

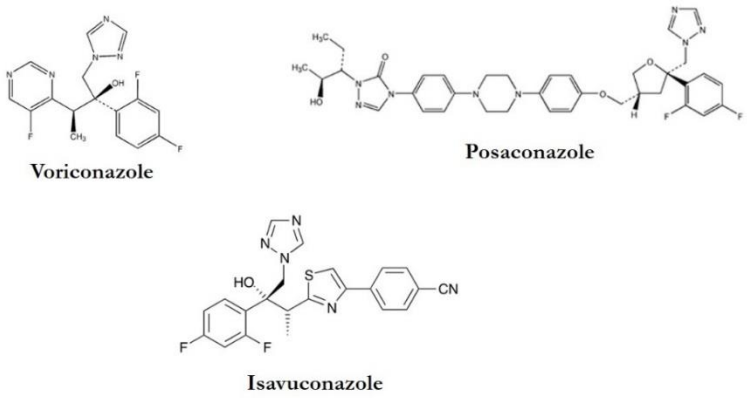


Figure 3. Structure of triazole compounds.

Voriconazole is formulated as an oral tablet, an oral suspension, and an intravenous solution (complexed with sulfobutylether β-cyclodextrin). The bioavailability for both oral formulations is quite high, greater than 90%. Absorption is not affected by gastric acidity and is optimal in the fasted state (Purkins *et al.*, 2003). Levels in the cerebrospinal fluid and ocular compartments are greater than 50% of the serum concentrations, so infections of the central nervous system (CNS) and eye (Lutsar *et al.*, 2003) can be treated with this drug. Voriconazole has been chosen as a first-line therapy for aspergillosis, fusariosis and scedosporiosis (Walsh *et al.*, 2008; Tortorano *et al.*, 2014). However, drug interactions, pharmacokinetic variability, short- and long-term toxicities, β-cyclodextrin administration in impaired renal function, and recommendations for therapeutic drug monitoring (TDM) have been problematic for patients (Jin *et al.*, 2016).

Posaconazole is currently available as an oral suspension, a delayed-release tablet and an intravenous solution that is complexed with sulfobutylether β-cyclodextrin. Absorption and bioavailability differ between the two oral formulations. For the oral solution, absorption highly depends on food intake and is best promoted by high-fat meals (Courtney

et al., 2004). Data from clinical investigations and animal studies show that posaconazole penetrates poorly into the cerebrospinal fluid and ocular compartments. For this reason, posaconazole is not recommended as the first option for treating endophthalmitis or infections of the CNS (Riddell IV *et al.*, 2011; Walsh *et al.*, 2008). However, posaconazole is chosen as alternative or salvage treatment of refractory aspergillosis, fusariosis, mucormycosis and scedosporiosis (Walsh *et al.*, 2008; Tortorano *et al.*, 2014; Cornely *et al.*, 2014).

Isavuconazonium, the water-soluble prodrug of isavuconazole, is available as an oral capsule and an intravenous solution. In contrast to the intravenous formulations for voriconazole and posaconazole, the isavuconazonium intravenous formulation does not contain the sulfobutylether β-cyclodextrin vehicle that may accumulate with renal insufficiency. The oral formulation is highly bioavailable and absorption is not significantly affected by food intake or gastric acidity (Pettit & Carver, 2015). The distribution of isavuconazole has not been extensively studied but drug levels in the cerebral spinal fluid and eye compartments are predicted to be low (Pettit & Carver, 2015). Dose reductions are not required for patients with renal insufficiency or dialysis. In a recent study, this drug proved to be non-inferior to voriconazole in the treatment of suspected invasive mould disease, with substantially fewer drug-related adverse events and discontinuations (Maertens *et al.*, 2016).

1.2.1.3 Echinocandins

Lipopeptide echinocandins, which include caspofungin, micafungin and anidulafungin (Figure 4), are the most recent class of antifungal drugs which target the synthesis of cell wall components by acting as non-competitive inhibitors of the β-1,3 glucan synthase required for β-glucan

synthesis (Figure 1) (Perlin, 2011; Shapiro *et al.*, 2011). Defects in the synthesis of cell wall components affect the integrity of fungal cells and result in cell wall stress. Consequently, echinocandin-treated cells become osmotically sensitive, form pseudohyphae, and have separation defects, reduced sterol contents and thicker cell walls. Echinocandins are generally non-toxic to mammalian cells because they act on specific cell wall synthesis pathway unique to fungal cells (Sanglard *et al.*, 2009; Perlin, 2011; Shapiro *et al.*, 2011).

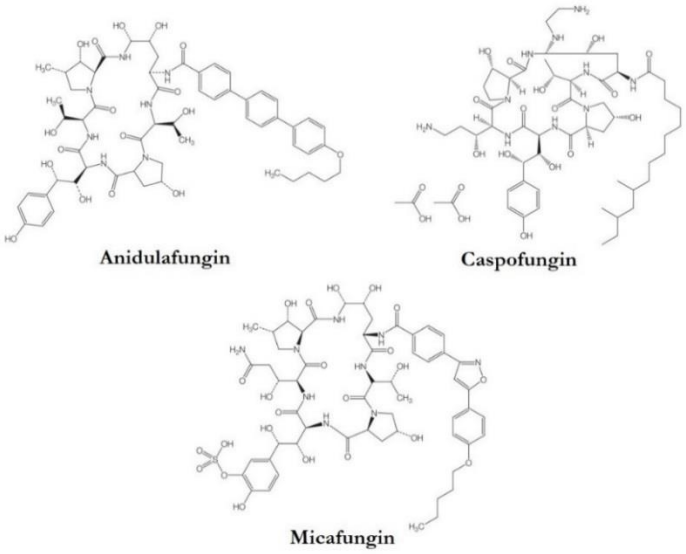


Figure 4. Structure of echinocandins.

Caspofungin, micafungin, and anidulafungin have very similar activities. They are highly active against many *Candida* spp., and somewhat active against many *Aspergillus* spp., although in this case the activity is fungistatic (Castanheira *et al.*, 2016). The spectrum of activity for echinocandins does not include *Cryptococcus* spp., species of the Mucorales order, *Fusarium* spp. or *Scedosporium* spp. (Almyroudis *et al.*, 2007; Pfaller *et al.* 2013). Some of the favorable attributes of echinocandin drugs are their

excellent side-effect profiles and few drug–drug interactions. However, only parenteral formulations are available for this drug class because it is poorly absorbed through the gastrointestinal system. Echinocandins have limited distribution to the CNS. Low concentrations are found in the cerebrospinal fluid and eye (Nett & Andes, 2016). Therefore, they are not ideal agents for infections involving these compartments. Echinocandins are generally well-tolerated and patients experience very few side effects (Nett & Andes, 2016).

Echinocandins can be effectively used for preventing invasive fungal infection, empirically treating fungal infection, and treating candidiasis (Pappas *et al.*, 2016; Nett & Andes, 2016), but they are recommended only as second-line treatment for aspergillosis (Walsh *et al.*, 2008). Because of their similar activities, caspofungin, micafungin and anidulafungin are generally used interchangeably.

1.2.1.4 Antifungal combination therapy

Antifungal combination therapy has emerged as one of the more effective strategies for clinicians. Achievement of synergy is one of the major theoretical justifications of combination therapy, since the desired therapeutic effect can be gained with lower quantities of each substance than when acting alone, thus reducing any toxic side effects, improving safety and tolerability, increasing the spectrum of antimicrobial activity, and preventing treatment failure when antimicrobial resistance is suspected.

On the basis of *in vitro* data, animal models, and clinical experience, recommendations on how to use combination antifungal therapy have been made in the clinical practice guidelines of the Infectious Disease Society of America (IDSA). For example, for HIV-associated cryptococcal meningitis, amphotericin B in combination with flucytosine or fluconazole is

commonly recommended (Perfect *et al.*, 2010). For initial therapy of invasive pulmonary aspergillosis, combination therapy is not endorsed as a first-line treatment option but may be considered in salvage situations (Walsh *et al.*, 2008). Caspofungin plus voriconazole is mentioned as an option for CNS aspergillosis, but few clinical data are available to support this strategy (Walsh *et al.*, 2008). Additionally, a randomized, controlled trial comparing voriconazole monotherapy and combination therapy with anidulafungin found that outcome tended to be better in the combination therapy group in a post hoc subgroup analysis. However, this difference did not meet statistical significance so it could not be said to be superior (Marr *et al.*, 2015). The data supporting the use of combination therapy for the treatment of aspergillosis is still, then, limited.

In the consensus guidelines for the management of rare mould infections such as fusariosis and scedosporiosis (Tortorano *et al.*, 2014), combined therapy is not recommended as first-line treatment. However, it is often considered because of the high antifungal resistance rates and, therefore, the substantial morbidity and mortality related to these infections. The use of combination antifungal therapy in these cases is mostly determined on a case-by-case basis, taking into account the *in vitro* data, animal models, and clinical experience available.

1.2.2 Opportunistic fungal infections

The opportunistic mycoses treated in this thesis are aspergillosis, infections caused by *Fusarium solani*, scedosporiosis and infections caused by *Lomentospora prolificans* and *Scopulariopsis* spp., since these are some of the most common opportunistic infections due to filamentous fungi and mortality rates are high.

1.2.2.1 Aspergillosis

Aspergillosis includes an extensive spectrum of diseases caused by fungi of the genus *Aspergillus*, mainly *Aspergillus fumigatus, A. flavus, A. niger* and *A. terreus*, with clinical manifestations that range from colonization to allergic bronchopulmonary aspergillosis, invasive infections involving the lungs, skin, and/or sinuses, or CNS, and disseminated infections (Segal, 2009). The prevalence of invasive aspergillosis (IA) has steadily increased in recent decades, mostly due to the advent of solid organ and hematopoietic stem cell transplantation (HSCT), and the increased use of chemotherapy and immunosuppression (Oliveira-Coelho *et al.*, 2015). Although diagnosing IA is now more effective, largely because of the introduction of biomarkers such as the detection of galactomannan in clinical practice (Morrissey *et al.*, 2013), successful treatment is still a challenge. Indeed, established infection is difficult to eradicate, and the associated mortality rates range from 40 to 90% (Neofytos *et al.*, 2009; Parody *et al.*, 2009; Ramos *et al.*, 2011). Failure to recover from neutropenia is often associated with a fatal outcome of invasive pulmonary aspergillosis (Walsh *et al.*, 2008).

A. fumigatus is the etiological agent in over 90% of cases of IA and it is thought to be the most common inhaled fungal pathogen (Espinosa & Rivera, 2016). The most severe disease caused by *A. fumigatus* is IA, in which fungal hyphae invade tissue and, in some cases, haematogenously spread to other organs (Latgé, 1999).

A randomized controlled trial demonstrated that voriconazole is better than deoxycholate amphotericin B as a primary treatment for IA. For this reason, it is recommended for the primary treatment of IA in most patients (Walsh *et al.*, 2008). In addition, a randomized trial comparing two

doses of liposomal amphotericin B showed similar efficacy in both arms, suggesting that liposomal therapy can be considered as an alternative primary therapy in some patients. In patients whose aspergillosis is refractory to voriconazole, there is very little data available to guide management. Therapeutic options include changing the antifungal class using an amphotericin B formulation or an echinocandin such as caspofungin; further use of azoles should take into account host factors and pharmacokinetic considerations (Walsh *et al.*, 2008). Recently, isavuconazole has been indicated for the treatment of aspergillosis, based on the results of a large randomized, controlled trial comparing isavuconazole and voriconazole for the treatment of IA and other mould infections (Maertens *et al.*, 2016). For all subjects and the subset with aspergillosis, both all-cause mortality and treatment success were similar and isavuconazole met non-inferiority criteria.

The TDM of azoles, particularly voriconazole, is required in the treatment of IA. It can be used to ensure suitable exposure to a given dose in a particular patient and it has been demonstrated that therapeutic failure is more common in patients without monitorization than in those who are monitored (Seyedmousavi *et al.*, 2013). Additionally, in a randomized trial investigating the use of TDM, more patients discontinued voriconazole due to adverse events in the non-monitorized group than in the TDM group (Park *et al.*, 2012). In the case of posaconazole, current TDM recommendations suggest that a trough concentration >0.7 μg/ml, 4–7 days after initiating prophylaxis, is an appropriate target and a trough concentration of 1.25 μg/ml seems to be needed in order to obtain a 75% of treatment response (Seyedmousavi *et al.*, 2013). Finally, on the basis of predictable and linear pharmacokinetics (Schmitt-Hoffmann *et al.*, 2006), there is no evidence to suggest that TDM is required for isavuconazole.

It is not clear what role combination therapy plays in the treatment of IA as a primary or salvage therapy. Combination therapy involving anidulafungin plus voriconazole, or caspofungin with other antifungal agents was effective and generally well tolerated as salvage therapy in patients with IA (Singh *et al.*, 2006; Maertens *et al.*, 2006; Raad *et al.*, 2015; Marr *et al.*, 2015; Barchiesi *et al.*, 2015). Combination therapy was associated with favourable outcomes in many patients with an expected poor prognosis. Although no firm conclusions about the use of combination therapy relative to monotherapy can be drawn from the efficacy results of these studies, the outcomes with combination therapy are probably no worse than those previously reported with monotherapy regimens.

1.2.2.2 Scedosporiosis and infections caused by *Lomentospora prolificans*

Scedosporium spp. are usually found in soil, contaminated water and urban environments. Of this genus, *S. apiospermum* is the most prevalent species. It causes diseases in humans and is commonly found in temperate climates but less frequently in tropical climates (Cortez *et al.*, 2008). *S. apiospermum* opportunistic infections are usually secondary to conidia inhalation, traumatisms or penetrating wounds and surgery. The fungus has also been shown to cause invasive disease, which above all affects the CNS, in immunocompetent patients after near drowning. Therefore, the respiratory tree, including nasal and paranasal sinuses, and the skin are the origin of most invasive fungal infections caused by *Scedosporium* spp., although the infection usually disseminates and affects such organs and locations as bones, articulations, eyes, brain and kidney. Disseminated infections caused by this fungus are mainly diagnosed in immunocompromised patients, mainly oncohaematologic, patients treated

with corticosteroids, HSCT recipients or solid organ transplant recipients (Pemán & Salavert, 2014), are very difficult to treat and are frequently fatal. Even in immunocompetent patients, the antifungal therapy needs to be supplemented with the surgical excision of affected tissues.

Lomentospora prolificans is a fungus that is phylogenetically close to the genus *Scedosporium,* and which causes similar affectations than *S. apiospermum*. However, *L. prolificans* has proved to be more virulent. Since it was discovered in 1974, this fungus has undergone several taxonomic changes. Until 2014 it was regarded as a species of the genus *Scedosporium*, but in this year, *S. prolificans* was found to be phylogenetically different from the other *Scedosporium* species so it was renamed *L. prolificans* (Lackner *et al.*, 2014).

While the geographical distribution around the world of *S. apiospermum* is more uniform, invasive fungal infections (IFIs) caused by *L. prolificans*, curiously, have been described in southern Europe (particularly Spain) (Nenoff *et al.*, 1996; Berenguer *et al.*, 1997; García-Ruiz *et al.*, 1998; del Palacio *et al.*, 2001; Idigoras *et al.*, 2001; Simarro *et al.*, 2001; Husain *et al.*, 2005; Marco de Lucas *et al.*, 2006; Rodriguez-Tudela *et al.*, 2009), California and the southern United States (Husain *et al.*, 2005), United Kingdom (Gosbell *et al.*, 2003), Australia (Howden *et al.*, 2003; Gosbell *et al.*, 2003; Li *et al.*, 2008; Tong *et al.*, 2007; Ananda-Rajah *et al.*, 2008; Kelly *et al.*, 2016), and more recently in Japan (Ohashi *et al.*, 2011; Nishimori *et al.*, 2014; Uno *et al.*, 2014; Ochi *et al.*, 2015). Some of the cases in Japan were related to the tsunami in 2011 (Hatakeyama *et al.*, 2012). However, the causes of this peculiar geographical distribution are unknown (Cortez *et al.*, 2008; Pemán & Salavert, 2014).

Both *S. apiospermum* and *L. prolificans* are highly resistant to antifungals, which makes treatment extremely challenging. In fact, these infections are usually associated with poor outcomes and mortality rates around 70-100% (Johnson *et al.*, 2014; Idigoras *et al.*, 2001; Berenguer *et al.*, 1997; Kelly *et al.*, 2016; Marco de Lucas *et al.*, 2006; Ochi *et al.*, 2015; Nishimori *et al.*, 2014). Monotherapy with echinocandins is not effective against infections by either species, while voriconazole and amphotericin B show variable activity against *S. apiospermum* and no efficacy against *L. prolificans*. Voriconazole is the treatment of choice for scedosporiosis and infections by *L. prolificans* (Tortorano *et al.*, 2014), while antifungal combinations are not recommended as first-line therapy but are used in most cases. *In vitro* and *in vivo* studies have shown that voriconazole plus terbinafine or micafungin, or amphotericin B plus micafungin have synergic activity and efficacy against *S. apiospermum* (Cortez *et al.*, 2008; Pemán & Salavert, 2014). In the case of *L. prolificans*, voriconazole alone or in combination with terbinafine showed efficacy in some cases (Gosbell *et al.*, 2003; Howden *et al.*, 2003; Studahl *et al.*, 2003; Bhat *et al.* ,2007; Tong *et al.*, 2007; Ochi *et al.*, 2015) but therapeutic success is alarmingly low.

1.2.2.3 Fusariosis

Fusarium solani is a hyaline mould widely distributed in nature and causes a broad spectrum of human infections. The clinical consequences depend on the immune status of the host and how the micro-organism enters the body (mainly by conidia inhalation and trauma) (Nucci & Anaissie, 2007). Onychomycosis and keratitis are the most frequent diseases caused by this fungus in the immunocompetent population, while in immunocompromised patients, especially those with acute laeukemia and HSCT recipients, invasive disease is the typical manifestation, often with

haematogenous dissemination (Nucci *et al.*, 2015). The most challenging and life-threatening manifestation is disseminated disease, which has an estimated mortality rate up to 75% (Stempel *et al.*, 2015). Risk factors for dissemination include prolonged and profound neutropenia, use of corticosteroids, haematologic malignancy, HSCT, burns, and T cell deficiency. Pre-existing *Fusarium* onychomycosis may be a risk factor and this syndrome should be strongly suspected in any neutropenic patient with fever and onychomycosis. The presentation of disseminated disease can mimic aspergillosis, but there is a greater propensity of skin lesions and positive blood cultures (Nucci & Anaissie, 2009). Fusariosis produces a disseminated maculopapular or nodular rash, commonly with necrosis and ulceration of the lesions (O'Connell *et al.*, 2011; Nucci & Anaissie, 2007). This rash is present in 60–90% of cases (Baer & Pappas, 2009) and about 60% of patients with disseminated fusariosis have positive blood cultures (Baer & Pappas, 2009; Nucci & Anaissie, 2009).

Treatment of fusariosis includes the administration of voriconazole, as first-line therapy, and liposomal amphotericin B as an alternative, while posaconazole is recommended only as salvage therapy (Tortorano *et al.*, 2014).

Recently, Nucci *et al.* confirmed that the outcome of invasive fusariosis has improved in the last decade. This improvement has been possible due to important changes in therapeutic practices: a reduction in the use of deoxycholate amphotericin B and an increase in the use of voriconazole and combination therapy (Nucci *et al.*, 2014).

1.2.2.4 Infections caused by *Scopulariopsis* spp.

The genus *Scopulariopsis* contains both hyaline and dematiaceous moulds, and their teleomorphs belong to the genus *Microascus*. These fungi

are usually saprobic and are commonly isolated from the soil, air, plant debris and moist indoor environments (de Hoog *et al.*, 2001; Sandoval-Denis *et al.*, 2013). In immunocompetent patients, *Scopulariopsis* is associated mainly with nail infections, but it occasionally causes cutaneous lesions in both immunocompetent and immunosuppressed individuals mostly following trauma or surgery and invasive diseases such as endocarditis (Migrino *et al.*, 1995; Jain *et al.*, 2011), sinusitis (Jabor *et al.* 1998), brain abscess (Baddley *et al.*, 2000; Patel *et al.*, 1994), deep cutaneous infections (Sellier *et al.*, 2000; Wu *et al.*, 2009), localized pulmonary infections (Petit *et al.*, 2011; Ustun *et al.*, 2006), and disseminated infections (Steinbach *et al.*, 2004; Salmon *et al.*, 2010; Miossec *et al.*, 2011; Neglia *et al.*, 1987; Beltrame *et al.*, 2009; Szental *et al.*, 2010; Swick *et al.*, 2010; Krisher *et al.*, 1995). The latter are almost invariably fatal, mainly due to the underlying conditions, delayed diagnosis, and of the fact that this fungus is highly resistant to conventional antifungal agents. Although *S. brevicaulis* is the most prevalent species, other species of the genus such as *S. brumptii, S. acremonium, S. flava, Microascus niger, M. cinereus, M. cirrosus, M. manginii* and *M. trigonosporus* have also been associated with human disease (Sandoval-Denis *et al.*, 2013).

There is no recommendation for the treatment of infections by *Scopulariopsis*. The scarcity of infections caused by these fungi explains the lack of consensus on the best antifungal regimen. European guidelines recommend any antifungal in combination with surgery (Tortorano *et al.*, 2014) and in most cases antifungal combinations are used although outcomes are often fatal (Miossec *et al.*, 2011).

1.3 Determination of *in vitro* susceptibility

To combat the problem of fungal infections and the limited availability of therapeutic options, it is important to develop an *in vitro*

system that can predict the success or failure treatment. However, the immense variety of opportunistic species involved in human infections, the difficulty in standardizing these metholodologies and the difficulty to translate the results to the clinical setting are important problems that have yet to be solved.

1.3.1 Single susceptibility

In vitro antifungal susceptibility testing has not developed as much as antibacterial testing but its design and performance are similar and its aims are the same. The main objective of *in vitro* susceptibility testing is to detect the resistant isolates in a susceptible population or the development of resistance during treatment. For this purpose, reproducible susceptibility methods and clinical breakpoints (CBPs) are needed so that results can be interpreted. The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antifungal Susceptibility Testing (EUCAST) have developed reproducible methods for determining the drug susceptibility of yeasts and filamentous fungi. The CLSI has standardized two methods for determining antifungal susceptibility: broth microdilution for yeasts (document M27-A3) and filamentous fungi and dermatophytes (document M38-A2); and the agar-diffusion method for yeasts (documents M44-A2 and M44-S2) and filamentous fungi (documents M51-A and M51-S1). EUCAST has also standardized a microdilution method for yeasts (document EDef 7.2) and for filamentous fungi (EDef 9.1). The CLSI and EUCAST methodologies differ in some points: the glucose concentration in the culture medium, the shape of the well bottom, the size of the inoculum, the length of incubation and the interpretation of results. Despite these differences, the results have proved to be comparable and are used worldwide (Alastruey-Izquierdo *et al.*, 2015).

Regardless of their advantages, the standardized broth microdilution methods used for antifungal susceptibility testing are time-consuming and cumbersome for clinical laboratories. Some commercially available methods, including manual, semi-automated and automated, do not require complex handling and are cost-effective, alternative methods for testing antifungal agents *in vitro*. The most used are: Sensititre® YeastOne®, Etest®, Vitek2® and Neo-Sensitabs™. In this thesis, disk diffusion and Etest® methodologies were used in addition to the reference microdilution method. For this reason, both techniques will be briefly discussed.

In 2010, CLSI published a reference method (M51-A) for the disk diffusion antifungal susceptibility testing of non-dermatophyte filamentous fungi (Clinical and Laboratory Standards Institute, 2010b). The method is a simple, rapid and cost-effective alternative approach for testing the susceptibility of opportunistic moulds to triazoles, amphotericin B and caspofungin. The disk diffusion assay does not generate a minimal inhibitory concentration (MIC) of the drug, only a zone of inhibition. However, the document M51-S1 sets the ECVs so that the results can be interpreted (Clinical and Laboratory Standards Institute, 2010b).

Etest® consists of a predefined gradient of antifungal drug concentrations on a plastic strip that is used to determine the MIC. When the strip is applied to an inoculated agar surface, the antifungal agent is immediately transferred to the agar matrix and, after an incubation time, an inhibition ellipse centered along the strip is formed. The MIC is read directly from the scale at the point where the edge of the ellipse intersects the strip. However, it is important to bear in mind that, like all tests evaluating antimicrobial susceptibility, the formulation of the medium and, in this case, the depth of the agar can strongly influence MIC results.

MICs are regarded as the "gold standard" for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MICs are defined as the lowest concentration that inhibits the growth of microorganisms (Clinical and Laboratory Standards Institute, 2008). However, when testing echinocandins, the minimum effective concentration (MEC) has been found to provide more consistent and reproducible susceptibility data than the conventional MIC reading. MEC corresponds to the lowest concentration of drug that leads to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the growth control well (Clinical and Laboratory Standards Institute, 2008).

Even though the main goal of antifungal susceptibility testing is to select the best treatment for a given isolate, these methods are also very important for detecting resistant strains. The two main factors are: the development of secondary resistance and the selection of species that are intrinsically resistant. CBPs have been developed for some fungal species and antifungals in both CLSI and EUCAST methods. These CBPs categorize fungal isolates as susceptible, resistant and intermediate. They are determined by correlating the *in vitro* susceptibility testing with therapeutic efficacy, MIC distribution, pharmacokinetic and pharmacodynamic parameters and antifungal resistance mechanisms. However, due to the lack of *in vivo* data, we do not have CBPs for all pathogenic species and all the antifungal drugs (Pfaller *et al*., 2013). In the absence of CBPs, epidemiological cutoff values (ECVs) have been determined and correspond to the highest concentration that divide the wild-type population from those isolates with some mechanism of resistance (non-wild type). Those isolates inhibited by a concentration

higher than the ECV are classified as isolates with reduced susceptibility but they can only be regarded as resistant to a drug when the CBP has been determined. Although ECVs do not predict therapy outcome as clinical breakpoints do, they may help to detect antifungal resistance.

1.3.2 Combinations

The study of interactive effects between molecules has a long history. For antimicrobial drugs, the use of paired and triple combinations of inhibitory agents in the clinic often begins with tests *in vitro* that show positive interactions inhibiting the growth of target microorganisms. *In vitro* systems that use the fungal strain that caused the infection are needed to help to predict drug interaction in clinical practice. Furthermore, although the *in vitro* data are obtained from simplified systems, they could be combined with *in vivo* data to design proper, feasible and less costly clinical trials, particularly when there is previous clinical experience of antifungal monotherapies, by optimizing, for instance, the dosing of regimens in combination (Hollander *et al.*, 1998; Meletiadis *et al.*, 2002).

Such methodologies as disk-diffusion, Etest® and time-kill studies have been used for testing the *in vitro* interactions of antifungal drugs (Lewis *et al.*, 2002; Svetaz *et al.*, 2016). However, the most common approach is the checkerboard microdilution method, and other more sophisticated designs that give more reliable information have also been developed. Most of these define an index that is useful for interpreting the results and defining the type of interaction (Meletiadis *et al.*, 2003; Meletiadis *et al.*, 2005; Boik *et al.*, 2008; Te Dorsthorst *et al.*, 2002).

The checkerboard assay is performed in 96-well microplates in which each row and each column contains two-fold serial dilutions of drugs

A and B, respectively, at concentrations ranging from 0 μg/ml to slightly higher than their MIC. Each well has a unique combination of the two substances. Then, a quantified inoculum of the fungus is added to each well and the microplate is incubated at a set temperature for a suitable time for each fungus.

The microdilution checkerboard technique has been used to analyze various *in vitro* interactions of two or more drugs against various fungal species. The standard approach in the field of medical microbiology is to calculate the fractional inhibitory concentration index (FICI). It is concluded that there is synergy or antagonism when the concentrations of the drugs in a combination with the same effect as the MIC are lower or higher, respectively, than the MIC of the single-acting drugs. Despite the simplicity of this model, describing correctly, reliably and precisely the multi-variate phenomenon of drug interaction does present several problems. These include the choice of the MIC endpoints, the sensitivity to intra-experimental errors, the imprecise approximation of the real FICI when off-scale MICs are present and the difficulty of statistically interpreting the results (Svetaz *et al.*, 2016). Additionally, MICs describe a static situation in which the concentration of a drug remains constant over time. *In vivo*, drug concentrations fluctuate over time and these fluctuations depend on the site of infection. So, the fungus can be exposed to different concentrations at each time point and the antifungal activity of the drugs *in vivo* changes. Pharmacokinetic data, then, should be taken into account when assessing *in vivo* antifungal activity. This can be done with *in vitro* pharmacodynamic models that simulate human pharmacokinetics by taking into account the between-patient variability in pharmacokinetics for the drugs (Meletiadis *et al.*, 2002; Meletiadis *et al.*, 2006; Elefanti *et al.*, 2013; Siopi *et al.*, 2015).

According to Odds, a FICI ≤ 0.5 indicates synergism; a FICI > 4.0 indicates antagonism and a FICI in the range of > 0.5 – 4.0 indicates no interaction or indifference (Odds, 2003). However, other authors consider other limits for FICI (Berenbaum, 1978; Martinez-Irujo *et al.*, 1996; Meletiadis *et al.*, 2010) in basis of the correlation between the *in vitro* results and the *in vivo* outcome in animal models of fungal diseases.

Although antifungal drugs may interact differently under different conditions in *in vitro* systems, even when the same *in vitro* methodology is used results can depend on the way that the nature and the intensity of drug interactions are assessed (Meletiadis *et al.*, 2003). For this reason, further investigation is required to find the best model for the interaction of antifungal drugs.

1.4 Animal models as a tool for studying drug efficacy

In the first phases of the development of new antifungals, *in vitro* studies are useful for determining the extent to which they inhibit the growth of fungal pathogens. These studies will determine the therapeutic potential of the new drug. However, before clinical studies are performed in humans, it is essential that pre-clinical studies be performed in animal models.

Over the past four to five decades, researchers have developed a plethora of animal models for investigating fungal pathogenesis, host immune responses, and the antifungal properties of chemical and biological compounds. Although laboratory mice are most commonly used to model clinical syndromes associated with pathogenic fungi, other vertebrate hosts like rats, guinea pigs, rabbits, and zebrafish have gained popularity, since each model has both distinct advantages (e.g. repeated body fluid sampling and drug administration in rabbits, availability of genetically defined strains

in mice, non-invasive imaging of the infection process in transparent zebrafish larvae) and limitations (Capilla *et al.*, 2007; Hohl, 2014).

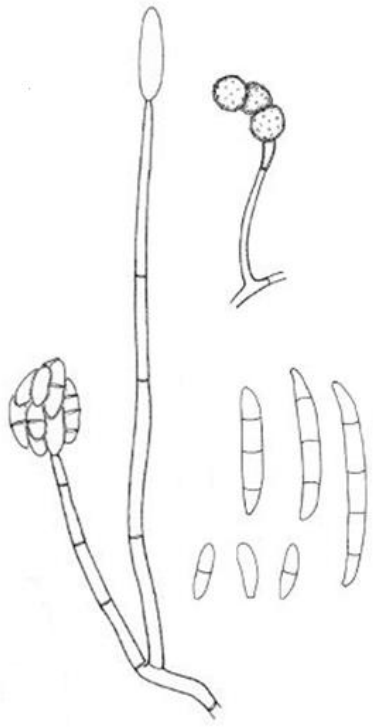
Another important issue is that the fungal infection has to mimic, as closely as possible, what occurs in humans. For this reason, the route of infection is a critical variable in vertebrate animal models of fungal disease. In most instances, the inoculum should be administered via the physiological route of infection. This allows us to understand the mechanisms involving the infectious process, the host's resistance to the pathogenic process and, potentially, the cure for the infection. So, there are a wide range of strategies for generating the different infections. For example, *Cryptococcus neoformans* infection is thought to be acquired by inhalation, and germination is followed by pulmonary infection, haematogenous dissemination and meningitis. Murine models have been developed using pulmonary, intravenous and intracranial routes of infection (Graybill, 2000). There are also disseminated, by direct intravenous inoculation, and pulmonary models of aspergillosis, when infection is either by inhalation of conidia blown up from a culture plate, by intranasal infection with a known inoculum, or by direct placement of the inoculum in the trachea.

In addition to the route of infection, animal models make it possible to model the immune deficiencies seen in patients with serious diseases. These may markedly alter the outcome of infection. In fact, some models cannot be used if the animals are not immunosuppressed.

Animal models give the first indications as to the optimal route of treatment and some idea about dose ranging, clearance, and maximum tolerated doses. Pre-clinical studies also show the effect of combination therapies. Combinations of drugs can be used to enhance antifungal activity

and to test for toxicity or increased/decreased clearance of antifungal agents. A number of interactions which have seemed to be quite promising for augmenting antifungal activity *in vitro* have been less evident *in vivo*. Therefore, parameters that can influence the outcome of combination studies include inoculum size, route of infection, immune status of the host, whether the therapy is combination or serial, and the route of drug administration.

It will be apparent from the above that while animal testing is crucial to the development of antifungal drugs, there are some important caveats. First, pharmacokinetics differs greatly among species, and the results of dosing in murine studies cannot be extrapolated directly to clinical applications. Drugs are cleared at different rates and perhaps by different mechanisms. Potential toxicities can only be screened in crude measures in small animals, such as mice. Multiple species need to be examined for toxicity, and only the longer term studies, often involving more than six months of therapy, can show such toxicities as carcinogenicity. The toxic effects seen in animals may or may not predict similar effects in patients, but they should be taken seriously and bring some note of caution to clinical trials. It is critical that both efficacy and tolerance be determined in animal models before clinical trials are launched. Additionally, differences in the biochemical pathways of laboratory mice and humans must also be considered when experimental murine data are extrapolated to human treatment strategies (Graybill, 2000; Capilla *et al.*, 2007; Hohl, 2014).



2. INTEREST AND OBJECTIVES

2. Interest and objectives

The incidence of haematogenous disseminated opportunistic mycoses has been increasing in recent years because of the raising number of immunocompromised patients. These emerging infections are a worldwide health problem because, despite the development of new antifungal agents and their formulations, the morbidity and mortality of invasive fungal diseases are still high. Hence, appropriate diagnosis and early aggressive antifungal therapy are necessary if patients are to have the best chance of a successful outcome. Therefore, the main objective of this thesis has been to develop new experimental treatments for invasive fungal infections caused by emerging opportunistic fungi.

Aspergillus fumigatus is the most prevalent filamentous fungus to cause infecion and is usually susceptible to the antifungal drugs available. However, the prolonged use of azoles to treat fungal infections, together with their fungistatic activity, has promoted the selection and emergence of drug resistant fungal strains of that species. Therefore, novel antifungal drugs need to be developed or therapeutic strategies improved if drug resistance problems are to be solved. In the clinical setting, the use of combination therapy has become a potential alternative for treating invasive fungal infections by improving clinical efficacy of existing drugs such as azoles and reducing their side effects to the host by lowering the given doses. For this reason, one of the objectives of this thesis was to find new therapeutic alternatives for treating refractory aspergillosis. The specific objectives were:

- ✓ To test the *in vitro* activity of two antifungal combinations (namely, posaconazole plus amphotericin B and posaconazole plus anidulafungin) against *A. fumigatus*.

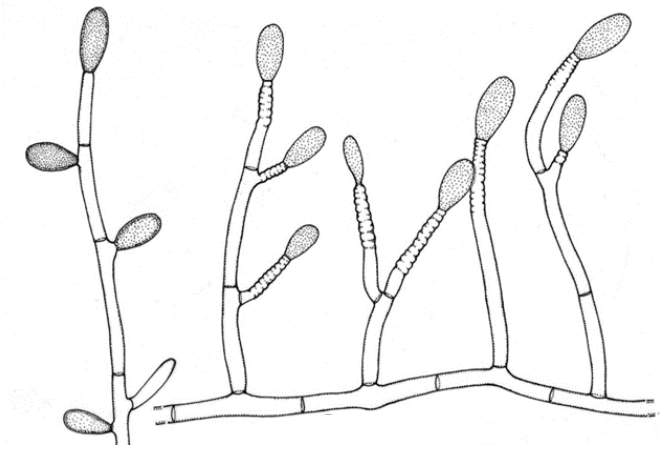
- ✓ To evaluate the *in vivo* efficacy of the aforementioned combinations in a murine model of disseminated aspergillosis.

Scedosporium apiospermum is mainly characterized by its neurotropism. The central nervous system (CNS) infections are the most difficult to treat and mortality is almost 100%. As the importance of determining antifungal susceptibility in cases of *S. apiospermum* infection is largely unknown, another objective of this study was to determine the possible correlation between *in vitro* susceptibility and *in vivo* efficacy of voriconazole against *S. apiospermum* strains with different susceptibilities to this drug. Additionally, since the use of microdilution methods seems to be restricted to reference laboratories because they are laborious and time-consuming, simpler, quicker and cost-effective methods should be tested. Our specific objectives were:

- ✓ To compare the *in vitro* susceptibility of 31 *S. apiospermum* strains to voriconazole by three methodologies (namely, CLSI microdilution, disk diffusion, and Etest®).
- ✓ To evaluate the efficacy of voriconazole against these strains in a murine model of disseminated scedosporiosis.
- ✓ To evaluate the correlation between the mentioned *in vitro* methods and the *in vivo* outcome.
- ✓ To propose "tentative cutoffs" of voriconazole in experimental scedosporiosis.

Combinatory therapies are now attracting considerable interest because of the high number of patients who are poorly responsive to antifungal single-drug therapy. Combination therapy can theoretically enhance the antifungal activity of individual drugs by expanding their spectrum of action, minimising resistance, and reducing the duration of

treatment. However, it has been reported that the therapeutic outcome is very poor even if two drugs are used in combination. Therefore, another objective was to test the *in vitro* activity of amphotericin B, voriconazole and anidulafungin administered separately or in double or triple combinations against four species of multiresistant fungi (namely, *Fusarium solani, Lomentospora prolificans, Scopulariopsis brevicaulis* and *Scopulariopsis brumptii*).



3. MATERIALS AND METHODS

3. Materials and methods

3.1 Conservation of the strains

During the development of the present thesis, a total of 73 strains were studied i.e. 3 *Aspergillus fumigatus,* 32 *Scedosporium apiospermum*, 8 *Fusarium solani*, 11 *Lomentospora prolificans*, 11 *Scopulariopsis brevicaulis* and 8 *Scopulariopsis brumptii*. All of the strains were conserved as submerged cultures in liquid paraffin, sterile water and also lyophilized. Working strains were subcultured into potato dextrose agar plates (PDA; Pronadisa, Madrid, Spain) until evident growth or sporulation.

3.2 *In vitro* studies

3.2.1 Strains and inocula preparation

To evaluate the *in vitro* susceptibility to different antifungal drugs, the 73 strains belonging to six opportunistic fungal species of clinical interest were assayed (Table 1).

Table 1. Isolates included in the *in vitro* studies.

Species	Origin	Number of isolates
A. fumigatus	FMR	3
S. apiospermum	FMR	8
	HMM	22
	UANL	2
F. solani	FMR	8
L. prolificans	FMR	11
S. brevicaulis	FMR	11
S. brumptii	FMR	8

FMR, Facultat de Medicina de Reus, Universitat Rovira i Virgili, Reus, Spain
HMM, Section for Hygiene and Medical Microbiology, Innsbruck, Austria
UANL, Universidad Autónoma de Nuevo León, Monterrey, México

For the preparation of the inocula, the strains were grown on PDA or oatmeal agar (OA) and incubated at 30 – 35 ºC, during 7 – 10 days, depending on the species studied and until sporulation occurred. Then, the surface of the agar plates was flooded with 5 ml of saline solution, and with the aid of a Pasteur pipette, the sporulating mycelium was scraped. The obtained suspension was filtered through sterile gauze in order to remove clumps of medium and hyphal fragments. The resulting suspensions were mixed with a vortex and the conidia were adjusted by haemocytometer count to the desired concentration. Viability of the inocula was assessed by placing serial 10-fold dilutions onto PDA plates and incubated at 30 – 35 ºC for 48 – 96 h.

3.2.2 Antifungal agents

Four antifungal drugs i.e. amphotericin B, posaconazole, voriconazole and anidulafungin (Table 2) were used in order to determine the MIC or MEC against the strains included in the *in vitro* studies (Table 1).

Table 2. Antifungal drugs for *in vitro* susceptibility studies.

Drug	Purity	Conservation	Dissolvent	Manufacturer
Amphotericin B (AMB)	99.8%	4 ºC	DMSO	Sigma Chemical Co. St.Louis, USA
Posaconazole (PSC)	100%	RT	DMSO	Schering-Plough Res. Inst., Kenilworth, USA
Voriconazole (VRC)	100%	RT	DMSO	Pfizer Inc., Madrid, Spain
Anidulafungin (AFG)	100 %	-20 ºC	DMSO	Pfizer Inc., Madrid, Spain

RT, room temperature; DMSO, dimethyl sulfoxide

3.2.3 Antifungal susceptibility testing

MICs of amphotericin B, posaconazole, voriconazole and anidulafungin against the strains included in the Table 1 were determined by microdilution method. Additionally, in the study including *S. apiospermum*, Etest® and disk-diffusion methodologies were also employed.

3.2.3.1 Broth microdilution

3.2.3.1.1 Single susceptibility testing

Antifungal susceptibility testing was performed by a broth microdilution technique according to the CLSI document M38-A2 (Clinical and Laboratory Standards Institute 2008). To prepare the antifungal stock solutions, the drugs were weighed and dissolved in dimethyl sulfoxide (DMSO) to a concentration 100 times higher than the highest desired assayed concentration.

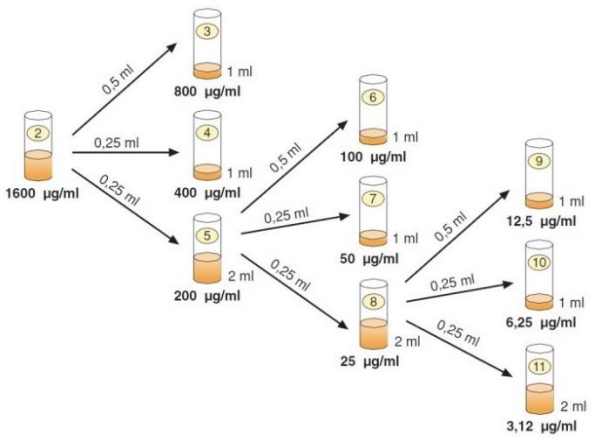


Figure 5. Scheme of antifungal drug dilutions in DMSO.

Then, a series of 2-fold dilutions were performed in DMSO following the scheme of the Figure 5, and then were diluted 1:50 in RPMI-1640 medium, reducing the final solvent concentration to 1% DMSO at each drug concentration.

One hundred microliters of each drug concentration assayed were dispensed onto rows of 96-multiwell microdilution plates with rounded bottom. Drug-free wells containing RPMI + 1% DMSO in the medium served as the growth control (positive control) and control of sterility of medium (negative control). Last row of each plate was reserved in aim to include a quality control strain (QC). Concentration ranges used were 0.03 to 16 μg/ml for all drugs tested.

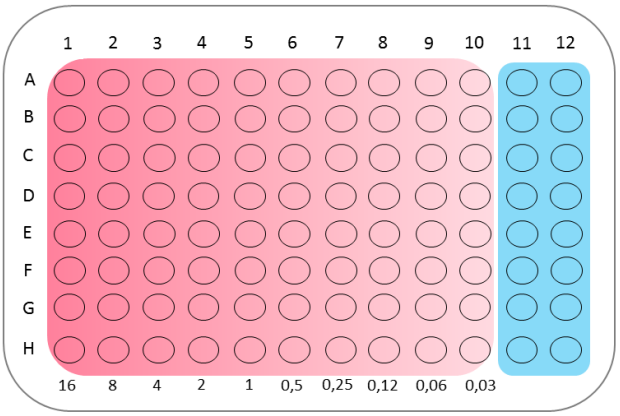


Figure 6. Schematic distribution of the different antifungal concentrations in a range of 0.03 to 16 μg/ml. Columns 11 and 12 are used as negative (-) and positive (+) controls, respectively.

Additionally, due to the high MICs of amphotericin B, voriconazole and anidulafungin against *F. solani, L. prolificans, S. brevicaulis* and *S. brumptii*, higher concentrations (until 256 μg/ml) were tested. Once the microplates were prepared, they were stored at -20 ºC until use and for a maximum of 6 months in order to ensure the potency of the drugs.

3.2.3.1.2 Antifungal combinations assays

In addition to the single susceptibility, due to the limited *in vitro* activity or *in vivo* efficacy of the drugs alone, the double combinations amphotericin plus posaconazole and anidulafungin plus posaconazole were tested against *A. fumigatus,* and the combinations amphotericin B plus voriconazole, amphotericin B plus anidulafungin, voriconazole plus anidulafungin and the triple combination of amphotericin B plus voriconazole plus anidulafungin were tested against the multiresistant species *F. solani, L. prolificans, S. brevicaulis* and *S. brumptii*.

Drug combinations were tested by using a broth microdilution checkerboard procedure. For the preparation of the stock solutions, the antifungals were dissolved in DMSO at four times the final concentration by following the dilution scheme of CLSI (Clinical and Laboratory Standards Institute, 2008). For the double combinations, a two-dimensional checkerboard with two-fold dilutions of each drug was used. In those studies regarding *A. fumigatus*, the final concentrations of AMB, PSC and AFG ranged from 0.12 to 8 μg/ml, from 0.002 to 1 μg/ml and from 0.002 to 0.125 μg/ml, respectively. In the case of the multiresistant species i.e. *F. solani, L. prolificans, S. brevicaulis* and *S. brumptii*, the concentrations of amphotericin B, voriconazole and anidulafungin ranged from 1 to 64 μg/ml (0.06 to 4 μg/ml against *F. solani*), 0.5 to 256 μg/ and 2 to 128 μg/ml, respectively.

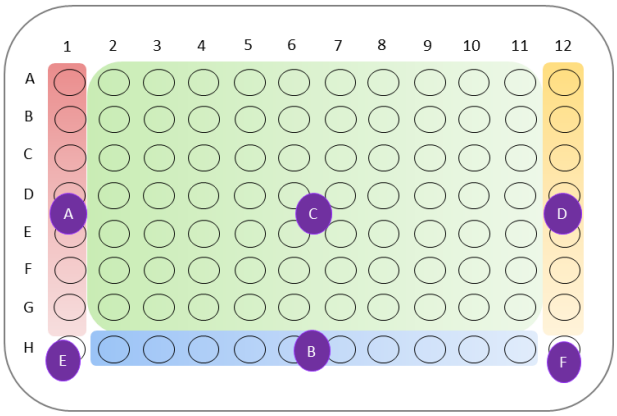


Figure 7. Representation of the distribution of antifungal compounds in a 96-well microplate testing a double combination. Six zones are defined: A and B, zones to determine the minimal inhibitory concentration (MIC) of each antifungal alone; C, zone of antifungal combination; D, defined as the zone of positive control without antifungal; E and F, defined as the zone of negative controls.

In the column 1 (A - G), 100 µl of a single drug were dispensed in order to determine the MIC of this drug alone. In the same way, in the row H (2 - 11) of each microplate, 100 µl of the second drug were dispensed in order to determine its MIC. The wells of the column 12 (A12 – G12) were used as positive controls and the wells H1 and H12 served as negative controls (Figure 7).

The triple combination was tested by a three-dimensional checkerboard technique in the following way: A checkerboard with two-fold dilutions of amphotericin B and voriconazole was set up as described above for the double combinations and then anidulafungin was added, at a constant concentration, in each plate. The concentrations of anidulafungin were 0.06, 0.25, 1 and 4 µg/ml.

3.2.3.2 Etest®

In order to compare different *in vitro* methodologies, MICs of voriconazole against *S. apiospermum* were determined by Etest®. The technique was performed following the manufacturer's instructions. Briefly, RPMI-1640 agar plates were inoculated by dipping a sterile cotton swab into the conidial suspension, adjusted to 1 – 5x10^6 CFU/ml, and streaking it across the surface of the agar in three directions. The agar surface was allowed to dry at ambient temperature for 15 minutes. After this time, the Etest® strips were placed onto the inoculated agar and the plates were incubated at 35 ºC during 72 h.

3.2.3.3 Disk diffusion assay

In parallel to the other susceptibility techniques, in the study including *S. apiospermum,* the susceptibility of the 32 strains was also determined by disk-diffusion. For this purpose, conidial suspensions of each *S. apiospermum* strain were prepared as described above and were adjusted by hemocytometer to a concentration of 1 – 5 x 10^6 CFU/ml. Then, non-supplemented Mueller Hinton agar plates were inoculated in three directions with the aid of a cotton swab dipped in the conidial suspensions. After the plates were allowed to dry, a 6 mm paper disk containing 1 μg of voriconazole was dispensed in the center of each plate and the plates were incubated at 35 ºC during 72 h.

3.2.3.4 Minimal fungicidal concentration

The minimal fungicidal concentration (MFC) was determined by subculturing 20 μl from each well that showed complete inhibition or an optically clear well relative to the growth control onto PDA plates. The

plates were incubated at 35 °C until growth was observed in the control subculture.

3.2.3.5 Reading and interpretation of the results

MICs of amphotericin B, posaconazole and voriconazole against *A. fumigatus, F. solani, L. prolificans, S. brevicaulis* and S. *brumptii* were determined at 48 h, while MIC of voriconazole against *S. apiospermum* was determined at 72h.

MEC of anidulafungin against *A. fumigatus* was determined at 24 h while for the multiresistant species, it was determined at 48 h, as recommended by the document M38-A2 of CLSI (Clinical and Laboratory Standards Institute, 2008).

3.2.3.5.1 Microdilution method

Single susceptibility

MICs of amphotericin B, posaconazole and voriconazole were visually read with the aid of an inverted mirror and corresponded to the 100% of growth inhibition. In the case of anidulafungin, MEC was determined with the aid of an stereomicroscope and corresponded to the minimum concentration producing an abnormal hyphal growth (small, rounded and compact hyphal forms) in comparison to the control hyphal growth.

Antifungal combinations

For the combination amphotericin B plus voriconazole, 100% of growth inhibition or MIC-0 was chosen as endpoint. However, the most appropriate endpoint for echinocandins against moulds appears to be the MEC, which corresponds to MIC-2 (50% growth inhibition) (Arikan *et al.*,

2002; Pastor & Guarro, 2005). For this reason, considering that the endpoint for each of the combined drugs should be the same, in those combinations containing anidulafungin i.e. amphotericin B plus anidulafungin, voriconazole plus anidulafungin and amphotericin B plus voriconazole plus anidulafungin, the MIC-2 was used.

Fractional inhibitory concentration indexes (FICIs) were calculated in order to evaluate the interaction of the different *in vitro* combinations. The FICIs of the triple combination were calculated as follows:

$$\text{FICI} = \frac{\text{MIC (A) in combination}}{\text{MIC (A)}} + \frac{\text{MIC (B) in combination}}{\text{MIC (B)}} + \frac{\text{MIC (C) in combination}}{\text{MIC (C)}}$$

For the double combinations, the third term of the equation was omitted. Drug interactions were defined as synergistic if the lowest FICI was ≤ 0.5, indifferent (i.e., no interaction) if the lowest FICI was > 0.5 and ≤ 4, and antagonistic if the highest FICI was > 4 (Odds, 2003). For the calculations, the high off-scale MICs were converted to the next highest concentration.

3.2.3.5.2 Etest®

Etest® MIC of voriconazole was determined after 72 h at 35 ºC and was defined as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip (Figure 8).

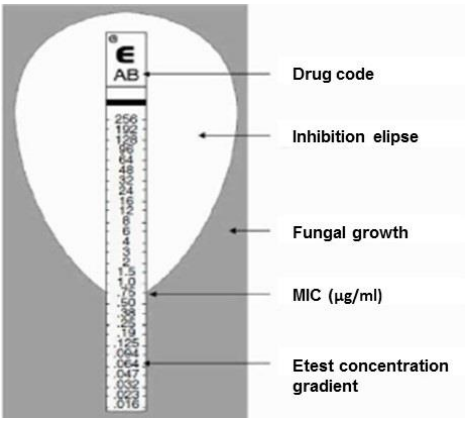


Figure 8. The Etest strip containing a voriconazole gradient is placed onto the inoculated agar plate. After incubation, the MIC corresponds to the place where the edge of the inhibition ellipse intersects with the side of the Etest.

3.2.3.5.3 Disk diffusion

After 72 h at 35 ºC, zone diameters were measured to the nearest whole millimeter at the point where there was a prominent reduction of growth (80%). Strains were categorized as susceptible (IZD ≥ 17 mm), intermediate (IZD from 14 to 16 mm) and resistant (IZD ≤ 13 mm). These tentative breakpoints were used to determine the categorical agreement between the disk diffusion and microdilution MIC endpoints (Espinel-Ingroff *et al.*, 2007).

3.3 *In vivo* studies

3.3.1 Strains

The strains used for the *in vivo* studies are listed in Table 3.

Table 3. Strains used in the *in vivo* studies.

Species	Strain	Origin
A. fumigatus	FMR 7739	Environmental (operating room), Spain
	FMR 10528	Clinical, Spain
	FMR 13142	Clinical (blood), Spain
S. apiospermum	FMR 6922	Environmental (garden soil), Spain
	FMR 8763	Clinical, Spain
	FMR 8869	Clinical (sphenoidal sinus), USA
	FMR 13011	Clinical, Austria
	FMR 13015	Clinical, Austria
	UANL-OC149	Environmental (urban garden), Mexico
	HMM 10-31	Clinical, Germany
	HMM 10-34	Clinical, Austria
	HMM 11-33	Clinical, Austria
	HMM 11-47	Clinical, Austria
	HMM 11-86	Clinical, Netherlands
	HMM 11-96	Clinical, Netherlands
	HMM 12-03	Clinical, Netherlands
	HMM 12-09	Clinical, Netherlands
	HMM 12-14	Clinical, Netherlands
	HMM 12-36	Clinical, Netherlands

FMR, Facultat de Medicina de Reus, Spain
HMM, Section for Hygiene and Medical Microbiology, Innsbruck, Austria
UANL, Universidad Autónoma de Nuevo León, Mexico

3.3.2 Animals

Male OF-1 mice (Charles River, Criffa S. A., Barcelona) weighing 28 – 30 g were used for the development of disseminated infection models. Animals were housed in standard boxes with corncob bedding and free access to water and food. After infection and all along the experimental period, animlas were supervised twice a day to monitor the clinical course of the infection, observing with special attention to the following aspects:

- Lose of weight
- Secretions (nasal, diarrhoea...)

- Behaviour
- Fur coat and posture
- Comatose condition

Those animals that showed impaired physical appearance, lost more than 20% of body weight, showed unkempt fur, alterations in proprioception or severe reduction of mobility were euthanized by anoxia with CO_2 in order to minimize their suffering.

All animals' care procedure were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

3.3.3 Immunosuppression

Two different immunosuppression regimens were used:

- **Combination of cyclophosphamide plus 5-fluorouracil:** cyclophosphamide (Genoxal® Baxter S. L., Valencia, Spain) was administered i.p. at a concentration of 200 mg/kg and 5-fluorouracil (Ferrer Farma, S. A., Barcelona, Spain) at a dose of 150 mg/kg i.v. Both drugs were administered one day prior to the infection.
- **Cyclophosphamide:** repeated doses of cyclophosphamide were also used in some experiments. In these cases, 200 mg/kg of this drug were administered i.p. every 5 days, starting two days before the infection.

3.3.4 Infection

In order to establish the murine models of systemic aspergillosis and scedosporiosis, mice were challenged i.v. via the lateral vein of the tail. Inocula of *A. fumigatus* and *S. apiospermum* were prepared as described in

section 3.2.1 and adjusted to the desired concentration by haemocytometer count. Viability of inocula was always checked by placing serial dilutions of the inocula on PDA.

3.3.5 Antifungal therapies

Antifungal agents used in the experimental treatments are shown in Table 4. Treatments started 24 hours after infection (day +1) and were administered daily for 7 days.

Table 4. Drugs, doses and its administration in the mice.

Drug	Origin	Dosing (mg/kg/day)	Route of administration
Amphotericin B	Fungizone (Bristol-Myers Squibb, Wien, Austria)	0.3, 0.8	intravenous
Anidulafungin	Ecalta® Pfizer, Ltd., UK	10	intraperitoneal
Posaconazole	Noxafil® Schering-Plough, Madrid, Spain	40	oral
Voriconazole	Vfend® Pfizer, Ltd., UK.	25, 40	oral
Ceftazidime	Combino Pharm S. L., S. Joan Despi, Spain	5	subcutaneous

To prevent bacterial infections due to the state of immunosuppression of the animals, 2 days before the infection and during the experiment, ceftazidime at 5 mg/kg was administered.

3.3.6 Parameters of efficacy

In order to establish the success or failure of the treatment, two parameters of efficacy were considered i.e. survival at 20 or 30 days and fungal burden in different organs.

Those animals presenting physical and/or behaviour alterations (see section 3.3.2), or those mice that survived to the observation time (20 – 30 days), were euthanized by CO_2 inhalation.

To determine the fungal load, the target organs i.e. lung and kidneys in the case of *A. fumigatus* infection and brain and kidneys for *S. apiospermum* infection, were aseptically removed, weighed and mechanically homogenized in 1 ml of sterile saline. Serial 10-fold dilutions were placed on PDA and incubated for 48- 96 h at 30-35 ºC depending on the fungus studied. The numbers of CFU/g of tissue were calculated as follows:

$$\frac{CFU}{g} = \frac{\frac{\text{CFU}}{\text{ml}} \text{ x (organ weight} + 1 \text{ ml} *)}{\text{organ weight}}$$

*** volume of saline used for the organs homogenization, considering that 1 g of tissue occupies a volume of 1 ml.**

3.3.7 Bioassay

The bioassay for determining voriconazole levels in serum was performed in the studies of disseminated aspergillosis in order to correlate the levels of voriconazole with the MIC strains and the efficacy of the treatment. For that purpose, the first step was to make a standard curve of voriconazole with a range of known concentrations of voriconazole (from 0.125 to 16 µg/ml). *Candida parapsilosis* ATCC 22019 was used as indicator microorganism and was cultivated in PDA for 24 h at 35°C. An inoculum was obtained suspending 5 colonies in sterile saline and adjusting by haemocytometer to a concentration of $2x10^6$ CFU/ml. Three hundred microliters of this inoculum were dispensed in sterile tubes containing 22.5 ml of yeast nitrogen base (YNB) agar at a temperature of 40 ºC approximately. The composition of the YNB medium was:

- 3.45 g YNB
- 5 g triptose peptone
- 2.5 g dextrose
- 7.5 g agar
- 500 ml sterile distillate water

After homogenizing YNB medium with the inoculum, the mixture was dispensed in 100 x 15 mm Petri dishes. Once the medium was solidified, wells of 4 mm were done and filled with 20 µl of each concentration of voriconazole previously prepared. The plates were incubated at 35 °C. The diameters of the zones of growth inhibition were measured after 24 hours and then the standard curve and its correlation coefficient were calculated. All assays were performed in duplicate.

In order to calculate the concentration of drugs in serum from treated animals, blood was extracted by cardiac puncture from animals previously anesthetized with inhalatory isoflurane. Then, the sera were processed in the same way that was obtained the standard curve. Once the diameters were measured, these were extrapolated to the standard curve in order to calculate the serum voriconazole concentration.

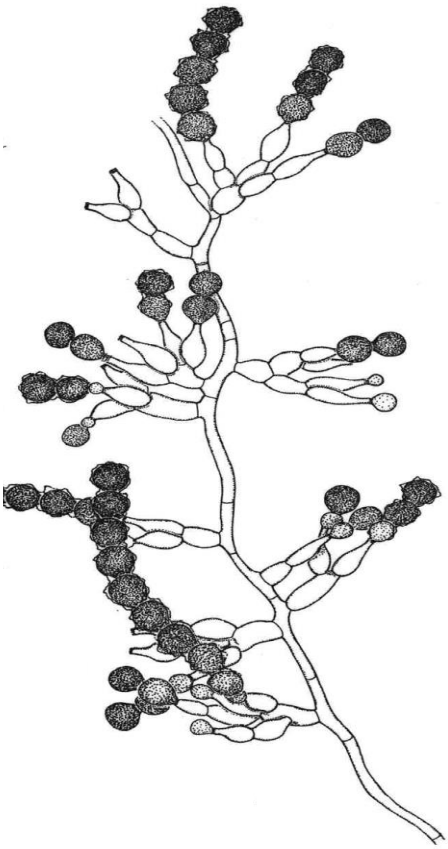
3.3.8 Statistical analyses

Statistical analyses were done using GraphPad Prism version 6.0 for windows (Graphpad Prism, San Diego, USA).

Survival: Kaplan Meier method calculates the Mean Survival Time (MST) to one group of mice, taking into account the animals left alive at the end of the study and also the days in which the animals that do not survive at the end of the observation period. Then, the log-rank test is used

to test whether the difference between survival times between two groups is statistically different or not.

Fungal burden: in order to compare the burden tissue between control and treated groups, Mann-Whitney U test was used. That is a non-parametric test that do not compare means but it sorts the data of the two groups which are being compared and determines a range. In this test, therefore there is no data with a normal distribution and allows to work with the original data or with logarithms, getting the same results.



4. RESULTS

4.1 *In vivo* synergy of amphotericin B plus posaconazole in murine aspergillosis

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In Vivo Synergy of Amphotericin B plus Posaconazole in Murine Aspergillosis

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***Aspergillus fumigatus* is the main mold causing invasive fungal infection that shows high mortality rates. Therapeutic failure and the increase in drug resistance make it necessary to explore alternative treatments for this infection. We have evaluated the efficacy of amphotericin B at 0.8 mg/kg or 0.3 mg/kg of body weight combined with 40 mg/kg of posaconazole against three *A. fumigatus* isolates in a murine model of disseminated infection. The combination of the polyene and the azole led to a greater increase in survival and a significantly greater reduction in tissue burden than monotherapies.**

Aspergillus fumigatus is the most common mold causing invasive fungal infection (IFI) in immunocompromised patients (1), especially in those with hematological malignancies, with high mortality rates (2–4). Voriconazole (VRC) is the first-line therapy for the treatment of aspergillosis, but in patients with infections that are refractory to this drug, therapy options include other azoles such as itraconazole or posaconazole (PSC), lipid formulations of amphotericin B (LAMB), or echinocandins (5). Because of the relatively limited efficacy of the current antifungal treatments, an exploration of alternative strategies against this difficult-to-treat infection is crucial. Combinations of antifungal agents are not common therapies but might be good alternatives for infections by resistant organisms or when the standard treatments fail (6–11). Synergistic interactions of two drugs with different targets on the fungal cell can be more effective than each drug working alone. In addition, combined therapies can allow lower doses to be administered, with lower toxicity, faster cure, and probably lower costs. Since the efficacies of different antifungal combinations have been demonstrated by several studies in patients with aspergillosis (6, 7, 12), we were interested in evaluating the *in vivo* efficacy of the combination of amphotericin B (AMB) plus PSC against *A. fumigatus*. This combination had already been tested in a murine model of invasive aspergillosis caused by *Aspergillus flavus* (13), although no improvement over the PSC monotherapy was observed. Another study demonstrated the efficacy of suboptimal doses of VRC plus anidulafungin in a murine model of *A. fumigatus* infection (14), suggesting that combined therapies might have an important role as alternative treatments against systemic aspergillosis, allowing a reduction of the doses administered. One of the isolates tested in the present study (FMR 10528) had already been used in previous studies but showed a poor *in vivo* response to VRC when administered at 25 mg/kg of body weight despite having a low MIC (15, 16). The goals of this study were (i) to evaluate the efficacy of the combination of AMB plus PSC against isolates of *A. fumigatus* in a murine model of disseminated aspergillosis, comparing the results with those of the corresponding monotherapies and VRC, (ii) to investigate the presence of *CYP51A* gene mutations that might explain the poor *in vivo* response of such an isolate, and (iii) to perform adaptation experiments that can assess the ability of this isolate to develop azole resistance.

MATERIALS AND METHODS

Fungal isolates. Two clinical isolates (FMR 10528 and FMR 13142) and one environmental isolate (FMR 7739) of *A. fumigatus* were used in this study. Fungi were grown on potato dextrose agar (PDA). The MICs were previously determined in triplicate following the CLSI guidelines (17). The MICs of AMB and PSC were 2 μg/ml and 0.5 μg/ml, respectively, for the strain FMR 7739 and 1 μg/ml and 0.25 μg/ml, respectively, for the strains FMR 10528 and FMR 13142. The MIC of VRC was 0.25 μg/ml for the three strains.

Inocula for both *in vitro* drug interaction testing and *in vivo* assays were prepared from 5-day-old cultures incubated at 37°C. Conidia were harvested with a sterile pipette by flooding the plates with sterile saline containing 0.025% Tween 20. The suspensions were adjusted to the desired concentrations by hemocytometer counting, and the viability was assessed by placing 10-fold dilutions on PDA plates.

***In vitro* antifungal interaction testing.** The interaction testing was carried out using a two-dimensional checkerboard microdilution method with 2-fold serial dilutions of AMB and PSC, ranging from 0.12 to 8 μg/ml and from 0.002 to 1 μg/ml, respectively. Readings were taken 48 h after incubation at 35°C using an inverted mirror and the MIC_0 (100% growth inhibition) as the endpoint criterion. The fractional inhibitory concentration index (FICI) was used to classify drug interactions, which were defined as synergistic if the FICI was ≤0.5, antagonistic if the FICI was >4, and indifferent if the FICI was >0.5 but ≤4 (18). Tests were carried out in duplicate.

Infection. For the *in vivo* studies, male OF-1 mice (Charles River, Criffa S.A., Barcelona, Spain) weighing approximately 30 g were used. Animals were housed under standard conditions with water and food *ad libitum*. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

One day prior to the infection, the animals were immunosuppressed by an intraperitoneal (i.p.) injection of 200 mg/kg of cyclophosphamide (Genoxal; Laboratories Funk S.A., Barcelona, Spain) and a single intravenous (i.v.) injection of 150 mg/kg of 5-fluorouracil (Fluorouracilo; Ferrer Farma S.A., Barcelona, Spain) (19).

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TABLE 1 Primers used for the amplification of *CYP51A* of *A. fumigatus*

Primer	Sequence
Cyp51 AF F1	5′-CACCCTCCCTGTGTCTCCT-3′
Cyp51 AF R1	5′-CCGATCACACCAAATCCTTT-3′
Cyp51 AF S1	5′-CTCAGCCGTGAGTTTGGAAC-3′
Cyp51 AF S2	5′-CCTCACAGCCAAAAGTCCTC-3′
Cyp51 AF S3	5′-ATTGTCCCAATTCCAAGCTG-3′
Cyp51 AF S4	5′-TCTCTGCACGCAAAGAA-3′

Groups of 16 animals, 8 for survival and 8 for the fungal load study and for determining drug serum levels, randomly chosen, were challenged i.v. via the lateral vein with 1×10^3 CFU/animal of the strain FMR 13142 or 1×10^4 CFU of the strains FMR 7739 and FMR 10528. Inoculum sizes were adjusted for each strain in order to obtain a similar degree of infection in all the cases, causing 100% of the animals to die within 9 days. After challenge, the mice were checked daily for 30 days.

Treatments. All treatments started 1 day after infection, and the animals were treated daily for 7 days. The treatments consisted of AMB at 0.8 mg/kg (amphotericin B deoxycholate; Xalabarder Pharmacy, Barcelona, Spain) administered i.v. (20), PSC (Noxafil; Schering-Plough Ltd., Hertfordshire, United Kingdom) at 20 mg/kg given orally by gavage (p.o.) twice a day (BID) (21), or VRC at 25 mg/kg (Vfend; Pfizer S.A., Madrid, Spain) administered p.o. (22). The combined therapies consisted of AMB plus PSC at the given doses, with the exception of animals infected with the strain FMR 7739, which received AMB 0.3 mg/kg in the combination due to the good efficacy obtained after the monotherapy with AMB at 0.8 mg/kg. From 2 days before the infection, animals receiving VRC were given 50% grapefruit juice instead of water. The control animals received no antifungal treatment. In order to prevent bacterial infections, mice received subcutaneous injections of 5 mg/kg/day of ceftazidime.

Tissue burden and bioassay. The mice included in the tissue burden study ($n = 8$) were euthanized on day 6 postinfection, in order to compare the fungal load with that of the control group. Five animals from each group were also used to determine drug serum concentrations by bioassay. For the bioassay, 2 h after the 6th dose, the animals were anesthetized by inhalation of isoflurane, and approximately 1 ml of blood was extracted by cardiac puncture, the serum being obtained by blood centrifugation. The concentrations of PSC, VRC, and AMB from serum samples were determined by bioassay, using *Candida parapsilosis* ATCC 22019, as previously described (23). After blood extraction, animals were euthanized by cervical dislocation, and the kidneys and lungs were aseptically removed, weighed, and homogenized in 1 ml of sterile saline. Serial 10-fold dilutions of the homogenates were placed on PDA plates and incubated for 48 h at 37°C to determine CFU per gram of tissue.

Amplification and sequencing of *CYP51A*. Genomic DNA of each *A. fumigatus* isolate was extracted from 3-day-old cultures (24). PCRs were carried out in a 25-μl volume, containing 10.5 μl of water, 12.5 μl of *Taq* Kapa 2G Robust Ready (Kapa Biosystems Inc., Wilmington, MA, USA), 0.5 μl of each primer (10 μM), and 50 ng of genomic DNA. Table 1 lists the primers used for amplification and sequencing of the *CYP51A* gene. The amplification took place in a thermal cycler for 1 cycle of 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 58°C, and 2 min at 72°C, followed by 1 final cycle of 10 min at 72°C. The PCR products were analyzed by electrophoresis on 1% agarose gel.

The *CYP51A* gene sequences of 10 known wild-type *A. fumigatus* isolates were used to generate a consensus sequence (data not shown). This sequence served as a negative control (*CYP51A* without mutations) and was used for comparison with the sequences of the three isolates tested. Lasergene SeqMan (DNAStar, Madison, WI, USA) was used for generating the consensus wild-type *CYP51A* sequence and for checking the quality of the sequences, and Mega 6 (25) was used for alignment and gene comparison.

***In vitro* and *in vivo* adaptation experiments.** An *in vitro* adaptation experiment (26, 27) was carried out to investigate the poor *in vivo* efficacy of azoles against the strains FMR 7739 and FMR 10528, despite their low MICs. Each strain was passaged 3 times at 1-week intervals on PDA containing PSC or VRC at a concentration corresponding to half of the MIC. The susceptibilities to PSC and VRC of both strains before antifungal exposure and of their 3 subcultures exposed to azoles were determined by a microdilution method (17). The assay was carried out in duplicate. In addition, *A. fumigatus* strains were recovered from the lungs and kidneys of those animals infected and treated for 6 days with the azole monotherapies, and the MICs were determined.

Statistical analysis. The mean survival time (MST) was estimated by the Kaplan-Meier method and compared among groups using the log rank test. The tissue burdens from the control and treated groups were compared using the Mann-Whitney U test. All statistical analyses used GraphPad Prism 6.0 for Windows. *P* values of ≤0.05 were considered statistically significant.

Nucleotide sequence accession numbers. The *CYP51* sequences from FMR 7739, FMR 10528, and FMR 13142 have been deposited in the NCBI database and are available under GenBank accession numbers KT070084, KT070085, and KT070086, respectively.

RESULTS

***In vitro* interaction testing.** The effects of the *in vitro* interaction between AMB and PSC were indifferent for the three strains, with FICIs ranging from 0.56 to 0.73 (data not shown).

***In vivo* studies.** Untreated animals began to die on day 4 to 5 postinfection, and on day 10 no animals were alive. For the strain FMR 7739, AMB at 0.8 mg/kg significantly increased the survival of the animals compared to that of the controls ($P < 0.0001$) and of the other monotherapy groups ($P \leq 0.0085$), all animals being alive at the end of the experiment (day 30 postinfection). Therefore, for the combined therapy, a suboptimal dose of AMB was tested, i.e., AMB at 0.3 mg/kg, which also prolonged the survival significantly with respect to that of the control group ($P = 0.0384$). In contrast, neither PSC nor VRC by itself was able to increase survival ($P = 0.3926$ and $P = 0.227$, respectively). The combination significantly increased survival compared to that of the animals treated with AMB at 0.3 mg/kg, PSC, or VRC ($P \leq 0.0313$).

With the strain FMR 10528, an increase in survival was only observed with AMB at 0.8 mg/kg and the combination ($P \leq 0.029$). In addition, the combined therapy worked better than the monotherapies ($P \leq 0.0418$). In the case of the strain FMR 13142, all of the therapies, including the combination, significantly increased the survival of the animals ($P \leq 0.0269$) (Fig. 1).

In the fungal load study, the combination of AMB plus PSC showed efficacy in reducing the numbers of CFU in the two organs and in all strains studied. This was even better than the monotherapies with either azole in all strains, with the exception of the lungs from the animals infected with the strain FMR 13142, where the combination equaled the efficacy of VRC. The combination also improved the efficacy of AMB 0.8 in the lungs of the animals infected with the strain FMR 7739 and in the kidneys of those infected with FMR 10528.

AMB at 0.8 mg/kg and VRC were able to reduce the tissue burdens in the two organs from animals infected with each of the three strains ($P \leq 0.0002$ and $P \leq 0.0298$, respectively), with the only exception being VRC against the strain FMR 10528 in lungs ($P = 0.1044$). PSC reduced the fungal burden of the kidneys of the animals infected with each of the three strains ($P \leq 0.0463$) and in the lungs of the animals infected with the strain FMR 10528 ($P = 0.0002$) (Fig. 2).

The serum concentrations of AMB at 0.8 mg/kg, PSC at 40

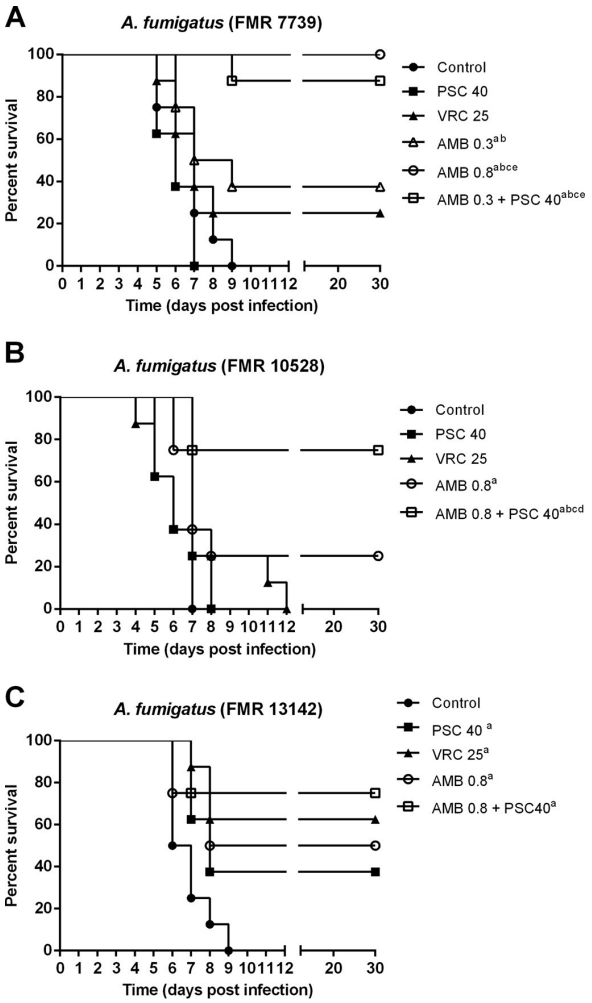

FIG 1 Cumulative mortality of immunosuppressed mice infected with *A. fumigatus* strains FMR 7739 (A), FMR 10528 (B), and FMR 13142 (C). AMB 0.8, amphotericin B at 0.8 mg/kg once a day (QD); PSC 40, posaconazole at 20 mg/kg BID; VRC 25, voriconazole at 25 mg/kg QD. $^{a}P \leq 0.05$ versus control; $^{b}P < 0.05$ versus PSC 40; $^{c}P < 0.05$ versus VRC 25; $^{d}P < 0.05$ versus AMB 0.8; $^{e}P < 0.05$ versus AMB 0.3.

mg/kg, and VRC at 25 mg/kg were higher than the MICs, with values of 4.28 ± 0.31, 6.34 ± 0.90, and 9.99 ± 0.71 μg/ml, respectively.

Amplification and sequencing of *CYP51A*. No mutations were found in the *CYP51A* gene sequences of the three strains tested, such sequences being identical to that of the wild-type consensus sequence.

***In vitro* and *in vivo* adaptation experiments.** With two of the strains grown on PDA plates containing PSC or VRC, no increases in MICs were observed. Only the strain FMR 10528 showed the VRC MIC two dilutions higher than before drug exposure. However, important morphological changes were observed. The colony growth rate decreased in the three strains tested. There was also a noticeable reduction of sporulation and change in the pigmentation of the colonies of the strain FMR 10528, from green to pale green.

The MICs of VRC and PSC against the isolates recovered from treated animals were the same or one dilution higher than that obtained originally. These isolates also showed a reduction in the growth rate and sporulation and also a change in the colony color.

DISCUSSION

Due to the important increase in the azole resistance of *Aspergillus* and the associated therapeutic failure, finding alternatives to the current therapies is crucial. In the present study, we tested the combination of AMB plus PSC in a neutropenic model of disseminated aspergillosis, using three *A. fumigatus* strains. In previous studies conducted in animal models, VRC at 25 mg/kg demonstrated poor efficacy against systemic aspergillosis by one of the strains included in the present study, i.e., strain FMR 10528 (15, 16). Now, we can corroborate the lack of efficacy of VRC administered at 25 mg/kg, and, in addition, we have also found a therapeutic failure of PSC administered at 40 mg/kg against two of the three strains assayed. It is worth mentioning that no correlation was found between the two parameters of efficacy used, i.e., survival and fungal burden. Azoles were not able to improve survival in those animals infected with the strains FMR 7739 or FMR 10528, but there was a reduction in fungal burden in at least one organ.

PSC is known to show good efficacy against *A. fumigatus* infections, which decreases when the fungus harbors mutations in the *CYP51A* gene (28, 29); however, in the present study, no mutations in the *CYP51A* gene were found. In order to explain the lack of efficacy, azole adaptation assays were carried out to determine

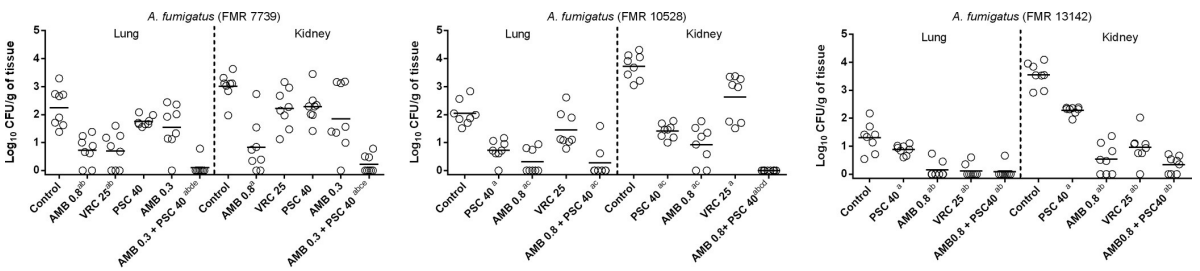

FIG 2 Effect of antifungal treatments on colony counts of *A. fumigatus* strains FMR 7739 (A), FMR 10528 (B), or FMR 13142 (C) in lungs and kidneys of immunosuppressed mice. AMB 0.8, amphotericin B at 0.8 mg/kg once a day (QD); PSC 40, posaconazole at 20 mg/kg BID; VRC 25, voriconazole at 25 mg/kg QD. Horizontal lines indicate median values. $^{a}P < 0.05$ versus control; $^{b}P < 0.05$ versus PSC 40; $^{c}P < 0.05$ versus VRC 25; $^{d}P < 0.05$ versus AMB 0.8; $^{e}P < 0.05$ versus AMB 0.3.

the ability of the strains to develop resistance to the drugs, as continuous contact with a compound is known to be able to result in tolerance and development of resistance to it, as seems to occur with *A. fumigatus* and azoles (30–32).

In a previous study, Salas et al. (15) demonstrated that *A. fumigatus* strains varied greatly in their *in vivo* responses to VRC, particularly those isolates with MIC values of ≥0.25 μg/ml. This has been observed in the present study for both VRC and PSC, suggesting a strain-to-strain variability. In the present study, all three strains show MICs for VRC of 0.25 μg/ml and for PSC of 0.25 to 0.5 μg/ml, all values being below the epidemiological cutoff values (ECVs) (33). Different studies suggest that MICs and ECVs are useful predictors of azole efficacy *in vivo* (15, 29). However, other studies suggest that the pharmacokinetics (PK) and particularly the determination of the area under the concentration-time curve (AUC)/MIC correlate better with efficacy than MICs, the AUC/MIC values being >25 and >100 for VRC and PSC, respectively, predictors of successful outcome (34, 35). Although the PK parameters have not been determined in the present study, previous studies have demonstrated the efficacy of VRC and PSC administered at 25 mg/kg and 40 mg/kg, respectively, against *Aspergillus* spp. in murine models (14, 15, 22, 28, 29, 36) and the fact that multiple dosing of VRC at 20 mg/kg in mice resulted in an AUC_{0-24} of 58.1 h · mg/liter, leading to efficacy against invasive aspergillosis by a strain with a MIC of 0.25 μg/ml (14). Other studies testing PSC and VRC doses similar to those used in our experiment have shown AUC values indicative of correct exposure (28, 37–39).

Little information is available on the *in vitro* interaction of AMB and PSC against *A. fumigatus*. Perkhofer et al. (40) reported *in vitro* indifference of this combination against 88% of the *Aspergillus* isolates studied. Although we found indifferences in the *in vitro* studies, such a combination has shown significant *in vivo* efficacy. *In vitro* results are not always found to be predictive of synergistic effects in animal models or in the clinical setting, which is why other authors have reviewed the categorization of drug interactions, i.e., synergy, indifference, or antagonism, based on the FICI. Meletiadis et al. found a better correlation between the FICI and the outcome if a FICI of <1 was considered indicative of synergy (41) instead of a FICI of ≤0.5. Our results show a good correlation between the *in vitro* and the *in vivo* data when we considered the synergistic effects at a FICI of <1; however, more studies are necessary to extrapolate the meaning of the FICI to the outcome.

This study has some limitations. In the combined therapy, AMB was administered at 0.8 mg/kg except against the infection by one strain (FMR 7739), for which AMB was used at 0.3 mg/kg due to the good efficacy it showed as a monotherapy at 0.8 mg/kg. Moreover, we used different inoculum sizes for each strain in order to obtain the same degree of infection.

Overall, our results demonstrate that the combination AMB plus PSC shows efficacy against *A. fumigatus*, improving the efficacy of the monotherapies with azoles and AMB in some cases, and might represent an alternative when the recommended treatment fails, with a possible reduction of the dose and, consequently, the toxicity and the cost. More studies testing more strains are needed to determine more accurately the role of such a combination in the treatment of invasive aspergillosis.

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4.2 Synergistic effect of anidulafungin combined with posaconazole in experimental aspergillosis.

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Synergistic effect of anidulafungin combined with posaconazole in experimental aspergillosis

Running title: Anidulafungin plus posaconazole against *Aspergillus fumigatus*

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Abstract

Clinical and experimental data have shown discrepancies on the efficacy of combinations between triazoles and echinocandins. In this study, anidulafungin plus posaconazole have shown efficacy against a murine systemic infection by three strains of *Aspergillus fumigatus*. The combination increased mice survival and reduced burden in the kidneys over the corresponding monotherapies and voriconazole. Clearance of kidneys was observed in 62% to 100% of animals (strain dependant). We observed good *in vitro* - *in vivo* correlation when a cutoff <1 was indicative of in vivo synergy. Our results showed that the comination could be a therapeutical option, especially against infections refractory to the first line therapy.

Keywords: *A. fumigatus*, posaconazole, anidulafungin, combined therapy, murine model

Aspergillus fumigatus is a cosmopolitan opportunistic pathogen, responsible for a broad-spectrum of human diseases, including systemic infections[1]. Voriconazole (VRC) is the first-line therapy for treating invasive aspergillosis (IA), while posaconazole (PSC) together with echinocandins constitute the alternative treatment. Despite that, fatal outcome due to aspergillosis remains as high as 80%[2]. Synergy between two antifungals can allow lower doses, thus reducing drug toxicity. Because of the safety profile and tolerability of PSC and the echinocandin anidulafungin (AFG)[3], combination of both drugs might be a promising alternative for the treatment of aspergillosis.

The aim of this study was to explore the therapeutic potential of AFG+PSC in the treatment of experimental IA in comparison to the respective monotherapies and VRC.

Two clinical isolates of *A. fumigatus* from our collection (Facultat Medicina Reus, FMR 10528 and FMR 13142) and one from environmental origin (FMR 7739), were used. The minimum effective concentration (MEC), able to cause abnormal growth of hyphae, and minimum inhibitory concentrations (MIC), corresponding to the 100% of the fungal growth inhibition, were determined following the recommendations of the document M38-A2[4]. MECs of AFG and MICs of PSC were 0.03 μg/ml and 0.5 μg/ml, respectively, against strain FMR 7739 and 0.015 μg/ml and 0.25 μg/ml against strains FMR 10528 and FMR 13142, respectively. MICs of VRC were 0.25 μg/ml against all the strains tested.

Inocula for both *in vitro* and *in vivo* assays were prepared as previously described[5]. The *in vitro* activity of the combination AFG+PSC was performed by using a checkerboard microdilution method with two-fold serial dilutions of AFG and PSC, ranging from 1 to 0.002 μg/ml and from

0.5 to 0.008 μg/ml, respectively, and the fractional inhibitory concentration indexes (FICI) were calculated as previously described[6]. *In vitro* experiments were performed in triplicate.

The combination AFG+PSC showed FICIs of 0.62, 0.75 and 1 for the strains FMR 10528, FMR 13142 and FMR 7739, respectively, similar to previous results when exploring the antifungal activity of triazoles with echinocandins against *Aspergillus* spp.[7,8]. Respective concentrations of AFG and PSC in combination were 0.015 and 0.25 μg/ml for the strain FMR 7739, 0.002 and 0.125 μg/ml for FMR 10528 and 0.004 and 0.125 in the case of the strain FMR 13142.

Groups of sixteen OF-1 male mice (Charles River, Criffa S.A., Barcelona, Spain), 8 for tissue burden and 8 for survival studies, were immunosuppressed with 200 mg/kg of cyclophosphamide (Genoxal; Laboratories Funk S.A., Barcelona, Spain), given intraperitoneally (i.p.), and a single intravenous injection of 150 mg/kg of 5-fluorouracil (Fluorouracilo; Ferrer Farma S.A., Barcelona, Spain) one day before infection[9]. Animals were infected intravenously via the lateral vein of the tail with 0.2 ml containing $1x10^4$ conidia of the strains FMR 7739 and FMR 10528 or with $1x10^3$ conidia of the strain FMR 13142. The inocula sizes were chosen to cause all animals to die within 8 – 10 d and to greatly affect the tissues, as observed by histopathology and CFUs recovery in previous virulence studies (data not shown). All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Treatments started one day post-infection and lasted for 7 days. PSC (Noxafil; Schering-Plough Ltd., Hertfordshire, United Kingdom) was administered at 20 mg/kg, orally by gavage (p.o.) twice a day (BID); AFG (Ecalta, Pfizer Ltd, Kent, United Kingdom) at 10 mg/kg i.p once daily (QD) and VRC (Vfend; Pfizer S.A., Madrid, Spain) at

25 mg/kg p.o. QD. Animals treated with VRC were given 50% diluted grapefruit juice starting 2 days before the first dose and throughout the experimental period in order to increase VRC serum levels[10]. Combined therapy consisted of AFG i.p. at 10 mg/kg QD + PSC p.o. at 20 mg/kg BID. Control animals received PBS i.p as placebo. In order to prevent bacterial infections, all animals, including those of the control groups, received 5 mg/kg/day of ceftazidime subcutaneously. Efficacy was evaluated through survival prolongation and fungal load reduction. The animals included in the tissue burden assay were euthanized by anoxia with CO_2 on day 5 post-infection. Kidneys were aseptically removed and processed as previously described[5]. Experiments were done in duplicate.

Survival curves were compared using the log rank test. In tissue burden studies, colony counts were $\log_{10}$-transformed and compared by the two-tailed Mann–Whitney U-test. All statistical analyses were performed using GraphPad Prism 6.0 for Windows (GraphPad Software, Inc. La Jolla, CA, USA). *P* values ≤ 0.05 were considered statistically significant.

The efficacy of the monotherapies, including VRC, was low, i.e., both azoles only increased survival against one strain (FMR 13142) ($P \leq 0.045$) while AFG improved survival in animals infected with strains FMR 7739 and FMR 13142 ($P \leq 0.038$), but not against FMR 10528 ($P = 0.142$). It is noteworthy that survival did not correlate with MICs, since infections by strains with the same MIC resulted in different outcome, but did with fungal burden reduction. AFG reduced CFUs in those animals challenged with strains FMR 10528 and FMR 13142 (MEC = 0.015 μg/ml) ($P \leq 0.0008$) but not against the strain FMR 7739, which was the strain with the highest MEC (MEC = 0.03 μg/ml) ($P = 0.42$). As observed here, variable efficacy of AFG in reducing tissue fungal burden has been previously

reported in animal models of aspergillosis [11]. In addition, both azoles reduced fungal load in animals infected with all three strains ($P \leq 0.009$).

Based on the traditionally considered interaction ranges[12], the FICI values obtained showed an indifferent effect of the combination AFG + PSC against the three strains studied. However, Meletiadis *et al.*, recently proposed FICIs < 1 as indicative of synergism[13], which would mean that in our case the combination AFG+PSC was synergistic against two strains (FMR 10528 and 13142) and indifferent for the other (FMR 7739). This interpretation of the FICI values correlates better with the *in vivo* results because the combination PSC + AFG showed high efficacy against all the tested strains, increasing survival ($P \leq 0.003$) (Figure 1) as well as reducing fungal loads ($P \leq 0.0002$) (Figure 2) over the controls. However, an *in vitro* indifferent effect was obtained with the combination against the strain FMR 7739 but the *in vivo* outcome showed a synergistic effect highlighting the difficulty of translating *in vitro* to *in vivo* results. Moreover, only the combined therapy sterilized kidneys from all animals infected with the strain FMR 10528 and 5 and 6 out of 8 (62% and 75%) of those infected with the other strains (Figure 2).

Although some studies have tested combinations of echinocandins with azoles in murine models of aspergillosis [14–16], the efficacy of AFG + PSC has not been evaluated before. Some discrepancies on the efficacy of combinations between triazoles and echinocandins have been reported [14–16]. Although *in vivo* synergy has been demonstrated against *A. fumigatus*[14,15], in a neutropenic rat model of IA, no advantage over VRC was obtained[16]. Similarly, variable results with VRC combined with echinocandins have been reported against IA in the clinical setting[17–19]. In a recent study, a moderate benefit of AFG+VRC over VRC in patients with haematological malignancies was demonstrated[20].

In conclusion, the results from this study indicate that AFG+PSC showed *in vivo* efficacy against *A. fumigatus*. However, more isolates are needed to be investigated to demonstrate the usefulness of this combination against IA.

Conflict of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the manuscript.

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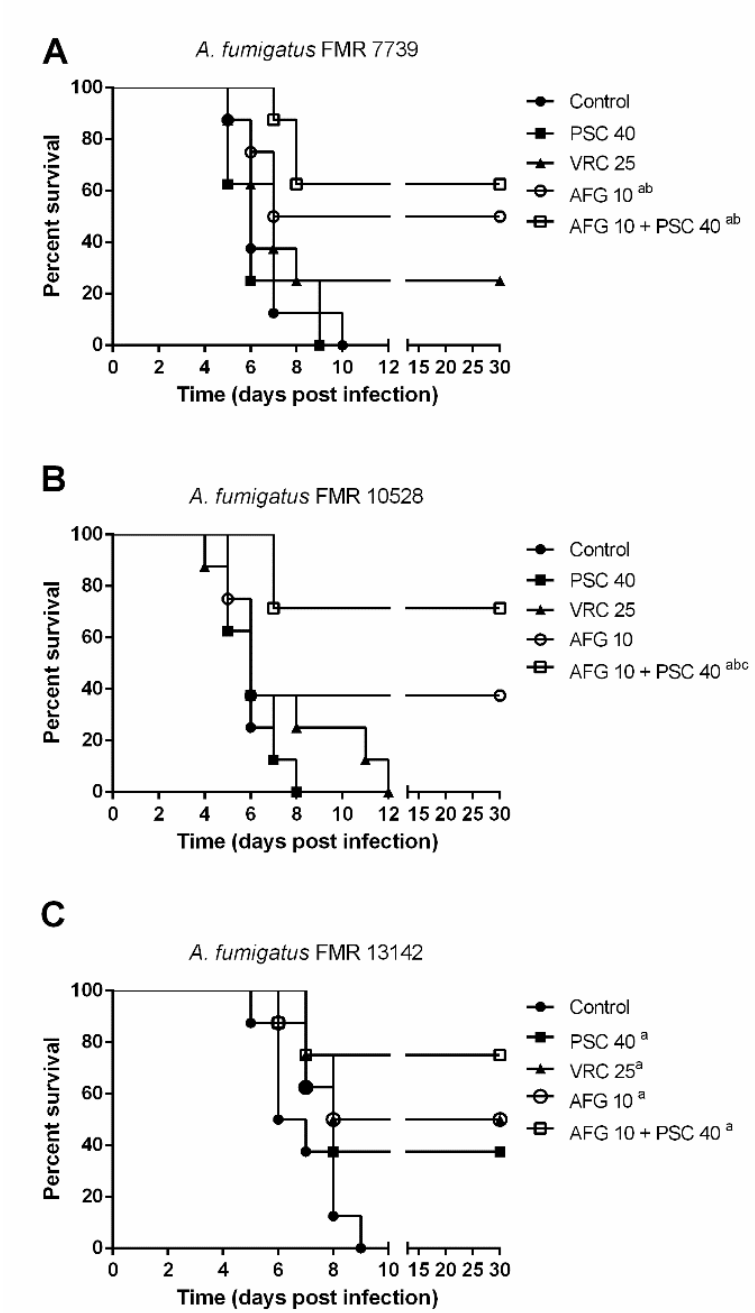
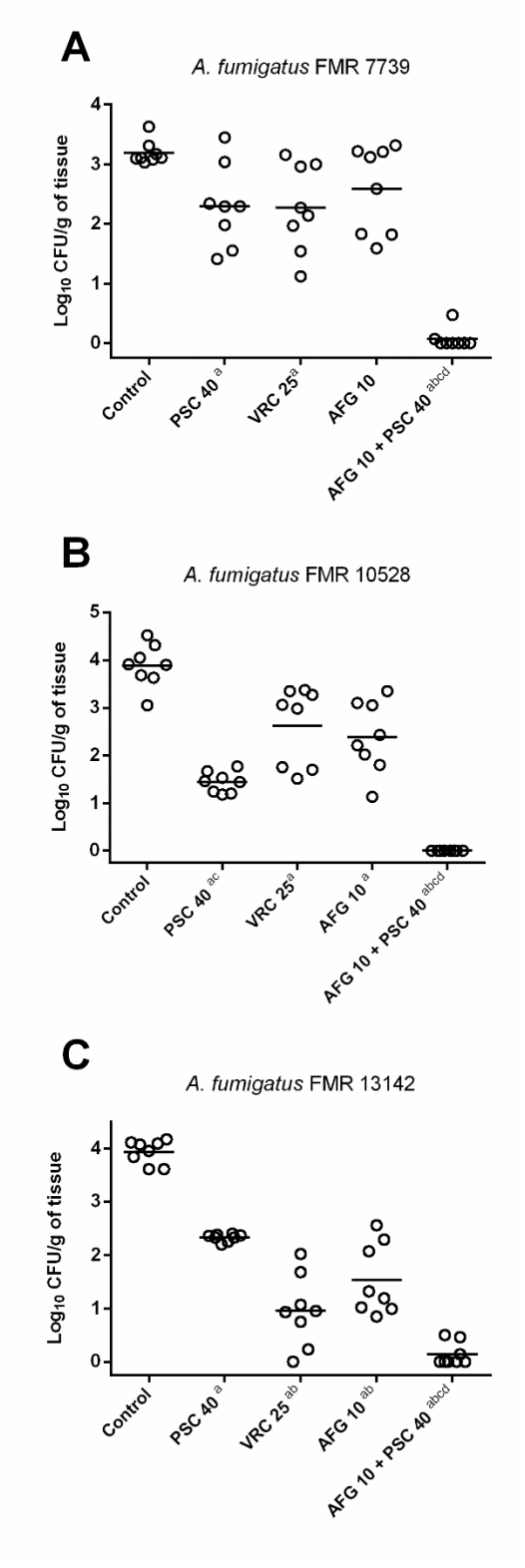
FIGURES

Figure 1. Cumulative mortality of immunosuppressed mice infected with *A. fumigatus* FMR 7739 (A), FMR 10528 (B) and FMR 13142 (C). PSC 40, posaconazole 20 mg/kg BID; VRC 25, voriconazole 25 mg/kg QD; AND 10, anidulafungin 10 mg/kg QD. [a] $P \leq 0.0448$ versus control, [b] $P \leq 0.0321$ versus PSC 40, [c] $P \leq 0.0037$ versus VRC 25

Figure 2. Effect of antifungal treatments on colony counts of *A. fumigatus* FMR 7739 (A), FMR 10528 (B) or FMR 13142 (C) in kidneys of immunosuppressed mice. PSC 40, posaconazole at 20 mg/kg BID; VRC 25, voriconazole at 25 mg/kg QD; AND 10, anidulafungin at 10 mg/kg QD. Horizontal lines indicate median values. [a] $P \leq 0.0096$ versus control; [b] $P \leq 0.0191$ versus PSC 40; [c] $P \leq 0.0068$ versus VRC 25; [d] $P \leq 0.0221$ versus AND 10.


A
A. fumigatus FMR 7739
Percent survival
Time (days post infection)
Control
PSC 40
VRC 25
AFG 10 ab
AFG 10 + PSC 40 ab
B
A. fumigatus FMR 10528
Percent survival
Time (days post infection)
Control
PSC 40
VRC 25
AFG 10
AFG 10 + PSC 40 abc
C
A. fumigatus FMR 13142
Percent survival
Time (days post infection)
Control
PSC 40 a
VRC 25a
AFG 10 a
AFG 10 + PSC 40 a


A
A. fumigatus FMR 7739
Log10 CFU/g of tissue
0
1
2
3
4
Control
PSC 40 a
VRC 25a
AFG 10
AFG 10 + PSC 40 abcd
B
A. fumigatus FMR 10528
Log10 CFU/g of tissue
0
1
2
3
4
5
Control
PSC 40 ac
VRC 25a
AFG 10 a
AFG 10 + PSC 40 abcd
C
A. fumigatus FMR 13142
Log10 CFU/g of tissue
0
1
2
3
4
Control
PSC 40 a
VRC 25 ab
AFG 10 ab
AFG 10 + PSC 40 abcd

4.3 Voriconazole minimal inhibitory concentrations (MICs) are predictive for the outcome of disseminated scedosporiosis.

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Voriconazole minimal inhibitory concentrations (MICs) are predictive for the outcome of experimental disseminated scedosporiosis.

Running title: Voriconazole MICs can predict outcome in disseminated scedosporiosis

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Synopsis

Background: Scedosporiosis is associated with a mortality rate of up to 90% in patients suffering from disseminated infections. Recommended first line treatment is voriconazole, but epidemiological cut off values and clinical breakpoints are lacking.

Objectives: We aimed to correlate voriconazole treatment response in mice suffering from disseminated scedosporiosis with MIC values determined with CLSI, Etest®, and disk diffusion assay.

Methods: Voriconazole MICs of 31 *S. apiospermum* strains were tested using CLSI broth microdilution, EtestR (Biomerieux), and disk diffusion. Groups of mice were challenged intravenously with one out of 16 *S. apiospermum* strains (voriconazole MIC range: 0.125mg/L - 8.0mg/L) and were i.v. treated with 40mg voriconazole/kg orally by gavage once daily. Efficacy of voriconazole was evaluated by the reduction of fungal burden in brain.

Results: An agreement of 84% was reached for CLSI microdilution broth and disk diffusion and of 93.5% for CLSI and Etest®. Correlation of CLSI MICs and *in vivo* outcome was good, as mice challenged with strains with a MIC ≤2mg/L responded and those challenged with the strain with a MIC >4mg/L failed to respond to voriconazole therapy.

Conclusions: CLSI broth microdilution and EtestR deliver comparable results that enable a prediction of *in vivo* outcome. When CLSI microdilution was used, voriconazole MICs ≤ 2mg/L were suggestive of susceptibility, MICs = 4mg/L suggested intermediate susceptibility, while the isolate with a MIC = 8mg/L was found resistant, based on the fungal burden in brain.

Keywords: Scedosporiosis, *Scedosporium apiospermum*, voriconazole, broth microdilution according to CLSI, EtestR, disk-diffusion

Introduction

Scedosporiosis and fusariosis are the most frequent mould infections, after aspergillosis.[1] In temperate climate zones, scedosporiosis is commonly caused by members of the '*Scedosporium apiospermum* species complex' (pooling the former species: *Pseudallescheria ellipsoidea, P. fusoidea, P. angusta,* and *P. boydii*).[2] These fungi cause a broad spectrum of diseases in both immunocompetent and immunocompromised patients.[3] Their special features are a pronouncedneurotropism and resistance against most systemic antifungals (amphotericin B, itraconazole, flucytosine, and echinocandins). In immunocompetent patients scedosporiosis is predominantly localized, but the majority of immunosuppressed patients (>50%) suffer from disseminated infections (mortality rates: 58%-75%).Particularly high mortality rates (>90%) are seen in patients with central nervous system (CNS) involvement; in immunosuppressed, *Scedosporium* disseminates via haematogenous spread, while CNS scedosporiosis is seen in immunocompetent patients, exclusively after near-drowning.[3,4] Although neither species–specific epidemiological cut off values (ECVs) nor clinical breakpoints (CBPs) have been established for *Scedosporium* spp., case reports[3] and currently available treatment guidelines5 suggest voriconazole (with or without surgery) as first line treatment for scedosporiosis. Voriconazole reaches high CNS-substance levels.[4] But, a comprehensive evaluation of voriconazole treatment outcome in experimental disseminated scedosporiosis and a correlation with MIC values is lacking. Therefore, the objectives of this study were to determine if *in vivo* efficacy of voriconazole correlates with MIC values and to suggest a species-specific experimental tentative cut-off value. In addition, we aimed to evaluate the performance of the commercial antifungal susceptibility tests (Etest® and disk diffusion test [DDT]) in comparison to

the gold standard method (CLSI), to offer alternatives for routine laboratories.

Materials and methods

In vitro antifungal susceptibility testing

Twenty-eight clinical isolates and three of environmental origin were used in the *in vitro* studies. All isolates were previously identified by sequencing the ITS region and β-tubulin gene. The sequences were blasted against two in-house databases and ISHAM ITS database (http://its.mycologylab.org/). The isolates were subcultured on potato dextrose agar (PDA) for 10 days at 30 ºC and inocula were prepared as previously described.[6] Voriconazole MIC values were determined using: i) broth microdilution according to CLSI M38-A2 document (international gold standard),[7] ii) disk diffusion using non-supplemented Mueller Hinton agar and 6-mm-diameter paper disks containing 1.0 μg of voriconazole,[8] and iii) Etest® (BioMerieux, S. A., Spain), according to the manufacturer's instructions and as previously described.[9] The strains *Paecilomyces variotii* ATCC MYA-3630 and *Aspergillus fumigatus* ATCC MYA-3626 were used as quality controls. All *in vitro* tests were incubated at 35 ºC for 72h. Microdilution MICs were determined according to CLSI using an endpoint of complete growth inhibition.[7] For disk diffusion, inhibition zone diameters (IZDs) were measured to the nearest whole millimeter at the point at which there was a prominent reduction of growth (80%). Slight trailing around the edges was ignored. Etest® MIC corresponded to the gradient concentration where the inhibition ellipse intersected the plastic strip. Etest® MICs were rounded up to the next higher CLSI concentration for comparison. Strains were categorized as susceptible, intermediate or

resistant in basis of their response to voriconazole considering as endpoint the reduction of fungal burden in brain (see next section). Disk diffusion tests were read as follows: susceptible (IZD ≥ 17 mm), intermediate (IZD from 14-16 mm), and resistant (≤ 13 mm, respectively). The results of Etest® and CLSI microdilution method were analyzed for their reproducibility by providing essential agreement values within ± 1 and ± 2 CLSI dilution steps, as previously described.[10] The minimal fungicidal concentration (MFC) was also determined by subculturing 20 µl from each well that showed complete growth inhibition onto potato dextrose agar (PDA) plates. The MFC was the lowest drug concentration at which approximately 99.9% of the original inoculum was killed. Each isolate and method was tested in triplicate.

Murine model and ethics

Sixteen strains with different susceptibility for voriconazole (based on CLSI MIC values) were selected for murine studies (Table 1). Groups of 8 OF-1 male mice (Charles River, Criffa S.A., Barcelona, Spain) were immunosuppressed two days before the infection and every 5 days with 200 mg/kg of cyclophosphamide. On the day of the infection, mice were challenged intravenously with $5x10^3$–$1x10^4$ conidia (inoculum size was adjusted to the virulence of each strain that was tested in a previous study evaluating survival as endpoint; see supplementary material Table S1) and treated with voriconazole at 40 mg/kg/d orally by gavage during 7 days as reported in a similar murine model (Table S2).[11] Animals received grapefruit juice during and two days before initiation of treatment.[12] Brain fungal burden was the study endpoint, for comparability all animals (control and treated groups) were euthanized after the first animal died in the control

group (Table 1). Brains were worked up as previously described[11] for cfu determination/g tissue. All animals were housed under standard conditions and care procedures were supervised and approved under the procedure number 8249, by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

Statistical analysis

Brain fungal burdens of the control and treated groups were compared using the Mann-Whitney U test. All statistical analyses used GraphPad Prism 6.0 for Windows. *P* values of < 0.05 were considered statistically significant.

Results

MIC ranges were 0.125-8.0 mg/L for broth microdilution, 0.094-> 32.0 mg/L for Etest®, and IZDs ranged from 0- 40.0 mm. The susceptibility results for each strain are presented in Table 1. Based on CLSI, 87.1% of the *S. apiospermum* strains were susceptible, 6.5% intermediate and 3.2% resistant. While with Etest®, 96.8% were susceptible and 3.2% resistant. Using DDT, 87.1% of strains were susceptible and 12.9% resistant. The essential levels of agreement (CLSI MICs) were 87.1% and 93.5%, respectively, while agreement between microdilution and disk-diffusion was 90.4%. MFCs of all strains exceeded 16.0 mg/L confirming the fungistatic activity of voriconazole against *S. apiospermum*. Voriconazole showed a statistically significant reduction of fungal burden in brain, compared to the control, in thirteen of the sixteen strains tested, which coincided, with only one exception, with those showing MICs $\leq$ 2mg/L by microdilution CLSI testing (Table 1). Mice challenged with *S. apiospermum* strains with MICs >

2mg/L failed to respond to therapy in two (FMR 8869, MIC = 4 mg/L and HMM 12-09, MIC = 8mg/L, respectively) out of three cases. Mice challenged with UANL-OC149 (MIC = 4 mg/L) showed a significant reduction in brain burden, when receiving voriconazole treatment. By using DDT, voriconazole showed efficacy against all the strains showing an IZD ≥ 18mm with only one exception (HMM 10-35, IZD=20 mm) and also against two strains considered resistant *in vitro* (UANL-OC149 and HMM 11-47; IZDs = 0 mm). With respect to the Etest®, voriconazole showed efficacy against the strains with MICs between 0.094 mg/L and 1.5 mg/L with two exceptions (strains HMM 10-35 and FMR 8869, MICs 0.38 mg/L and 0.5 mg/L, respectively) and did not against HMM 12-09 (MIC > 32 mg/L).

Discussion

Mortality rates among patients suffering from disseminated scedosporiosis remain high depending on the underlying disease (highest in HSCT recipients) and the site of infection (CNS) and can be up to 90%.[3,13] ECVs and CBs have not been established for the interpretation of voriconazole MICs, the currently suggested drug of first line treatment. Due to the rareness of the disease, these will remain difficult to establish. In contrast to *Scedosporium*, ECVs for amphotericin B and some azoles have been established for most clinically relevant moulds such as *Aspergillus* spp., *Fusarium* spp. and some Mucorales.[14,15] The present study has some limitations that require careful interpretation of the results. MIC values for voriconazole and *S. apiospermum* >1 mg/L are rare, as MIC90 < 2mg/L.[16] Therefore, only limited set of voriconazole-resistant strains was tested.

Our results and the results of other murine models of scedosporiosis demonstrated the efficacy of voriconazole against *S. apiospermum ,S. boydii,* and *S. aurantiacum* strains with MICs between 0.25mg/L and 1mg/L.[11,17,18] Similar to our results, in a guinea pig model azole did not show efficacy against one *S. apiospermum* strain with a MIC of 8.0 mg/L.[19]

In the clinical setting, the relevance of susceptibility determination is largely unknown. Most *S. apiospermum* isolates have voriconazole MIC < 2mg/L. Considering our results, a therapeutic failure for strains with >2mg/L is likely, however, alternative therapeutics are lacking due to the multi-resistance of these fungi. Posaconazole is one alternative which demonstrated efficacy in treating brain abscesses due to *S. apiospermum.*[20] Surgical debridement should be considered whenever possible.[5]

Overall, our results suggest that voriconazole was able to reduce fungal burden in the brain of 92.3% of all mice challenged with strains with voriconazole MICs ≤ 2 mg/L. Therefore, we propose ≤ 2 mg/L and > 4 mg/L as species-specific tentative cut-off values for *S. apiospermum.* Etest® and CLSI broth microdilution were found to be suitable methods to generate reliable voriconazole MICs for *S. apiospermum* strains.

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Transparency declarations

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TABLES

Table 1. *In vitro* antifungal susceptibility results of 31 isolates of *S. apiospermum* using 3 antifungal susceptibility methods and results of *in vivo* efficacy of voriconazole in mice.

Strain	MIC (mg/L)[a]		IZD (mm)[b]	log_{10}cfu/g of brain of control mice	log_{10}cfu/g of brain of treated mice	Day of euthanasia
	CLSI	Etest®				
HMM 12-14	0.125	0.094	40.0	4.287 ± 0.12	1.493 ± 0.51*	+7
HMM 12-03	0.25	0.094	35.0	4.37 ± 1.12	1.63 ± 1.25*	+7
FMR 13015	0.25	0.125	30.0	6.83 ± 0.05	5.29 ± 0.53*	+5
FMR 13011	0.25	0.125	35.0	6.11 ± 0.61	2.68 ± 1.01*	+7
HMM 12-04	0.25	0.125	35.0	ND	ND	ND
HMM 12-34	0.25	0.094	30.0	ND	ND	ND
HMM 11-33	0.5	0.19	25.0	5.25 ± 1.25	2.66 ± 0.65*	+6
HMM 12-36	0.5	0.38	20.0	4.99 ± 0.6	2.94 ± 0.33*	+7
HMM 11-96	0.5	0.25	25.0	3.33 ± 1.8	0.87 ± 0.58*	+5
HMM 11-92	0.5	0.19	24.0	ND	ND	ND
HMM 11-97	0.5	0.19	22.0	ND	ND	ND
FMR 8856	0.5	0.25	25.0	ND	ND	ND
HMM 11-93	0.5	0.25	20.0	ND	ND	ND
HMM 11-87	0.5	0.25	25.0	ND	ND	ND
HMM 12-33	0.5	0.25	22.0	ND	ND	ND

HMM 11-36	0.5	0.38	25.0	ND	ND	ND
HMM 10-35	1.0	0.38	20.0	4.14 ± 1.01	2.82 ± 1.5	+5
HMM 11-86	1.0	0.75	25.0	5.43 ± 0.68	2.69 ± 1.45*	+5
HMM 10-34	1.0	1.5	30.0	3.14 ± 0.49	0.68 ± 0.15*	+7
HMM 11-34	1.0	0.25	25.0	ND	ND	ND
HMM 12-06	1.0	0.75	22.0	ND	ND	ND
FMR 9155	1.0	0.5	20.0	ND	ND	ND
HMM 10-38	1.0	1.5	24.0	ND	ND	ND
FMR 6922	2.0	1.0	20.0	5.18 ± 0.68	3.85 ± 0.61*	+5
HMM 10-31	2.0	1.0	18.0	5.75 ± 0.92	3.49 ± 1.4*	+5
HMM 11-47	2.0	1.5	0.0	4.2 ± 0.33	3.5 ± 0.35*	+7
UANL-PL071	2.0	1.0	20.0	ND	ND	ND
FMR 9167	2.0	1.0	22.0	ND	ND	ND
UANL-OC149	4.0	0.75	0.0	6.3 ± 0.22	5.13 ± 0.13*	+5
FMR 8869	4.0	0.5	0.0	4.64 ± 0.64	3.55 ± 0.88	+7
HMM 12-09	8.0	> 32.0	0.0	5.47 ± 0.67	5.1 ± 0.84	+5

HMM, Section for Hygiene and Medical Microbiology, Medical University of Innsbruck, Austria; FMR, Facultat de Medicina de Reus, Spain; UANL, Universidad Autónoma de Nuevo León, Mexico; MIC, minimum inhibitory concentration; IZD, Inhibition zone diameter; S, susceptible; R, resistant; MFC, minimal fungicidal concentration.

* Voriconazole reduced significantly fungal burden in brain

[a] Microdilution method was performed according to the document M38-A2 from CLSI (Clinical and Laboratory Standards Institute 2008) and Etest® (Biomérieux) according to manufacturer's instructions. Strains were considered susceptible if MIC ≤ 1 mg/L, intermediate if MIC = 2 mg/L and resistant if MIC ≥ 4 mg/L. (A Espinel-Ingroff *et al.* 2007)

[b] Disk diffusion method was performed following the recommendations of the document M51-A of CLSI. (Clinical and Laboratory Standards Institute 2010a) Strains were classified as susceptible if IZD ≥ 17 mm, intermediate if IZD was between 14 and 16 mm, and resistant if IZD was ≤ 13 mm. (A Espinel-Ingroff *et al.* 2007)

* Reduction in brain of treated mice was statistically significant in comparison to the control group ($p \leq 0.05$).

SUPPLEMENTARY MATERIAL

Table S1. Virulence study and survival data expressed as range of days of mice mortality and mean survival time, and kidney fungal burden from mice treated with voriconazole at 40 mg/kg and without treatment.

strain	inocula (cfu/animal)	mortality (range in days) (control group) (MST)	mortality (range in days) (treated group) (MST)	FB in kidney (control group)	FB in kidney (treated group)
HMM 12-14	$5x10^3$	no mortality at day +20	ND	ND	ND
	$1x10^4$	5 - 13 (9.5)	11 - 15 (12.33)*	5.27 ± 0.51	3.45 ± 1.1*
HMM 12-03	$1x10^4$	7 - 14 (9.38)	8 - 20 (13.63)*	5.321 ± 0.34	2.7 ± 1.02*
FMR 13015	$7x10^3$	5 - 7 (5.33)	7 - 10 (8.66)*	5.77 ± 0.35	4.55 ± 0.5*
FMR 13011	$5x10^3$	no mortality at day +20	ND	ND	ND
	$1x10^4$	6 - 17 (9)	6 - 20 (14.6)*	5.46 ± 0.28	4.13 ± 0.28*
HMM 11-33	$5x10^3$	6 - 17 (9.25)	12 - 20 (15.63)*	5.12 ± 0.73	3.54 ± 1.1*
HMM 12-36	$5x10^3$	7 - 20 (16.5)	ND	ND	ND
	$1x10^4$	5 - 8 (6.17)	7 - 11 (8.6)*	4.64 ± 0.91	3.425 ± 0.55*
HMM 11-96	$5x10^3$	7 - 20 (16.13)	ND	ND	ND
	$7x10^3$	5 - 10 (6.67)	10 - 13 (11.8)*	5.547 ± 0.56	2.37 ± 0.64*
HMM 11-86	$5x10^3$	11 - 20 (17.5)	ND	ND	ND
	$7x10^3$	5 - 11 (8.33)	11 - 13 (11.5)*	4.85 ± 0.82	3.38 ± 1.11
HMM 10-34	$5x10^3$	7 - 12 (10.38)	13 - 20 (17.63)*	5.15 ± 0.69	4.28 ± 1.1*
FMR 6922	$5x10^3$	6 - 20 (7)	6 - 20 (11.5)	5.194 ± 0.6	3.8 ± 0.93*

	$5x10^4$	4 - 8 (5.4)	ND	ND	ND
HMM 10-31	$5x10^3$	5 - 14 (6.5)	6 - 20 (11.63)	5.27 ± 0.75	4.83 ± 0.5
HMM 11-47	$5x10^3$	12 - 20 (17.38)	ND	ND	ND
	$7x10^3$	5 - 11 (9)	10 - 18 (13..63)*	4..11 ± 0..22	3..79 ± 0..56
UANL OC-149	$5x10^3$	5 - 7 (5.66)	6 - 11 (8)*	5.24 ± 0.94	4.01 ± 0.55*
FMR 8869	$5x10^3$	7 - 11 (8.83)	5 - 20 (14.6)*	5.48 ± 0.36	4.5 ± 0.7*
HMM 12-09	$5x10^3$	5 - 8 (7.6)	4 - 12 (8.28)	5.22 ± 0.72	4.48 ± 0.5
	$5x10^4$	3 - 5 (4)	ND	ND	ND

MST, mean survival time; ND, not determined

Those inocula painted in brown were not used due to the fast or too low mortality.

Table S2. Review on the literature of voriconazole doses administered in mice and serum levels achieved.

dose (mg/kg)	VRC concentration (mg/L)	time of drug determination	mouse strain	reference
20	1.8 4.4 5.8	Day +5 Day +7 Day +10	ICR	Sugar & Liu 2000. PMID: 10892988
5 10 20 40	1.9 (Cmax); 0.15 (Cmin) 5 (Cmax); 0.07 (Cmin) 12.3 (Cmax); 0.06 (Cmin) 40.1 (Cmax); 0.24 (Cmin)		CD-1	Seyedmousavi *et al* 2013. DOI: 10.1093/jac/dks402
20 40	5.8 ± 0.94 8.2 ± 2.25	24h after 12th dose	OF-1	Fernandez-Silva *et al* 2013. DOI: 10.1111/myc.12112
10 25 40	2.89 ± 0.55 5.37 ± 0.32 7.77 ± 0.84	24h after 10th dose	OF-1	Sanchis *et al* 2016. DOI: 10.1016/j.diagmicrobio.2015.09.010
25	9.99 ± 0.71	2h after 6th dose	OF-1	Martin-Vicente *et al* 2015. DOI: 10.1128/AAC.01462-15.
60	6.71	4h after the 5th dose	OF-1	Rodriguez *et al* 2009. DOI: 10.1128/AAC.01477-08
15 BID	3.6 (peak level); 0.7 (2h after 3rd dose)	Day +3	CD-1	Majithiya *et al* 2009. DOI: 10.1093/jac/dkn431
5 20	1.6 ± 0.2 1.6 ± 0.2	Day +11	BALB/cByJ	Sugar 2001 (DOI: 10.1128/AAC.45.2.601–604.2001)

40	4.7 ± 0.2			
10	0.47 ± 0.10	1h after 1st dose	ICR/Swiss	Andes *et al* 2003. DOI: 10.1128/AAC.47.10.3165–3169.2003
20	1.67 ± 0.69			
40	6.9 ± 2.4			
10	1.61 (Cmax); 0.36 (Cmin)		CD-1	Mavridou 2010. DOI: 10.1128/AAC.00606-10
40	11.81 (Cmax); 4.58 (Cmin)			
80	35.86 (Cmax); 23.01 (Cmin)			
25	5.65 ± 0.95	24 h after the 10th dose	OF-1	Sanchis *et al* 2014. DOI:10.1128/AAC.03051-14

4.4 Has a triple combination better activity than doubles against multiresistant fungi? Experimental *in vitro* evaluation.

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Has a triple combination better activity than doubles against multiresistant fungi? Experimental *in vitro* evaluation

Running title: Triple combination against multiresistant fungi

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Abstract

We have evaluated the *in vitro* interactions of amphotericin B, voriconazole and anidulafungin in double and triple combinations against four species of multiresistant fungi, i.e. *Fusarium solani, Lomentospora prolificans, Scopulariopsis brevicaulis* and *Scopulariopsis brumptii* using fractional inhibitory concentration indices inferred by the checkerboard method. In general, amphotericin B combined with anidulafungin was the most synergistic against all the species tested, and especially against *F. solani* (87.5%). The triple combination was also highly synergistic against *F. solani* and *S. brevicaulis*. The triple combination was more effective than the double combinations in some cases, but not against all the strains, suggesting that the administration of three drugs is not always useful in the treatment of infections due to multiresistant fungi.

Keywords: antifungal resistance, antifungal combinations, *F. solani, L. prolificans, S. brevicaulis, S. brumptii, in vitro,* FICI

1. Introduction

Fungal opportunistic infections have increased over the past two decades as a result of the rising number of immunocompromised patients. *Candida* and *Aspergillus* are the major human fungal pathogens although many other species have recently been involved in human infections [1]. Among the moulds, clinically important species of *Fusarium*, *Scedosporium* and *Scopulariopsis* are intrinsically resistant to antifungal drugs, including the most recent ones such as voriconazole (VRC), posaconazole (PSC) or echinocandins. The infections by multiresistant fungi have increased in recent years and the poor outcome of monotherapies together with the high mortality rates make necessary to explore new therapies.

Fusarium solani is a hyaline mould, widely found in nature that causes a broad spectrum of human infections. Their clinical consequences depend on the immune status of the host and the portal of entry, mainly by conidia inhalation, followed by trauma [2]. Onychomycosis and keratitis are the most frequent fungal diseases caused by this fungus in the immunocompetent population, while in immunocompromised patients, especially in those with acute leukaemia and in HSCT recipients, often with haematogenous dissemination, is the typical manifestation. The most challenging and life-threatening disease is disseminated infection with an estimated mortality rate of up to 75%. Management of fusariosis has changed over the last decade, with an increasing use of VRC and combination therapies that have had a better outcome, although the mortality rate remains high [3].

Lomentospora prolificans (formerly *Scedosporium prolificans*) is a ubiquitous filamentous fungus present in soil, sewage and polluted waters. Diseases produced by this organism range from cutaneous and subcutaneous soft

tissue infections, with predilection for cartilage and joint areas, to profound and disseminated diseases. In the immunocompromised population, therapy with corticosteroids and neutropenia are important risk factors in the development of disseminated disease [4]. These infections are usually associated with poor outcomes and mortality rates of more than 75% [5](Kelly et al. 2016).

Scopulariopsis includes both hyaline and dematiaceous moulds. These fungi are usually saprobic and commonly isolated from soil, air, plant debris and moist indoor environments [6]. In immunocompetent patients, *Scopulariopsis* is associated mainly with nail infections, but it occasionally causes cutaneous lesions following trauma or surgery, and invasive diseases, such as endocarditis, sinusitis, brain abscess, deep cutaneous, localized pulmonary, and disseminated infections [7] in all types of patients. These are almost invariably fatal, mainly due to the underlying conditions, delayed diagnosis, and a high level of resistance of this fungus to conventional antifungal agents. Although *S. brevicaulis* is the most prevalent species, other species of the genus, like *S. brumptii* have been associated with human disease too [6].

In vitro studies have repeatedly shown that these species are resistant to almost all the current antifungal drugs [6,8]. VRC is recommended as the first-line treatment for fusariosis and scedosporiosis, but a treatment regimen has not been established for infections caused by *Scopulariopsis* spp. [9] because infections are rare. Most patients diagnosed with a fungal infection are usually treated first with amphotericin B (AMB), its lipid formulations or azoles. However, in many cases due to the worsening condition of the patients, clinicians quickly change the treatment and double combinations used as alternatives although, generally, results do not improve. Combined therapy is considered to increase efficacy, minimize

toxicity and lower the cost of the therapy by reducing the dosages of individual drugs. In this sense, some efficacy has been observed for VRC plus terbinafine (TBF) against *L. prolificans* but only against localized infections [10-12]. In the same way, AMB plus TBF and VRC plus caspofungin (CFG) showed some efficacy in a few cases of infection by *S. brevicaulis* [13-14].

The limited efficacy of the available antifungal drugs against these important fungal pathogens makes it crucial to find alternative therapies. For this reason, we considered it of interest to investigate the *in vitro* interactions among AMB, AFG and VRC in double, as well as in triple, combinations against relevant multiresistant fungi such as *F. solani, S. brevicaulis, S. brumptii* and *L. prolificans*. We chose three drugs belonging to some families of antifungals which have different mechanisms of action, hypothesizing that combinations might produce synergistic interactions against such pathogens. AMB belongs to the oldest class of antifungals (polyenes). It still has an important clinical role due to its broad spectrum and the rarity in developing resistance but its use is very limited due to a substantial nephrotoxicity. VRC is an orally available triazole with potent activity against a range of medically important fungal pathogens [9,15], although drug interactions and neural and hepatic toxicity are the primary concerns to its use [16]. Finally, anidulafungin (AFG) belongs to echinocandins, the most recent class of antifungals, and has several advantages over the other members of this family and other antifungals. It presents few adverse events, does not require dosing adjustments or induce cross-resistance with other classes of antifungals [17]. Echinocandins have good activity against *Aspergillus* spp. but they are inactive against *Cryptococcus* spp. and non-*Aspergillus* moulds. Their use in combination with AMB has shown good *in vitro* results [18,19].

2. Materials and methods

2.1. Drugs and strains

The *in vitro* activity of AMB (Sigma Chemical Co. St. Louis, USA), VRC (Pfizer Inc., Madrid, Spain) and AFG (Pfizer Inc., Madrid, Spain), was tested alone, and in double and triple combinations against 38 clinical fungal isolates, i.e. 11 *L. prolificans*, 8 *F. solani*, 11 *S. brevicaulis* and 8 *S. brumptii*. Three reference strains, *Candida krusei* ATCC 6258, *C. parapsilosis* ATCC 22019 and *Aspergillus fumigatus* ATCC MYA 3626, were included as quality controls.

The isolates were grown at 30 ºC on potato dextrose agar (PDA) until sporulation occurred in the case of filamentous fungi i.e., from 7 to 10 days depending on the species. Inocula were obtained by flooding the plates with sterile saline and conidia were harvested with a sterile pipette. The suspensions were adjusted to the desired concentrations by haemocytometer counts and viability assessed by placing 10-fold dilutions onto PDA plates.

2.2. Antifungal activity assays

Single susceptibility testing of the isolates was carried out following the broth microdilution method according to the CLSI document M38-A2 [20]. After 48 h at 35 ºC, MICs of AMB and VRC were visually read with the aid of an inverted mirror and corresponded to the 100% of growth inhibition, while the MEC of AFG was read with the aid of a stereomicroscope as the minimum concentration to produce an abnormal hyphal growth.

The activity of double combinations i.e, AMB + VRC, AMB + AFG and VRC + AFG, was tested by a two-dimensional checkerboard as previously

described [21]. Two-fold dilutions of each drug at concentrations ranging from 1 to 64 μg/mL for AMB (0.06 to 4 μg/mL against *F. solani*), 0.5 to 256 μg/mL for VRC and 2 to 128 μg/mL for AFG were used. The concentrations of the drugs were selected on the basis of the previously determined MICs and MECs.

The triple combination was tested by a three-dimensional checkerboard technique, i.e. a checkerboard with two-fold dilutions of AMB and VRC was set up as described above for the double combinations, the same concentration of AFG then being added to each plate. The concentrations of AFG tested were 0.06, 0.25, 1.0 and 4 μg/mL.

For the combination AMB + VRC, 100% of growth inhibition or MIC-0 was chosen as endpoint. However, the most appropriate endpoint for echinocandins against moulds has been determined to be the MEC, which corresponds to MIC-2 (50% growth inhibition) [18]. Therefore, considering that the endpoint for the combined drugs must be the same, in those combinations containing AFG i.e. AMB + AFG, VRC + AFG and AMB + VRC + AFG, we used the MIC-2.

The fractional inhibitory concentration indices (FICI) of the double combinations were calculated as follows: FICI = (MIC_{drugA} in combination/MIC_{drugA} alone) + (MIC_{drugB} in combination/MIC_{drugB} alone). For the triple combination, the third parameter MIC_{drugC} in combination/MIC_{drugC} alone, was added. Drug interactions were defined as synergistic if the lowest FICI was ≤ 0.5, indifferent (i.e., no interaction) if the lowest FICI was > 0.5 and ≤ 4, and antagonistic if the highest FICI was > 4. For the calculations, the high off-scale MICs were converted to the next highest concentration. Every isolate was assayed twice.

3. Results

3.1. Combinations against *F. solani*

Table S1 summarizes the MICs of AMB, AFG and VRC alone, the lowest FICIs and the corresponding MICs of the drugs in combination against the *F. solani* isolates. All the strains were highly resistant to VRC (16 to > 256 μg/mL) and AFG (≥ 128 μg/mL) but, by contrast, they showed lower AMB MICs (1 to 8 μg/mL). All the double combinations showed a high percentage of synergy against this species, AFG plus VRC and AMB plus AFG being the most active (87.5%). AMB combined with VRC showed 62.5% of synergy, but concentrations of VRC ≥ 16 μg/mL were needed to achieve that. The triple combination showed 87.5% synergy. Antagonism was not observed in any case.

3.2. Combinations against *L. prolificans*

Results of the *in vitro* susceptibility testing of all interactions for every *L. prolificans* strain are given in supplementary material (Table S2). The highest percentage of synergy was observed for the combination AMB + AFG (72.7%), while the lowest was for the combination of AMB + VRC (45.5%), for which very high concentrations of AMB and VRC were needed to achieve the lowest FICI in some strains. For example, for strain FMR 9799, maximum synergy was observed when 4 μg/mL of AMB was combined with 16 μg/mL of VRC; however, these concentrations are not recommended due to their possible toxicity (Table S1).

When AFG was combined with VRC, synergistic interactions were found against 5 of the 11 *L. prolificans* isolates tested (54.5%). The interaction between the three drugs was synergistic for 7 of them (63.6%) and indifferent for 4 strains (36.4%) (Table 1). In general, the most synergistic triple combination was with the lowest concentrations of AMB (1 μg/mL)

(Table S1). The benefit of the triple combination over the double ones was clearly demonstrated in the strains FMR 6641, FMR 6721 and FMR 9798, leading to the use of lower concentrations. However, this benefit was not always so evident, suggesting that, in general, the addition of a third antifungal might not improve treatment. Antagonism was not observed in any case.

3.3. Combinations against *S. brevicaulis*

The MICs of AMB, VRC and AFG against each strain of *S. brevicaulis*, and the lowest FICIs achieved with the double and triple combinations are summarized in Table S3. The double combination with the highest percentage of synergy was AFG + AMB (81.8%) (Table 1). AFG combined with VRC produced synergistic interactions against 8 of the 11 strains tested (72.7%) and indifference against 3 strains (27.3%) (Table 1). Interestingly, with this combination, antagonism was also observed against three strains of *S. brevicaulis* i.e. FMR 12246, FMR 12260 and FMR 12270, indicating that the same combination can give contrasting results depending on the concentrations used (data not shown) and as previously reported. For these isolates, indifference was obtained with AMB at concentrations of 4 - 16 μg/mL, whereas antagonistic interactions were observed with higher concentrations of AMB (≥ 32 μg/mL).

The triple combination was 81.8% synergistic, although it did not show any advantage over the AMB plus AFG in some cases. For example, against FMR 12258, the lowest FICI of the triple combination was achieved when AMB, VRC and AFG concentrations were 2, 4 and 4 μg/mL, respectively. However, the combination AMB + AFG was synergistic with 0.125 μg/mL of AMB plus 4 μg/mL of AFG, suggesting that the double combination is as effective as the triple.

3.4. Combinations against *S. brumptii*

Table S4 summarizes the MICs of the antifungal drugs and the FICIs of the combinations against the 8 *S. brumptii* strains. The double combination with the highest synergy was AMB plus AFG (62.5%), followed by AMB plus VRC (50%) and AFG plus VRC (37.5%). The triple combination was synergistic in 4 strains (50%), having, in most cases, the lowest FICIs with the lowest concentration of either AMB or VRC and being better than the double combinations (Table S4).

Overall, AMB plus AFG was the most synergistic combination against the four multiresistant species, being even better than the triple combination. On the contrary, the combination that showed less synergistic interactions was AMB plus VRC.

4. Discussion

The prevalence of infections caused by multiresistant fungi has increased in recent years and is becoming an important matter of concern due to their difficult management and poor outcome. Monotherapies usually fail in the treatment of these infections because of the limited range of activity of the current antifungals. Since antifungal compounds are not effective enough for most infections, the combination of surgery and antifungal drugs is a common choice [22]. A combination of two drugs is recommended for the treatment of some fungal diseases like cryptococcal meningoencephalitis [23] and there is little clinical experience with triple combinations, although they have sometimes been used as salvage therapy, as in some refractory aspergillosis [24-26]. Triple combinations might be useful for some multiresistant infections. In the present study, we chose four of the most resistant fungi, against which no therapy has been established. We found

high synergy for the combinations tested, especially for AMB + AFG, which, in some cases, was better than the triple combination.

Against *F. solani*, in particular, synergy was high for all the combinations tested, i.e. nearly 90% for AFG + VRC, AMB + AFG and the triple combination, suggesting a potential role of these combinations in the treatment of fusariosis. This agrees with the results of a previous *in vitro* study that reported additive to synergistic interactions between AMB and VRC against *F. solani* isolates [27] and some degree of efficacy of the same combination in a murine model of disseminated infection by this fungus [28]. However, for most of the strains tested, synergy was achieved at concentrations of VRC that were not in the range of the levels achievable in serum, i.e. 16 or 32 μg/mL (Table S1).

AMB was synergistic with AFG in this study, and with CFG against the 82% of *F. solani* strains tested in another study [27]. The latter combination was reported to be effective in reducing fungal burden in a murine model of disseminated *F. solani* infection [29]. However, the combination of AMB with micafungin (MFG) did not show *in vivo* efficacy against this fungus, suggesting that the effect of the combinations of AMB with echinocandins seem to depend on which echinocandin is tested [28]. AFG, when used alone, has poor *in vitro* activity against *F. solani* but the combination of VRC with AFG or MFG has shown synergy *in vitro* and in murine models [28, 30, 31].

In the clinical setting, it is of note that the outcome of invasive fusariosis has significantly improved since the recent use of VRC and combined therapies. Some clinical cases have reported favourable responses in patients with haematological malignancies treated with AMB or its lipid

formulations plus CFG or VRC, highlighting the potential of these combinations [32].

The combined activity of antifungal agents against *L. prolificans* has rarely been evaluated. It has been demonstrated that VRC in combination with AMB or AFG showed synergy in a small percentage of strains while AMB plus AFG produced indifferent interactions in all the cases [33]. Our results are quite different since a high rate of synergy was observed for this latter combination while around 50% of synergy/indifference was obtained for the other double combinations assayed. The present results correlate better with Yustes and Guarro, who reported synergistic interactions between AMB and MFG against 14 of 17 (82%) *L. prolificans* strains tested [19]. Previously, only a triple antifungal combination had been tested *in vivo* against *L. prolificans* [34]. In that study, AMB, MFG and VRC were tested alone, in double and in triple combinations in a murine model of disseminated infection after showing *in vitro* synergy only for the triple combination; however, the *in vivo* efficacy of that triple combination was worse than for MFG plus AMB or VRC [34]. Similar combinations have been used against *L. prolificans* infections with some therapeutic success with VRC plus CFG [35], a result that agrees with our *in vitro* interaction results, but not for the combinations VRC + LAMB and itraconazole (ITC) plus MFG [36]. Due to the small number of clinical cases, conclusions about the *in vitro – in vivo* correlation cannot be made.

Several antifungal combinations have been tested *in vitro* against *S. brevicaulis*, resulting in a high percentage of indifference for the combination AMB + VRC, and more than 50% of synergy for CFG + AMB [37], results that agree with ours. Combined antifungal therapy is often used for the treatment of *Scopulariopsis* infections with different outcomes, but combinations of more than two drugs are rarely used. An AMB lipid

complex in combination with ITC and VRC plus CFG have shown *in vivo* synergy in patients with haematological malignancies [13,38], but other studies report therapeutic failure of the latter combination and for LAMB with CSP, VRC or MFG [39]. To our knowledge, only one triple combination has been tested *in vitro* against *Scopulariopsis* and *Microascus* species. PSC, CFG and TBF showed synergy against 100% of the *S. brevicaulis* strains [40]. In our case, the triple combination achieved 81.8% synergy against *S. brevicaulis* and a modest 50% in the case of *S. brumptii*. Animal studies are needed to prove the *in vivo* efficacy of these combinations.

In the present study, we used a checkerboard method that, although it has not been standardized for testing moulds, has the advantage of simplicity in performance and interpretation. Some of our results are controversial in comparison to other *in vitro* studies testing the same double combinations. This can be due to many models and approaches having been described for testing *in vitro* drug interactions. It is also known that, even when the same methodology is used for testing the *in vitro* activities of drug combinations, variable conclusions might be made, depending on the way data is analyzed and interpreted.

In conclusion, our data demonstrates that powerful interactions are achievable with AMB, VRC and AFG against clinically relevant multiresistant fungi and their combinations show interesting results. We have also found that the triple combination is not always better than a double one. Further animal studies are required to demonstrate their possible efficacy.

Conflicts of interest

None.

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Ethical approval

Not required

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Table 1. MIC/MEC and FICI results of the interaction of double and triple combinations against *L. prolificans, F. solani, S. brevicaulis* and *S. brumptii*.

Species	Single susceptibility[a]						Interaction assay[b]							
	MIC-0 range (µg/ml)			MIC-2 range (µg/ml)			AMB+VRC		AFG+VRC		AFG+AMB		AMB+VRC+AFG	
	AMB	VRC	AFG	AMB	VRC	AFG	FICI range	No (%) of strains for which the combination showed synergism	FICI range	No (%) of strains for which the combination showed synergism	FICI range	No (%) of strains for which the combination showed synergism	FICI range	No (%) of strains for which the combination showed synergism
F. solani (n = 8)	1 - 8	16 - > 256	> 128	0,5 - 2	4 - 8	128 - > 128	0,16 - 0,31	5 (62,5)	0,13 - 0,63	7 (87,5)	0,14 - 0,51	7 (87,5)	0,2 - 0,66	7 (87,5)
L. prolificans (n = 11)	4 - > 128	32 - 64	> 128	2 - 64	4 - 16	4 - 16	0,19 - 1,03	5 (45,5)	0,16 - 1	6 (54,5)	0,07 - 0,56	8 (72,7)	0,17 - 0,64	7 (63,6)
S. brevicaulis (n = 11)	8 - 128	16 - 256	> 128	4 - 32	4 - 32	8 - > 128	0,31 - 1	2 (18,2)	0,04 - 0,75	8 (72,7)	0,02 - 2	9 (81,8)	0,14 - 0,63	9 (81,8)
S. brumptii (n = 8)	4 - 128	4 - 32	> 128	2 - 16	2 - 4	8 - 32	0,19 - 1	3 (37,5)	0,25 - 2,06	3 (37,5)	0,16 - 1,5	5 (62,5)	0,25 - 0,78	4 (50)

[a] MICs and MECs were determined following the recommendations of the document M38-A2 of CLSI [20]. [b] Interaction assay was performed as previously described [21]. AMB, amphotericin B; VRC, voriconazole; AFG, anidulafungin; FICI, fractional inhibitory concentration index.

Supplementary material

Table S1. MIC/MEC and FICI results of the interaction of double and triple combinations against 8 isolates of *F. solani*

Species	Strain	Single susceptibility[a]						Interaction assay[b]							
		MIC (μg/ml) of drug alone			MIC-2 (μg/ml) of drug alone			AMB+VRC		AFG+VRC		AFG+AMB		AMB+VRC+AFG	
		VRC	AMB	AFG	VRC	AMB	AFG (MEC)	Lowest FICI	Concentration at lowest FICI (AMB/VRC) (μg/ml)	Lowest FICI	Concentration at lowest FICI (AFG/VRC) (μg/ml)	Lowest FICI	Concentration at lowest FICI (AFG/AMB) (μg/ml)	Lowest FICI	Concentration at lowest FICI (AMB/VRC/AFG) (μg/ml)
F. solani	4391	16	1	> 128	4	0,5	> 128	1	0,5/8	0,31	64/0,5	0,28	8/0,125	0,25	0,125/1/0,06
	7238	> 256	2	> 128	8	1	> 128	0,31	0,5/32	0,27	4/2	0,19	16/0,125	0,2	0,06/1/4
	7242	> 256	1	> 128	4	0,5	> 128	0,28	0,25/16	0,31	16/1	0,16	8/0,06	0,39	0,125/0,5/4
	8340	128	2	> 128	8	1	> 128	0,25	0,25/16	0,19	16/1	0,51	2/0,5	0,39	0,06/2/4
	8634	32	8	> 128	4	2	> 128	0,63	4/4	0,63	16/2	0,27	2/0,5	0,66	1/0,5/4
	11950	256	2	> 128	8	1	128	0,25	0,25/32	0,27	2/2	0,27	2/0,25	0,28	0,125/1/4
	12773	> 256	2	> 128	8	1	> 128	0,16	0,25/16	0,16	8/1	0,14	4/0,125	0,27	0,125/1/4
	13932	> 256	1	> 128	8	1	> 128	0,53	0,5/16	0,125	16/0,5	0,14	4/0,125	0,5	0,25/2/0,06
	Synergism							62,50%		87,50%		87,50%		87,50%	
	Indifference							37,50%		12,50%		12,50%		12,50%	
	Antagonism							0%		0%		0%		0%	

[a] MICs and MECs were determined following the recommendations of the document M38-A2 of CLSI [20].
[b] Interaction assay was performed as previously described [21].
AMB, amphotericin B; VRC, voriconazole; AFG, anidulafungin; FICI, fractional inhibitory concentration index.

Table S2. MIC/MEC and FICI results of the double and triple combinations against 11 isolates of *L. prolificans*.

Species	Strain	Single susceptibility[a]						Interaction assay[b]							
		MIC-0 (µg/ml) of drug alone			MIC-2 (µg/ml) of drug alone			AMB+VRC		AFG+VRC		AFG+AMB		AMB+VRC+AFG	
		VRC	AMB	AFG	VRC	AMB	AFG (MEC)	Lowest FICI	Concentration at lowest FICI (AMB/VRC) (µg/ml)	Lowest FICI	Concentration at lowest FICI (AFG/VRC) (µg/ml)	Lowest FICI	Concentration at lowest FICI (AFG/AMB) (µg/ml)	Lowest FICI	Concentration at lowest FICI (AMB/VRC/AFG) (µg/ml)
L. prolificans	6641	32	> 128	> 128	16	32	8	0,51	2/16	0,63	4/2	0,53	4/1	0,125	1/1/0,25
	6647	64	16	> 128	16	8	8	0,19	2/4	0,38	2/2	0,31	2/0,5	0,26	1/2/0,06
	6720	32	8	> 128	8	4	16	0,63	1/16	0,19	4/0,5	0,16	4/0,125	0,63	1/1/4
	6721	32	128	> 128	16	64	16	0,56	8/16	0,5	4/4	0,07	1/4	0,17	2/2/0,25
	7250	32	64	> 128	8	8	8	0,52	1/16	1	4/4	0,375	2/1	0,38	1/2/0,06
	9796	32	128	> 128	8	64	8	0,28	4/8	0,75	4/2	0,52	4/1	0,63	8/1/1
	9797	32	> 128	> 128	8	32	16	0,31	8/16	0,375	2/2	0,25	4/0,125	0,34	1/0,5/4
	9798	32	128	> 128	8	16	8	1,03	4/32	0,75	4/2	0,38	2/2	0,26	2/1/0,06
	9799	64	128	> 128	4	32	4	0,28	4/16	0,75	2/1	0,56	2/2	0,51	1/2/0,06
	9800	32	64	> 128	8	8	16	0,31	4/8	0,375	2/2	0,27	4/0,125	0,44	1/0,5/4
	9801	64	4	> 128	16	2	16	0,75	2/16	0,16	2/0,5	0,38	4/0,25	0,64	1/2/0,25
	Synergism							45,50%		54,50%		72.7%		63,60%	
	Indifference							54,50%		45,50%		27.3%		36,40%	
	Antagonism							0%		0%		0%		0%	

[a] MICs and MECs were determined following the recommendations of the document M38-A2 of CLSI [20].

[b] Interaction assay was performed as previously described [21].

AMB, amphotericin B; VRC, voriconazole; AFG, anidulafungin; FICI, fractional inhibitory concentration index.

Table S3. MIC/MEC and FICI results of the interaction of double and triple combinations against of 11 isolates of *S. brevicaulis*

Species	Strain	Single susceptibility[a]						Interaction assay[b]							
		MIC-0 (μg/ml) of drug alone			MIC-2 (μg/ml) of drug alone			AMB+VRC		AFG+VRC		AFG+AMB		AMB+VRC+AFG	
		VRC	AMB	AFG	VRC	AMB	AFG (MEC)	Lowest FICI	Concentration at lowest FICI (AMB/VRC) (μg/ml)	Lowest FICI	Concentration at lowest FICI (AFG/VRC) (μg/ml)	Lowest FICI	Concentration at lowest FICI (AFG/AMB) (μg/ml)	Lowest FICI	Concentration at lowest FICI (AMB/VRC/AFG) (μg/ml)
S. brevicaulis	12216	256	64	> 128	16	16	> 128	0,5	16/0,5	0,19	16/2	0,06	8/0,5	0,2	1/2/4
	12246	16	16	> 128	4	4	8	0,75	4/8	0,75	2/2	2	8/4	0,63	1/1/1
	12257	64	16	> 128	16	8	> 128	0,51	8/0,5	0,14	4/1	0,16	8/1	0,5	2/2/1
	12258	64	32	> 128	16	16	> 128	0,75	8/32	0,19	16/2	0,02	4/0,125	0,39	2/4/4
	12260	64	128	> 128	32	32	8	0,53	4/32	0,75	4/8	0,5	2/8	0,32	4/8/0,06
	12262	64	16	> 128	32	8	32	1	8/32	0,31	2/8	0,25	4/1	0,5	2/8/0,06
	12270	64	32	> 128	32	16	8	0,75	8/32	0,63	4/4	0,31	2/1	0,41	4/4/0,25
	12272	64	16	> 128	16	8	> 128	0,75	8/16	0,04	2/0,5	0,02	2/0,125	0,2	1/1/4
	12273	32	8	> 128	8	4	128	0,52	4/0,5	0,13	8/0,5	0,09	8/0,125	0,34	1/0,5/4
	12275	64	64	> 128	8	16	16	0,31	4/16	0,5	4/2	0,53	8/0,5	0,56	1/2/4
	12276	128	32	> 128	16	16	> 128	0,63	16/16	0,09	16/0,5	0,19	16/2	0,14	2/0,5/4
	Synergism							18,20%		72,70%		81,80%		81,80%	
	Indifference							81,80%		27,30%		18,20%		18,20%	
	Antagonism							0%		0%		0%		0%	

[a] MICs and MECs were determined following the recommendations of the document M38-A2 of CLSI [20].

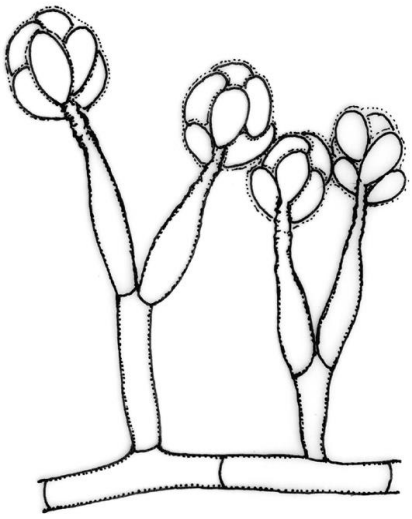
[b] Interaction assay was performed as previously described [21].

AMB, amphotericin B; VRC, voriconazole; AFG, anidulafungin; FICI, fractional inhibitory concentration index.

Table S4. MIC/MEC and FICI results of the interaction of double and triple combinations against 8 isolates of *S. brumptii*

Species	Strain	Single susceptibility[a]						Interaction assay[b]							
		MIC (µg/ml) of drug alone			MIC-2 (µg/ml) of drug alone			AMB+VRC		AFG+VRC		AFG+AMB		AMB+VRC+AFG	
		VRC	AMB	AFG	VRC	AMB	AFG (MEC)	Lowest FICI	Concentration at lowest FICI (AMB/VRC) (µg/ml)	Lowest FICI	Concentration at lowest FICI (AFG/VRC) (µg/ml)	Lowest FICI	Concentration at lowest FICI (AFG/AMB) (µg/ml)	Lowest FICI	Concentration at lowest FICI (AMB/VRC/AFG) (µg/ml)
S. brumptii	12210	16	16	> 128	4	8	16	0,625	2/8	1	8/2	0,5	4/4	0,31	4/0,5/1
	12215	32	16	> 128	2	2	32	0,19	2/2	2,06	2/4	0,19	4/0,12	0,78	1/0,5/1
	12229	16	16	> 128	4	8	8	0,75	4/8	0,75	4/1	1,5	4/8	0,66	1/2/0,25
	12240	8	16	> 128	4	8	32	1	8/4	0,25	4/0,5	0,31	8/0,12	0,25	1/0,5/0,06
	12241	16	4	> 128	2	4	8	0,5	1/4	0,5	2/0,5	0,63	4/0,5	0,51	1/0,5/0,06
	12248	4	16	> 128	4	8	16	0,56	1/2	0,38	2/1	0,16	2/0,25	0,25	1/0,5/0,06
	12256	8	128	> 128	4	16	16	0,56	8/4	1	8/2	0,63	2/8	0,5	2/0,5/4
	12263	32	64	> 128	4	4	8	0,25	8/4	0,75	4/1	0,38	2/0,5	0,53	1/1/0,25
	Synergism							50%		37,50%		62,50%		50%	
	Indifference							50%		62,50%		37,50%		50%	
	Antagonism							0%		0%		0%		0%	

[a] MICs and MECs were determined following the recommendations of the document M38-A2 of CLSI [20].
[b] Interaction assay was performed as previously described [21].
AMB, amphotericin B; VRC, voriconazole; AFG, anidulafungin; FICI, fractional inhibitory concentration index.



5. DISCUSSION

5. Discussion

5.1 *Aspergillus fumigatus*

Invasive aspergillosis (IA) remains the most frequent invasive mould disease in haematological patients, and the outcome of treatment is still poor even if antifungal therapy is used. This means that combination therapy is particularly interesting in patients with refractory infection or, even, as first-line therapy.

In our study, the interaction of amphotericin B with posaconazole showed FICIs between 0.56 and 0.73, and for the combination of anidulafungin plus posaconazole, the FICIs were 0.62, 0.75 and 1, for the strains FMR 10528, FMR 13142 and FMR 7739, respectively. Considering the traditional cutoffs, both combinations resulted in indifferent interactions for all the strains studied (Odds, 2003). These results agree with previous studies that also explored the antifungal interaction of triazoles with echinocandins or amphotericin B against *Aspergillus* spp. (Shalit *et al.*, 2003; Mavridou *et al.*, 2015; Ruíz-Cendoya *et al.*, 2008; Manavathu *et al.*, 2003). However, Meletiadis *et al.* proposed FICIs < 1 as indicative of synergism (Meletiadis *et al.*, 2010), which would mean that in our case the combination of amphotericin B plus posaconazole was synergistic against the three strains. In the case of the combination of posaconazole plus anidulafungin, the interaction would be synergistic against two strains and indifferent against the other. This interpretation of the FICI values correlates better with our *in vivo* results because both combinations showed high efficacy against all the strains tested: survival was higher and fungal loads lower than in controls (Martin-Vicente *et al.*, 2015) (Martin-Vicente *et al.*, unpublished).

As observed in the results of this thesis, *in vitro* and animal studies have favoured the use of novel triazoles combined with echinocandins to treat IA (Cacciapuoti *et al.*, 2006; Chandrasekar *et al.,* 2004; Elefanti *et al.*, 2013; Kirkpatrick *et al.*, 2002; Lepak *et al.,* 2013; Lewis & Kontoyiannis, 2005; Perea *et al.*, 2002; Petraitis *et al.*, 2003; Petraitis *et al.*, 2009; Ruíz-Cendoya *et al.*, 2008; Seyedmousavi *et al.*, 2013; Shalit *et al.*, 2003). However, *in vitro* studies and animal models are not always predictive of synergistic or additive clinical effects. *In vitro* studies have demonstrated synergy between caspofungin and voriconazole against clinical *Aspergillus* isolates (Perea *et al.*, 2002), and the combination was able to reduce the fungal burden of IA in animal tissues compared with novel triazoles or echinocandins as single agents (Kirkpatrick *et al.*, 2002; Petraitis *et al.*, 2003). On the basis of these findings, Marr *et al.* retrospectively evaluated the effectiveness of voriconazole, with and without caspofungin, as salvage therapy in a small number of HSCT patients (Marr *et al.,* 2004). The combination was associated with a lower mortality rate than voriconazole alone. Other studies have reported the use of voriconazole combined with caspofungin in primary and salvage therapy of IA (Maertens *et al.*, 2006; Singh *et al.*, 2006), but only one study has compared the three regimens (voriconazole, caspofungin or the combination of both) in patients with haematological malignancies (Raad *et al.*, 2015) (Table 5). In this latter 12-year retrospective study, responses and IA-associated mortality rates in patients who received either voriconazole, caspofungin or the combination as primary or salvage therapy were compared. It was observed that the combination of voriconazole and caspofungin did not result in better outcomes than when voriconazole was used by itself (Raad *et al.*, 2015). In the same way, some studies have demonstrated synergistic interactions between anidulafungin and voriconazole and this combination has been associated with an increase

in survival of experimental murine models of IA (Petraitis *et al.*, 2009; Seyedmousavi *et al.*, 2013). These results were translated to the clinical setting, where Marr *et al.* reported the conclusive results of the largest prospective Phase III trial of IA (Marr *et al.*, 2015). The trial compared the efficacy and safety of voriconazole alone or in combination with anidulafungin in patients with IA and showed that the combination of the two antifungals was associated with a reduction in early mortality. However, although the 6-week mortality was significantly lower in the combination arm, monotherapy showed a better overall clinical response (43% versus 32.6% for the combination) (Marr *et al.*, 2015).

Table 5. Summary of clinical studies evaluating combination therapies for invasive aspergillosis in adult patients.

Number of patients (underlying disease)	Therapeutic strategy	Combination therapy	Overall survival rate at 12 weeks, %	Reference
30 (HM)	Salvage therapy	CFG + LAMB	60	(Aliff *et al.* 2003)
48 (HM)	Salvage and first line therapy	CFG + LAMB	65	(Kontoyiannis *et al.* 2003)
47 (HM)	Salvage therapy	CFG + VRC VRC	63 32	(Marr *et al.* 2004)
112 (HM)	First line therapy	LAMB + ITC LAMB	9 24	(Kontoyiannis *et al.* 2005)
53 (HM)	Salvage therapy	CFG + ITC CFG + VRC CFG + AMB	55	(Maertens *et al.* 2006)
87 (SOT)	First line therapy	VRC + CFG LAMB	67.5[a]	(Singh *et al.* 2006)
30 (HM)	First line therapy	CFG + LAMB LAMB	100 80	(Caillot *et al.* 2007)
56 (HM)	Salvage therapy	CFG + LAMB CFG + VRC Other	66	(Rieger *et al.* 2008)
90 (HM)	Salvage (85%) and first line (15%) therapy	MFG + LAMB	24	(Kontoyiannis *et al.* 2009)
159 (HM)	Salvage therapy	Echinocandin + LAMB	38 39	(Mihu *et al.* 2010)

Number of patients (underlying disease)	Therapeutic strategy	Combination therapy	Overall survival rate at 12 weeks, %	Reference
31 (HM)	Salvage therapy	CFG LAMB CFG + VRC	33 NR	(Lellek *et al.* 2011)
61 (HM)	Salvage therapy	CFG + LAMB Triazole + LAMB VRC + echinocandin	57	(Rojas *et al.* 2012)
84 (HM)	Salvage and first line therapy	CFG + VRC CFG + LAMB VRC + LAMB Other combinations	63	(Candoni *et al.* 2014)
454 (HM)	First line therapy	VRC + AFG VRC	19.3[b] 27.5[b]	(Marr *et al.* 2015)
138 (HM)	Salvage and first line therapy	CFG + VRC CFG VRC	49 47 67	(Raad *et al.* 2015)

[a] survival at 90 days; [b] survival at 6 weeks. HM, haematological malignancies; SOT, solid organ transplant; AFG, anidulafungin; AMB, amphotericin B; CFG, caspofungin; ITC, itraconazole; LAMB, liposomal amphotericin B; MFG, micafungin; VRC, voriconazole

For combinations of echinocandins and amphotericin B, Arikan *et al.* demonstrated a synergistic or additive effect of caspofungin plus amphotericin B in, at least, one-half of the clinical *Aspergillus* isolates tested and observed no antagonism (Arikan *et al.*, 2002). In addition, Sionov *et al.* revealed that the combination of caspofungin and amphotericin B increased mice survival, and reduced fungal burden in lungs, spleen and kidneys more than either agent alone (Sionov *et al.*, 2006). These promising *in vitro* and animal data about caspofungin and amphotericin B, however, have been translated into controversial clinical results.

The prospective Combistrat trial compared the use of caspofungin in combination with liposomal amphotericin B (LAMB) with a LAMB monotherapy in 30 patients with haematologic malignancies

(Caillot *et al.*, 2007). By the end of treatment, the response rate was significantly higher for the combination arm (67%) than for the high-dose monotherapy (27%) and the survival rates at 12 weeks were 100 and 80%, respectively. However, Kontoyiannis *et al.* revealed that this combination was associated with a response rate of only 18% (Kontoyiannis *et al.*, 2003). In addition, Mihu *et al.* evaluated 159 patients with haematological malignancies and IA and found that the combination of LAMB and echinocandins did not improve response or reduce the mortality rate in comparison to when the drug was used alone in IA treatment (Mihu *et al.*, 2010).

It seems that the preferred combinations used in the treatment of IA are those that include an echinocandin with either amphotericin B or an azole. However, some combinations of amphotericin B plus an azole have also been tested. Previous studies have suggested that the interaction between amphotericin B and triazoles is antagonistic to IA (Kontoyiannis *et al.*, 2000; Lewis *et al.,* 2002; Meletiadis *et al.*, 2006; Polak *et al.,* 1982; Polak, 1987; Schaffner & Frick, 1985). For example, Meletiadis *et al.,* found *in vitro* and *in vivo* antagonism between LAMB and ravuconazole in the simultaneous treatment of experimental invasive pulmonary aspergillosis in persistently neutropenic rabbits (Meletiadis *et al.*, 2006). Likewise, antagonism was observed when amphotericin B was combined with ketoconazole (Polak, 1987; Polak *et al.*, 1982) or itraconazole (Kontoyiannis *et al.*, 2000; Lewis *et al.*, 2002). In contrast, our *in vivo* study demonstrated that the combination of amphotericin B plus posaconazole had good activity (Martin-Vicente *et al.*, 2015), although Najvar *et al.* tested the same combination but observed no benefit over posaconazole alone in a murine model of IA by *Aspergillus flavus* (Najvar *et al.*, 2004). As we did, Sandoval-Denis *et al.* found synergistic interactions between amphotericin B and

voriconazole in a murine model of disseminated aspergillosis (Sandoval-Denis *et al.,* 2013). In the clinical setting, in a retrospective analysis, the addition of itraconazole to LAMB did not show any benefit in a series of patients with haematologic malignancies and documented IA (Kontoyiannis *et al.*, 2005).

In general, combination therapy is well tolerated and there are only a few serious drug-related adversities, mainly attributed to the use of voriconazole (Maertens *et al.*, 2006). Additionally, echinocandins proved to be safe, since no patients discontinued these drugs because of toxicity. However, infusion-related reactions and increases in serum creatinine have been attributed to the use of high doses of amphotericin B lipid formulations (Caillot *et al.*, 2007).

In conclusion, although the combination of an echinocandin with amphotericin B or triazoles have produced good results *in vitro* and in animal models, the results in the clinical setting are highly heterogeneous, and the benefits and efficacy of this approach are unclear. For this reason, on the basis of the clinical data available, there is little evidence to suggest that combination antifungal therapy is effective against IA and it is not a common recommendation in the international guidelines (Ruhnke *et al.*, 2003; Walsh *et al.*, 2008; Maertens *et al.*, 2011). Nevertheless, bearing in mind that patients generally tolerate the therapy well and that the response rate is favourable, this strategy should be considered as salvage treatment in selected immunocompromised patients.

5.2 *Scedosporium apiospermum*

Scedosporium apiospermum is the species that most causes scedosporiosis and is responsible for high mortality rates (90–100%) in patients with disseminated disease and in those showing central nervous

system (CNS) involvement where surgery is not possible due to the presence of multiple abscesses (Buzina *et al.*, 2006; Rodriguez-Tudela *et al.*, 2009). Although voriconazole is the treatment of choice (Tortorano *et al.*, 2014), there are no epidemiological cutoff values (ECVs) or clinical breakpoints (CBPs) and the usefulness of determining MICs to predict efficacy remains unclear. For this reason, the susceptibility of 31 *S. apiospermum* strains was determined by three methodologies (namely, microdilution, disk-diffusion and Etest®) in order to evaluate the correlation between them and with the *in vivo* results in a murine model of systemic infection caused by *S. apiospermum* with high CNS involvement.

The essential agreement between disk diffusion and the CLSI microdilution method when testing voriconazole against *S. apiospermum* was 90.4%, which is considered acceptable. Good correlations between both methodologies were also observed for azoles against *Candida, Cryptococcus* (Barry *et al.*, 2002; Espinel-Ingroff *et al.*, 2007), *Aspergillus* and other relevant clinical moulds, including *S. apiospermum* (Espinel-Ingroff *et al.*, 2007).

Some studies suggest that Etest® is a useful method for determining the susceptibility of *Aspergillus* spp. to voriconazole and other antifungal drugs (Serrano *et al.*, 2003; Serrano *et al.*, 2004; Pfaller *et al.*, 2003; Badiee *et al.*, 2012). In our study we found a good correlation between this methodology and CLSI microdilution (93.5% agreement) (Martin-Vicente *et al.*, unpublished). These results are in accordance with those obtained in a recent study (Lamoth & Alexander, 2015) and suggest that Etest® is a suitable alternative procedure for testing the susceptibility of *S. apiospermum* to voriconazole.

Voriconazole was effective at reducing the fungal burden in the brain of mice infected with strains showing MIC $\leq$ 2 μg/ml, when

determined by microdilution. Previous experimental animal models have already demonstrated the efficacy of this drug against isolates of *S. apiospermum* with MICs between 0.25 and 1 μg/ml (Capilla *et al.*, 2003; Rodríguez *et al.*, 2010; Lackner *et al.*, 2014). In addition, and similar to our results, in a guinea pig model the azole proved not to be effective against one strain with a MIC of 8 μg/ml (Capilla & Guarro, 2004).

Overall, our results suggest the importance of determining MIC if correct treatment is to be established and show that voriconazole was able to reduce fungal burden in the brain of 92.3% of all mice challenged with strains with voriconazole MICs ≤ 2 μg/ml. Therefore, we propose ≤ 2 μg/ml and > 4 μg/ml as tentative cutoff values for *S. apiospermum*.

5.3 Multiresistant fungi

Recent data from haematological patients suggest that mortality attributable to invasive fungal infections has decreased, probably due to correct and timely diagnosis and to the availability of newer, better tolerated and more efficacious drugs (echinocandins, expanded spectrum triazoles, lipid formulations of amphotericin B) (Neofytos *et al.*, 2009; Candoni *et al.*, 2014). However, the efficacy of current antifungal therapies is still suboptimal in several fungal infections that affect particular patient populations. In addition, some opportunistic fungi have been described as intrinsically resistant to most of the antifungal agents approved for human therapy. Of these, species of *Mucor*, *Fusarium*, *Scedosporium/Lomentospora* and *Scopulariopsis* deserve special attention due to their higher MIC values and their ability to cause invasive infections, which usually lead to fatal outcomes (Perfect, 2012; Nucci *et al.*, 2014).

Little is known about the epidemiology of infections caused by multiresistant moulds in haematologic patients and any effective therapies. Data on this subject are limited to a small number of patients and anecdotal reports. However, although species belonging to the genera *Fusarium, Scedosporium* and *Scopulariopsis* are uncommon, clinicians need to know what steps to take in case of infection because of their aggressive clinical course and the lack of effective therapies.

In the present thesis, we aimed to test the activity of amphotericin B, voriconazole and anidulafungin in double and triple combinations against four multiresistant fungal species. *Fusarium solani, Lomentospora prolificans, Scopulariopsis brevicaulis* and *S. brumptii* are some of the most common non-*Aspergillus* moulds that cause disseminated diseases and are responsible for high mortality rates.

We demonstrated that against *F. solani*, in particular, synergy was high for all the combinations tested (i.e. nearly 90% for anidulafungin plus voriconazole, amphotericin B plus anidulafungin and the triple combination) suggesting that these combinations have a potential role in the treatment of fusariosis. This agrees with the results of a previous *in vitro* study that reported additive to synergistic interactions between amphotericin B and voriconazole against *F. solani* (Spader *et al.*, 2011) and some degree of efficacy of the same combination in a murine model of disseminated infection by this fungus (Spellberg *et al.*, 2006). However, for most of the strains tested, synergy was achieved at concentrations of voriconazole that were not in the range of achievable levels in serum (i.e. 16 or 32 μg/ml).

In our study, amphotericin B was synergistic with anidulafungin against 87.5% of the isolates tested, and in another study, the polyene

combined with caspofungin was synergistic in 82% of the *F. solani* strains tested (Spader *et al.*, 2011). The latter combination had already been reported as effective at reducing fungal burden in a murine model of disseminated *F. solani* infection (Ruíz-Cendoya *et al.*, 2008). However, Spellberg *et al.* observed that the combination of amphotericin B with micafungin did not show *in vivo* efficacy against this fungus, suggesting that the effect of the combinations of amphotericin B with echinocandins seems to depend on the echinocandin tested (Spellberg *et al.*, 2006). When used alone, anidulafungin has poor *in vitro* activity against *F. solani* but the combination of voriconazole with anidulafungin or micafungin has shown synergy *in vitro* and in murine models (Spellberg *et al.*, 2006; Shalit *et al.*, 2009; Philip *et al.*, 2005). This suggests that these combinations have an important role in the treatment of *F. solani* infections.

In the clinical setting, it should be noted that the outcome of invasive fusariosis has significantly improved since the recent use of voriconazole and combined therapies (Nucci *et al.*, 2014). The best results have been obtained for combinations of amphotericin B or its lipid formulations plus caspofungin or voriconazole in patients with haematological malignancies, which highlights the potential of these combinations (Durand-Joly *et al.*, 2003; Ho *et al.*, 2007; Liu *et al.*, 2011; Vagace *et al.*, 2007).

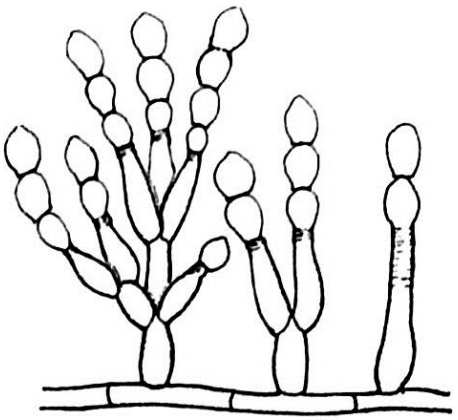
The combined activity of antifungal agents against *L. prolificans* has rarely been evaluated. It has been demonstrated that voriconazole in combination with amphotericin B or andulafungin shows *in vitro* synergy in a small percentage of strains while amphotericin B plus anidulafungin produces indifferent interactions in all cases (Cuenca-Estrella *et al.*, 2008). Our results are quite different since a high rate of synergy (72.7%) was observed for this latter combination while around 50% of

synergy/indifference was obtained for the other double combinations assayed. Our results correlate better with those of Yustes and Guarro, who reported synergistic interactions between amphotericin B and micafungin against 14 out of 17 (82%) *L. prolificans* strains tested (Yustes & Guarro, 2002). Previously, only a triple antifungal combination had been tested *in vivo* against *L. prolificans* (Rodríguez *et al.*, 2009). In that study, amphotericin B, micafungin and voriconazole were tested alone, and in double and triple combinations in a murine model of disseminated infection after showing *in vitro* synergy only for the triple combination. However, the *in vivo* efficacy of that triple combination was worse than for micafungin plus amphotericin B or plus voriconazole (Rodríguez *et al.*, 2009). Similar combinations have been used against *L. prolificans* infections and the voriconazole plus caspofungin combination has had some therapeutic success (Steinbach *et al.*, 2003). This result agrees with our *in vitro* interaction data, but not with the combinations voriconazole plus LAMB and itraconazole plus micafungin (Ochi *et al.*, 2015; Uno *et al.*, 2014). However, because there are so few clinical cases, no conclusions can be drawn about the *in vitro–in vivo* correlation.

Several antifungal combinations have been tested *in vitro* against *S. brevicaulis*, resulting in a high percentage of indifference for the combination amphotericin B plus voriconazole, and more than 50% of synergy for caspofungin plus amphotericin B (Cuenca-Estrella *et al.*, 2006). These results agree with ours. Combined antifungal therapy is often used to treat *Scopulariopsis* infections although combinations of more than two drugs are rarely used and outcomes are variable. Amphotericin B lipid complex in combination with itraconazole and voriconazole plus caspofungin have shown *in vivo* synergy in patients with haematological malignancies (Baddley *et al.*, 2000; Petit *et al.*, 2011), but other studies report the therapeutic failure

of the latter combination (Steinbach *et al.*, 2004; Yang *et al.*, 2012) and the combination of LAMB with caspofungin, voriconazole or micafungin (Salmon *et al.*, 2010; Iwen *et al.*, 2012). To our knowledge, only one triple combination has been tested *in vitro* against *Scopulariopsis* species: posaconazole, caspofungin and terbinafine. This combination showed synergy against 100% of the *S. brevicaulis* strains assayed (Yao *et al.*, 2015). In our case, the triple combination showed synergy in 81.8% of cases involving *S. brevicaulis* and a modest 50% in cases of *S. brumptii*. Animal studies are needed to prove the *in vivo* efficacy of the combinations tested.

In conclusion, it was demonstrated that there are significant interactions between amphotericin B, voriconazole and anidulafungin against the four species of multiresistant fungi tested. The percentage of synergism was highest for amphotericin B plus anidulafungin. The triple combination also showed synergism so, in theory, both the dose and the toxicity of the treatment could be reduced. However, this benefit was not observed for all the strains, suggesting that the use of three drugs would not always be better than the use of two.



6. CONCLUSIONS

6. Conclusions

Determination of the activity of posaconazole in combination with amphotericin B or anidulafungin against *Aspergillus fumigatus*.

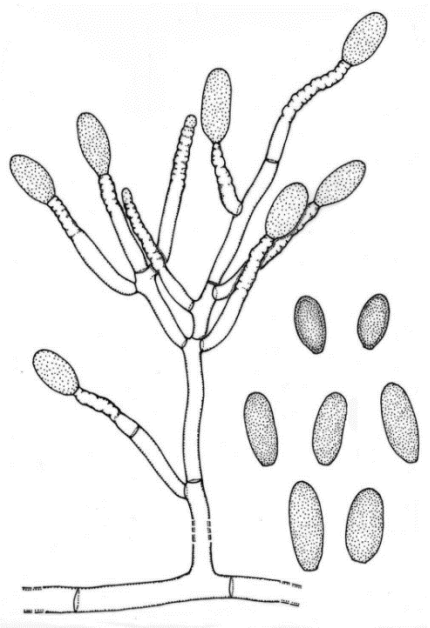
- ✓ The combinations of posaconazole with either amphotericin B or anidulafungin proved to be highly effective against murine aspergillosis. Additionally, the combination containing anidulafungin sterilized most of the kidneys of the animals challenged with all the strains assayed. Therefore, these combinations could be an alternative for treating invasive aspergillosis, with a possible reduction in the dose, the toxicity and the cost.
- ✓ The correlation between the *in vitro* activity and *in vivo* efficacy of the combinations against *A. fumigatus* was high.

Determination of the *in vitro* activity by comparing three methods, and of the *in vivo* efficacy of voriconazole against *S. apiospermum* strains with different susceptibilities to the drug.

- ✓ All three methods – microdilution, disk diffusion and Etest® – correlated to a similar extent with the *in vivo* outcome, indicating that they are useful for testing the antifungal activity of voriconazole against *S. apiospermum*.
- ✓ The voriconazole MIC values ≤ 2 μg/ml and > 4 μg/ml could be tentative cut-off values of this drug in *S. apiospermum* infections.

Determination of the *in vitro* activity of amphotericin B, voriconazole and anidulafungin in double and triple combinations against the multiresistant fungi *Fusarium solani, Lomentospora prolificans, Scopulariopsis brevicaulis* and *Scopulariopsis brumptii*.

- ✓ Amphotericin B plus anidulafungin showed the highest synergy, suggesting that this combination could play an interesting role in the treatment of infections by these multiresistant fungi.
- ✓ The triple combination also showed good synergy against the four fungal species, suggesting that the administration of three drugs can reduce the doses and consequently the toxicity of the therapy. However, these interactions were strain-dependent and should be evaluated in each case.



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