EVALUATION OF VIRULENCE AND NEW EXPERIMENTAL THERAPEUTIC STRATEGIES FOR EMERGING AND UNCOMMON MEDICALLY IMPORTANT FUNGAL PATHOGENS

Fabiola Vanessa Fernández Silva

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UNIVERSITAT ROVIRA I VIRGILI

Departament de Ciències Mèdiques Bàsiques

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Doctoral Thesis

Directed by Drs. Emilio Mayayo Artal, Josep Guarro Artigas and Javier Capilla Luque

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Certify that:

The present work entitled “Evaluation of virulence and new experimental therapeutic strategies for emerging and uncommom medically important fungal pathogens” presented by Fabiola Vanessa Fernández Silva to obtain the degree of Doctor by the *Universitat Rovira i Virgili*, has been carried out under their supervision at the *Unitats d´Anatomia Patològica i Microbiologia*, *Departament de Ciències Mèdiques Bàsiques*, and that it fulfills the requirements to obtain the European Doctorate mention.

Reus, June 27, 2013

Emilio Mayayo Artal Josep Guarro Artigas Javier Capilla Luque

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I. Introduction

1.1 Overview

Fungi are heterotrophic eukaryotic organisms that belong to the Fungi Kingdom (Webster and Weber 2009). As members of a different Kingdom they show some unique characteristics such as the presence of a cell wall rich in chitin and β-glucan and a plasmatic membrane with ergosterol. Despite being microscopic entities, fungi can produce macroscopic structures such as basidiocarp (commonly known as mushrooms) and ascomas which do not present a vascular system. The obtention of nutrients from the substrate is done by absorption (Webster an Weber, 2009, Kirk *et al*., 2008).

Fungi can grow in two basic forms; one of them is unicellular or yeast form which is morphologically defined as a single-celled fungus that reproduces itself by budding to form blastoconidia. The other morphology that fungi can show includes a filamentous or mould form which consists on filament-like cells called hyphae, which are cylindrical thread-like structures 2–10 μm in diameter. Hyphae grows apically and branching. Nonetheless, hyphal tips occasionally bifurcate (fork) giving rise to two parallel-growing hyphae. The combination of both, apical and branching/forking growth leads to the development of a mycelium, an interconnected network of hyphae. Hyphae can be either septate or coencytic. While septate hypae is divided into compartments separated by cross walls called septa, which are formed at right angles to the cell wall giving hypa its shape, coenocytic hyphae are not compartmentalized (Alexopoulus *et al*., 1996). Some fungi, called dimorphic fungi, show both morphologies yeast-like and filamentous depending on their growth conditions. They are isolated from environmental sources and generally limited to some geographical regions, principally from America (Brandt and Warnock, 2003). Fungi are worldwide distributed and can be found in a wide range of habitats ranged from nutrient-rich substrate to harsh environments (Calduch *et al*., 2004). In spite of

being considered the major decomposer microorganisms of the ecosystem due to their saprophytic nature fungi are able to establish different symbiotic relationships with other organisms (Webster and Weber, 2009).

Numerous fungal species have a commensal interaction with animals, being part of their microbiota or establishing mutualistic relationship with animals and plants. Fungi can also act as parasites of plants, other fungi and/or animals, including human. In fact not all fungi are pathogenic and the fungal infection is opportunistic. Both forms, filamentous and yeast are able to cause infections, which are called mycoses. In human hosts are able to cause wide range of diseases in normal and immunocompromised hosts (Walsh and Dixon, 1996; Richardson and Warnock, 2003). Opportunistic fungal infections are those that cause diseases among the immunocompromised population and rarely affect immunocompetent individuals, being *Candida* spp. and *Aspergillus* spp. the most frequently isolated pathogens (Ascioglu *et al*., 2002; Richardson and Lass-Flörl, 2008).

1.2 Fungal infection

The ability of fungi to cause diseases in human beings seems to be an accidental phenomenon, since pathogenicity among fungi is not necessary for the maintenance or dissemination of the species. In general, the development of human mycoses is primarily related to the immunological status of the host and to its environmental exposure, rather than to the infecting organism (Rex and Pfaller, 2002). A small number of fungi, traditionally named as **primary pathogens**, have the ability to cause infections in healthy humans by (a) having a unique enzymatic capacity (dermatophytes), (b) exhibiting thermal dimorphism (such as *Sporothrix* spp.) and (c) by having mechanism able to block the cell-mediated immune defences of the host (as occurs in *Histoplasma capsulatum's* infections). Thus, the basic

mechanism of fungal pathogenicity is its ability to adapt to the tissue environment and to withstand the activity of the host immune system (Casadevall and Pirofski, 2001).

Apart from the so-called primary pathogens many fungal species, traditionally considered as saprophytic, have been reported to be the cause of serious and even fatal illness. These fungal species are globally designed as **opportunistic fungi** (de Hoog *et al*., 2002). It is unprofitable to list all fungal species that can be considered as opportunistic since new species are frequently identified for the first time as causal agents, increasing the number of fungi involved in infections. Opportunistic infections occur mainly in patients with impaired immunity, especially in those cases where neutropenia occurs (Walsh *et al*., 2004).

Fungal infections or mycoses range from superficial infections, involving the outer layer of the stratum corneum of the skin, to systemic infections. Mycoses may be classified according to (i) the origin of infection (ii) the type of virulence of the fungi or to (iii) the site of the infection (Walsh and Dixon, 1996). When classified according to the route of acquisition a fungal infection may be designated as **exogenous** or **endogenous** in origin. While exogenous infections can be transmitted via airborne, cutaneous or percutaneous routes; endogenously-acquired fungal infections may be earned trough colonization. When fungal infections are classified according to the virulence of the fungi, fungal infections are denominated primary or opportunistic. A primary fungal infection is the one in which the pathogen may infect an immunologically normal host; whereas an **opportunistic fungal infection** requires some kind of agreement with the host defences' for its establishment. Finally, a more clinical classification recognizes four categories according to the site of the infection: superficial, cutaneous, subcutaneousand deep mycoses (Walsh and Dixon, 1996; de Hoog *et al*., 2002).

1.2.1 Superficial mycoses

Infections are considered superficial when affection of the stratum corneum of the skin or the hair shaft occurs. Since no living tissue is invaded there is no cellular response from the host. Superficial mycoses include tinea nigra, caused by *Hortaea werneckii,* piedra, *by Piedraia hortae o Trichosporn spp,* and pityriasis versicolor caused by *Malassezia* spp among others (de Hoog *et al*., 2002; Ameen, 2010; Arenas *et al*., 2012).

1.2.2 Cutaneous mycoses

Cutaneous mycoses are a group of diseases that affect deep layers of the epidermis causing the inflammation of adjacent structures. These mycoses are called **dermatomycoses** and are acquired through the contact with contaminated surfaces. The most common affections are those known as ringworm or tinea, caused by a particular group of fungi denominated **dermatophytes**, which includes the genera *Epidermophyton*, *Microsporum*, and *Trichophyton.* On the other hand, dermatomycoses can also be produced by a wide spectrum of non-related fungal species such as *Syctalidium dimidiatum* (Skin), *Scopulariospis brevicaulis* (onychomycoses), *Nattrasia mangiferae* (hyperquerathosis), *Fusarium* and *Aspergillus* (keratitis) and *Candida albicans* among others (Erbagci, 2002; de Hoog *et al*., 2002; Arenas *et al*., 2012).

1.2.3 Subcutaneous mycoses

These infections are usually acquired through traumatic implantation of fungal elements from a wide variety of ubiquitous saprophytic fungi. Subcutaneous mycoses affect deep tissues; including dermis and hypodermis, showing a subacute or chronic evolution. In these types of infections The fungal disease remains localized in the

inoculation area with slow peripheral growth although lymphatic or hematologic dissemination may occur (Arenas *et al*., 2012). The incidence and prevalence of subcutaneous infections varies geographically being more common in rural areas of underdeveloped countries. In addition, in some cases, subcutaneous infections have been considered occupational due to their prevalence in certain types of employment. Other factors such as climate (tropical or subtropical), gender, genetic background or nutritional deficiencies have been reported as risk factors. Within the subcutaneous infections chromoblastomycosis, eumycetoma and sporotrichosis are the most commom infections reported, and the least is lobomycosis (de Hoog *et al*., 2002).

The **chromoblastomycosis** is a fungal infection of the skin and the subcutaneous tissues characterized by the presence of nodular verrucous lesions, often localized at the lower extremities. The disease is usually chronic and is rarely life-threatening. It is normally caused by several species of dematiaceous or pigmented fungi such as *Fonsecaea* spp, *Phialophora verrucosa* and *Cladophialophora carrionii;* but, in a less common basis, *Exophiala spinifera*, *E. jeanselmei* and *E. dermatitidis* have also been reported to cause it. The chromoblastomycosis is the presence, in infected tissues, of single or multiple muriform cells also called sclerotic bodies (Clancy *et al*., 2000; Sharma, 2002; Ameen, 2009).

The **eumycetoma** is a suppurative, granulomatous and chronic subcutaneous infection. It can involve bone and lymph nodes by contiguous spread. It presents three clinical characteristics: tumor, sinuses and grains. The tumor results as a consequence of a progressive and relatively painless swelling. Sinuses are a disorder's characteristic; they can be absent in early stages, and develop later the sinuses with purulent material and grains. Grains are colonies of the causative agent

and can be black; when the agent involved is either *Exophiala jeanselmei, Madurella spp., Leptosphaeria spp., or Pyrenochaeta;* or white when the infection is caused by *Acremonium* spp., *Neotestudina rosatii or Pseudallescheria boydii* (McGinnis, 1996; Pelzer *et al*., 2000; Arenas *et al*., 2012).

The **lobomycosis** is a rarely reported infection**.** Like the eumycetoma is a chronic subcutaneous infection. Its characteristics are the presence of nodules, plaques and verrucoid or ulcerated lesions that can be either localized or disseminated throughout the skin, resulting into contiguous extension, autoinoculation or lymphatic spread. Lesions occur on exposed areas such as feet, legs, ears, arms or elbows, and less frequently on the face. The agent of lobomycosis, *Lacazia loboi*, is a yeast-like organism in tissues and an obligate pathogen (Fuchs *et al*., 1990; de Hoog *et al*., 2002; Belone *et al*., 2002).

The **sporotrichosis** is an acute or chronic subcutaneous mycosis that usually affects humans and mammals. *S schenckii*, now considered a complex of species, has largely been known as the etiologic agent of this disease (Marimón *et al*., 2007; López-Romero *et al*., 2011). This infection can be acquired through traumatic contact with live or decaying vegetation from handling cats with the disease or, trough scratches as recently has been reported (Messias *et al*., 2013). This infection is usually limited to the skin and to subcutaneous tissues, but may evolve as a serious disseminated disease with visceral and osteoarticular involvement in immunocompromised individuals, particularly people with AIDS (Bustamante and Campos, 2001; Bonifaz and Vasquez-Gonzalez, 2010; Barros *et al*., 2011).

1.2.4 Deep mycoses

Deep infections include those affecting internal organs, usually lungs, but can disseminate and become systemic when two or more not adjacent organs are

involved or disseminated whether spread through the blood vessels or the lymphatic system occurs. These mycoses can affect immunocompetent hosts, in this case **primary** pathogens such as *Histoplasma capsulatum, Coccidioides immitis complex, Blastomyces dermatitidis, Paracoccidioides brasiliensis and Penicillium marneffei* (de Hoog *et al*., 2002) are usually involved. However, deep mycoses are more frequent in immunocompromised patients, in this case the fungus involved are **opportunistic**, thus the resulting infections are called opportunistic fungal infections (Walsh and Dixon, 1996; Groll and Walsh, 2001; de Hoog *et al*., 2002; Walsh *et al*., 2004).

1.2.4.1 Opportunistic fungal infection

Opportunistic fungal infections are described as those infection caused by fungi not generally known for its pathogenic role. These opportunistic fungi are a heterogeneous group of fungi worldwide distributed (Walsh *et al*., 2004, Guarro, 2011). They are able to cause deep mycosis invading via the respiratory route, the alimentary routeor intravascular devices. Clinical manifestations of these infections highly depend on the immune status of the host. In an immunocompetent person, opportunistic infection may be minimally symptomatic. In sharp contrast, in patients with compromised host defences, such as human immunodeficiency virus (HIV) patients and transplant recipients receiving immunosuppressive therapy, primary infection may evolve into an instant disseminated disease involving the lungs, abdominal viscera, bones and/or central nervous system (Lockhart *et al*., 2012).

Over the past two and a half decades, the incidence of opportunistic fungal infections has increased dramatically (Enoch *et al*., 2006). Numerous factors have contributed to this raise, specially the growth of immunosuppressed or immunocompromised patients whose immune mechanisms have been impaired by primary disease states (e.g., AIDS, cancer), the increasing prevalence of chronic

medical conditions in aging population and the use of new and aggressive medical and surgical therapeutic strategies, including broad-spectrum antibiotics, cytotoxic chemotherapies and organ transplantation (Warnock, 2007).

Although treatments for these opportunistic infections have been already established, mortality and morbidity rates remain unacceptably high, especially in immunocompromised patients (Walsh *et al*., 2004). *Aspergillus fumigatus* and *Candida albicans* have been reported to be the most important fungi causing invasive fungal infections and, although these fungus are still the most frequently isolated, there has been an increase in the incidence of other less studied fungi such as *Sporothrix, Acremonium, Scedosporium* spp. and *Candida glabrata* among others, which are known as emerging opportunistic fungi (Enoch *et al*., 2006; Warnock, 2007; Chandrasekar, 2009). Contrary to *Aspergillus* spp., there is scarce data about the epidemiology, evolution and therapy of fungal infections caused by these emerging opportunistic fungi, thus their study has a great interest.

1.2.4.1.1 *Sporothrix* infection

Sporotrichosis, as mentioned above is an acute or chronic subcutaneous mycosis that affects humans and mammals. This mycosis is caused by different species of the hyphomycete genus *Sporothrix* (Bustamante and Campos, 2001; Rodrigues *et al*., 2013). It is a ubiquitous environmental fungus scatered around the world. In this sense, while is more frequently found in tropical and subtropical climetes of the American, Asian and Australian continents, is rarely found in Europe. It is considered endemic in some areas of Mexico, Costa Rica, Guatemala, Colombia, Brazil, Uruguay, South Africa, India and Japan and hyperendemic in the south central Peruvian highlands (Bustamante and Campos, 2001, López- Romero *et al*., 2011). Significant genetic variability has been observed in *S. schenckii* (Marimón

et al., 2006; de Meyer *et al*., 2008) suggesting that this fungus is not a single species but a complex of numerous cryptic species (Marimón *et al*., 2006). Recent studies have revealed that there is a high genetic variability among isolates that were morphologically identified as *S. schenckii* (Marimón *et al*., 2006; Marimón *et al*., 2007), which has led to the introduction of new species due to the main phenotypic and physiological characteristics of these species. Medically relevant *Sporothrix* species include *S. brasiliensis*, *S. schenckii*, *S. globosa*, *S. mexicana* and *S. luriei* (Marimón *et al*., 2006; Marimón *et al*., 2007; Marimón *et al*., 2008a). *S. schenckii* cryptic species are dimorphic fungi, which myceliar morphotype is found on environmental sources and when penetrates in the human hosts through skin abrasions produced by fungal-contaminated plants or animals or more rarely by inhalation.*Sporothrix* develop the yeast morphotype. These yeasts are fusiform or ovoid with single or multiple budding. Direct microscopic examination of wet mount slides with saline solution or distilled water of the purulent material of lymphocutaneous sporotrichosis' may reveal the yeast cells forming "asteroid bodies", described as a central cell surrounded by an extracellular eosinophilic material forming spicules (Mata-Essayag *et al*., 2013). Yeast cells have been also observed in humans and in experimental host tissues as elongated cells of different forms, generally described as "cigar bodies" (Arrillaga *et al*., 2009).

Previously to the reclassification of *S. schenckii* as complex, several studies reported that *S. schenckii* showed variability in its virulence and antifungal susceptibility (Dixon *et al*., 1992; Mesa-Arango *et al*., 2002; Marimón *et al*., 2008b) remaining unclear whether these differences were attributable to different species of the complex or to different isolates of the same species. *In vivo* studies, realized in an immunocompetent murine model using the new *S. schenckii* complex species, demonstrated that there were differences between the species from the complex,

being *S. brasiliensis* the most virulent species followed by *S. schenckii* and then *S. globosa;* while S*. mexicana* and *S. albicans* showed low or no virulence at all in this model (Arrillaga *et al*., 2009). Zoonotic transmission has also been described in isolated cases or in small outbreaks (Barros *et al*., 2004). It rarely can be acquired by spore inhalation (Lopez-Romero *et al*., 2011). Sporotrichosis is characterized by a wide range of cutaneous and systemic clinical manifestations (Kauffman *et al*., 2007). Localized cutaneous and subcutaneous infections are the most common forms of sporotrichosis, and it is characterized by ulcerative lesions, associated regional lymphangitis and lymphadenopathy. Extracutaneous sporotrichosis can be subclassified as unifocal forms, affecting one organ or system (lung, joints, bones and other organs), or as multifocal or systemic forms. Both are developed after conidia inhalation or hematogenous disseminations from a primary focus to others organs. The systemic form of sporotrichosis is usually associated with immunocompromised patients. Interestingly, in these cases, primary skin lesions are scattered or even absent. Reports of sporotrichosis in HIV-positive patients have been increasing worldwide, and although sporotrichosis is not the most common fungal infection in immunocompromised patients it is difficult to treat (Hardman *et al*., 2005). The recommended procedures for the management of sporotrichosis include local measures such as hyperthermia and systemic measures like the administration of a saturated solution of potassium iodide, azoles (itraconazole and fluconazole), Amphotericin B (AMB) or terbinafine. Itraconazole has become the drug of choice when treating lymphocutaneous and cutaneous forms. In those patients with intolerance to itraconazole, fluconazole is the recommended alternative. AMB is the first choice in the treatment of disseminated, but its renal toxicity limits its use and treatment outcome remains poor to date (Kauffman *et al*., 2007). There is no published clinical data on the use of other drugs as first line therapy. *In vitro* studies

have showed that azoles, as voriconazole (VRC) and posaconazole (PSC), show some *in vitro* activity against the species of the *S. schenckii*. However, their *in vivo* efficacy has not been demonstrated until now.

1.2.4.1.2 *Acremonium* infection.

Acremoniosis includes a broad spectrum of infections caused by fungi belonging to *Acremonium* genus. The *acremonium spp.* are cosmopolitan environmental fungi common in soil, plant debris and rotting mushrooms (Guarro *et al*., 1997). These fungi belong a complex and polyphyletic genus comprising approximately 150 species (Summerbell *et al*., 2011) some of them being pathogens of plants, insects and mammals including humans (de Hoog *et al*., 2002). *Acremonium* species are morphologically very similar to each other and can only be distinguished on the basis of subtle differences, making their identification difficult (Perdomo *et al*., 2011). Therefore, in most of the the etiological agent's clinical cases it has only been reported as *Acremonium* spp., which drastically reduced the value of the reports and the knowledge of the real incidence of the different *Acremonium* species in the clinical setting (Novick *et al*., 2003). A recent molecular study showed the prevalence of *Acremonium* in a large set of clinical isolates from the USA being *A. kiliense*, *A. sclerotigenum-A. egyptiacum* complex, *A. implicatum*, *A. persicinum* and *A. atrogriseum* the most common species (Perdomo *et al*., 2011). Summerbell *et al., (*2011) carried out a comprehensive review of the genus based on the ribosomal DNA sequences of a large number of species and *A. kiliense* was relocated to the genus *Sarocladium* as *Sarocladium kiliense.*

Acremonium spp. has been reported as the causative agent of a broad range of clinical manifestations. Localized infections, such as mycetoma (Venugopal and Venugopal, 1995, Castro and Piquero-Casals, 2008), keratitis (Weissgold *et al*.,

1998; Fridkin *et al*., 1996) or onychomycosis (Gupta *et al*., 2000, Gupta *et al*., 2012), mainly affect immunocompetent patients through trauma. Invasive infections generally affect immunosuppressed hosts such as those undergoing transplantation or those with AIDS, resulting in a high degree of fatality (Das *et al*., 2010), although, invasive infections in immunocompetent individuals have also rarely, been reported (Anadolu *et al*., 2001; Purnak *et al*., 2011). *Sarocladium* (*Acremonium) kiliense* in particular has been described as a cause of mycetoma, keratitis, endophthalmitis (Fridkin *et al*.,1996, Weissgold *et al*., 1998), endocarditis (Lacaz *et al*.,1981), continuous ambulatory peritoneal dialysis (CAPD)-associated peritonitis (Khan *et al*., 2011), and catheter-related practices. There is currently a lack of data about the antifungal susceptibility profile of *Acremonium* spp hence the appropriate treatment for*Acremonium* infections remains undetermined (Pfaller and Diekema 2004; Das *et al*., 2010). There are sparse in vitro studies of antifungal susceptibility and although resistance to AMB, itraconazole, and echinocandins have been reported, the newest triazoles, and especially VRC, seem to be active against *Acremonium* (Díaz-Couselo *et al*., 2011). Nowadays, AMB still remains the most common used drug in severe fungal infections by this opportunistic mould (Das *et al*., 2010; Khan *et al*., 2011); however, its poor *in vitro* activity and variable clinical results (Guarro *et al*., 1997; Perdomo *et al*., 2011) require therapeutic alternatives.

1.2.4.1.3 *Scedosporium* infection

Scedosporiosis includes a broad spectrum of infections caused by species belonging to *Scedosporium* genus (Guarro *et al*., 2006, Guarro and Pastor, 2009). The species responsible for these infections are ubiquitous ascomycetous fungi, commonly found in soil, manure, decaying vegetation, and polluted water (Cortez, 2008). These fungi tolerate a high saline content (5%)therefore they can survive in

polluted poorly aerated and highly osmotic environments (Guarro *et al*., 2006; Grenouillet *et al*., 2009; Harun *et al*., 2009a; Kaltseis *et al*.,2009). *Scedosporium's* life cycle includes two different stages: the anamorphic (*Scedosporium*) and the teleomorphic stage (*Pseudallescheria*). Traditionally two clinically relevant species have been included in this genus *S. apiospermum* (teleomorph *Pseudallescheria boydii*) and *S. Prolificans.* However, a study at molecular levels showed that *S. apiospermum* is a complex of at least eight phylogenetic species and that *S. apiospermum (Pseudallescheria apiosperma)* is different than *S. boydii (Pseudallescheria boydii)* (Gilgado *et al*., 2005; Gilgado *et al*., 2008; Harun *et al*., 2009b; Gilgado *et al*., 2010; Lackner *et al*., 2012b).At present, the species from the complex with the highest clinical prevalence are *S. apiospermum, S. boydii, S. prolificans,* and *S. aurantiacum* (Delhaes *et al*., 2008; Kaltseis *et al*., 2009; Harun *et al*., 2009; Heath *et al*.,2009; Harun *et al*., 2010).

Scedosporium was most commonly associated with traumatic, subcutaneous infections and asymptomatic pulmonary colonization, but in recent years, new clinical manifestation have emerged (Guarro *et al*., 2006; Cortez *et al*., 2008). Currently *Scedosporium* infections are among the most common deep mould infections being recognized as a significant pathogen, particularly in immunocompromised host. *Scedosporium* now account 25% of all non-*Aspergillus* mould infections in organ transplant recipients (Hussain *et al*., 2003, Hussain *et al*., 2005) and is one of the most frequent filamentous fungi colonizing the lungs of cystic fibrosis patients (Guarro *et al*., 2006; Harun *et al*., 2010). The dissemination of *Scedosporium* is mostly seen among in immunocompromised patients; however, disseminated diseases have also been reported in immunocompetent individuals, particularly in patients near drowning on polluted water. One interesting feature is its ability to affect the central nervous system developing highly fatal diseases. *Scedosporium* should

be considered, in the differential diagnosis, as a potential cause of infections, especially if pneumonia or brain abscess ensues (Lackner *et al*., 2012a).

One of the major challenges in the therapy of patients infected with *Scedosporium* is its resistance to most antifungal agents with the exception of VRC, and in lesser extent PSC and MFG, which show *in vitro* activity, at least against part of the *Scedosporium* population (Carrillo-Muñoz *et al*., 2001, Meletiadis *et al*.,2002, Gilgado *et al*.,2005, Gilgado *et al*.,2006, Alastruey-Izquierdo *et al*., 2007, Lackner *et al*., 2012a) and in some clinical cases (Mellinghoff *et al*., 2002; Apostolova *et al*., 2005; Porte *et al*., 2006). Immunorestitution has proved to be beneficial in animal models of systemic scedosporiosis in combination with antifungal therapy (Rodriguez *et al*., 2010). Considering the ineffectiveness showed by curren therapeutic strategies against *Scedosporium* infections, new strategies are required.

1.2.4.1.4 *Candida* infection

Candida infections or candidiasis show a wide spectrum of clinical presentations including superficial, cutaneous or invasive candidiasis among others (Walsh and Dixon, 1996). Nowadays, *Candida* spp are considered as one of the leading causes of invasive infections (Pappas *et al*., 2009). The expanding population of immunocompromised patients with intravenous catheters, parenteral nutrition, invasive procedures as well as the increasing use of broad-spectrum antibiotics, cytotoxic chemotherapies or transplantation along with other factors, contribute to the reported increase of these infections (Almirante *et al*., 2005; Horn *et al*., 2009).

Currently, more than 17 *Candida* species are known to be aetiological agents of human infections; however, more than 90% of invasive infections are caused by *Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis* and

Candida krusei. Although, *C. albicans* is the predominant species of invasive candidiasis, the incidence of non-albicans species is increasing worldwide (Pfaller *et al*., 2007a). As a matter of fact, *C. glabrata* has, undoubtedly, emerged as an important opportunistic fungal pathogen involved in the invasive candidiasis. In this sense recent surveillance studies have shown that *C. glabrata* is often the second or third most common cause of candidiasis after *C. albicans* and *C. parapsilosis* (Pfaller *et al*., 2007a; Pemán *et al*., 2012, Arendrup *et al*., 2013). What is more, *C. glabrata* infections are difficult to treat due to its intrinsic and acquired resistance to triazoles and polyenes, particularly to fluconazole and AMB respectively (Hitchcock *et al*., 1993 Lockhart *et al*., 2012). Consequently, *C. glabrata* is one of the causes of the high mortality rate in compromised, at-risk hospitalized patients. Currently, the Infectious Diseases Society of America (IDSA), the Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC) and the European Confederation of Medical Mycology (ECMM) recommend the echinocandins as the first line therapy for the invasive candidiasis caused by *C. glabrata* (Pappas *et al*., 2009, Aguado *et al*., 2010, Ullmann *et al*., 2013). However, resistance to this class of antifungals is an emerging problem for this *Candida* species as detailed in recent case reports and surveillance studies (Thompson *et al*.,2008; Kofteridis *et al*., 2010; Pfeiffer *et al*.,2010; Sun *et al*., 2010; Zimbeck *et al*.,2010; Lockhart *et al*., 2011; Pfalller *et al*., 2012). Phenotypic and genetic analyses of these resistant strains have revealed that reduced susceptibilities to the echinocandin class are associated with mutations in *fks-1* and/or *fks-2* genes which encode the active subunit of glucan synthase, the target of echinocandins (Park *et al*.,2005; Cleary *et al*., 2008; Garcia-Effron *et al*., 2009; Castanheira *et al*., 2010; Garcia-Effron *et al*., 2010; Costa de Oliveira *et al*.,2011; Perlin *et al*., 2011). Identification of these genetic changes, which can be associated with elevated MICs, has importance as they are potential sentinels

of treatment failure. Indeed, in many cases these changes have been linked to treatment failures (Cleary *et al*., 2008; Chapeland-Leclerc *et al*., 2010; Garcia-Effron *et al*., 2010; Costa-de-Oliveira *et al*., 2011). The lowest MIC values associated with these genetic changes have been used recently in the determination of epidemiological cutoff values (ECV) and Clinical breakpoint (CBP) to help laboratories and clinicians to identify strains harboring resistance mutations and therefore minimize the risk of therapeutic failure (Pfaller *et al*., 2010; Pfaller *et al*., 2011). However, since the change in the MIC associated with the adquisition of *fks* mutations can vary significantly (Shields *et al*., 2012), it will be important to study the impact of these mutations in MIC variations and in clinical outcomes.

1.3 Antifungal drugs

Unlike the development of antibacterial agents, progress in the development of antifungal drugs has been slow (Lattif and Swindell, 2010). The main reason for this delay is that, as eukaryotic organisms, fungi present few drug targets not present in mammalian cells, making the search for agents with selective fungal toxicity difficult. Throughout history many antifungal agents have been developed but only few show *in vitro* and *in vivo* activity and have been commercialized (Odds *et al*., 2003). In some cases, they cannot be used in humans because they do not diffuse properly in the tissues, were inactivated by enzymes or were too toxic for the host. Developments in the pharmacological treatment of fungal infections ranging from the use of antifungal considered "first generation" from product derived from certain microorganisms or from their metabolic activity, to the "second generation", derived from chemical synthesis (Steinbach and Perfect , 2003; Lattif and Swindell, 2010).

During the period from the beginning of the 20th century until after the Second World War, one of the first compounds used with antifungal activity was potassium

iodide (SSKI), which was the standard treatment for many cutaneous fungal infections including actinomycosis, blastomycosis, sporotrichosis and tinea. Unfortunately, its usage was limited by its very narrow antifungal spectrum activity. Thus, as fungal infections increased, so did the need for intravenous or oral antifungal agents. The first landmarks in the development of active and safe antifungal agents were the discovery, in 1939, of the antifungal activities of griseofulvin by Oxford (Oxford *et al*., 1939) and of the first azole, benzimidazole, by Wooley in 1944 (Sheehan *et al*., 1999). These were followed by Hazen & Brown´s subsequent discovery of the first polyene macrolide antifungal, nystatin, in 1950, which still has important implications in the modern antifungal therapy era (Elson, 1945). The discovery of AMB in 1955 and the subsequent reports of its use illustrate the rapid progress that the search of effective and safe antifungal agents was experimenting. .

The introduction of oral griseofulvin and topical chlormidazole in 1958 and the subsequent introduction of AMB in 1960 heralded the beginning of the modern era of antifungal therapy (Sheehan *et al*., 1999). Over the next decade, the first azole antifungals clotrimazole, miconazole and econazole, were developed and were followed by others such as ketoconazole, fluconazole and itraconazole.In recent years other drugs in this group such as VRC, PSC have been developed. Moreover, others like albaconazole and ravuconazole are still under clinical trials. Another group of antifungals developed between 1980 and 1990 are the allylamines, whose representatives are naftifine and terbinafine. Finally, during the first decade of the 2000 new drugs, belonging to the group of echinocandins, have been introduced being caspofungin (CSP), micafungin (MFG) and anidulafungin (AFG) the main representatives (Gubbins and Anaissie, 2009). The mechanism of action of antifungal drugs acts mainly on three different targets located in the fungal cell (figure 1):

- **Ergosterol or its synthesis pathway targeted by**: polyenes, azoles and allylamines.
- **Nucleic acid syntesis**: fluorcitosine.
- **Fungal cell wall**: echinocandins.

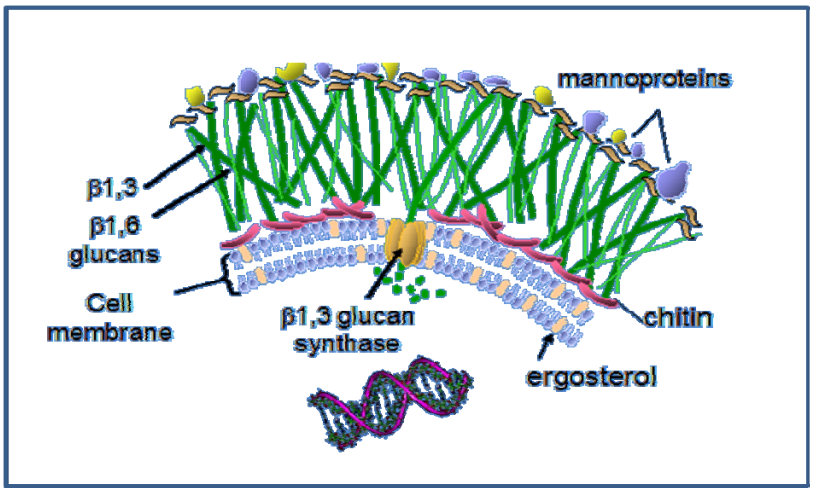

Figure 1. Target on a fungal cell.

1.3.1 Polyenes

Introduced in the 1950s this class of antifungal drug class represents the oldest family of antifungal drugs. They were developed from the fermentation of *Streptomyces spp* (Chandrasekar, 2011). AMB, synthesized from *Streptomyces nodosus* was marketed for clinical use in 1958 and is the most important antifungal of this group. AMB is a heptane macrolide with many hydroxyl groups, which confer the amphipathic nature of the compounds (figure 2). AMB is amphophilic and acts binding hydrophilic hydrogen bonds and hydrophobic, non-specific van de Waals forces to ergosterol in fungal cell membrances. The binding occurs within minutes of exposure and is followed by an increasing leakage of intracellular ions out of fungal cells (i.e., potassium) and extracellular ions into cells, which leads to a depolarization of the membrane and an increased permeability to protons and monovalent cations.

This osmotic disruption may not be the main mechanism of lethality in/of fungal cells, because polyenes also interfere with membrane-associated oxidative enzyme function, and is this what, in the second instance, is thought to be lethal (Gubbins and Anaissie, 2009; Drew, 2010a; Chapman *et al*.,2011).

AMB is available as an intravenous preparation formulated by combination with sodium deoxycolate (AMB-d). The utility of AMB-d is hindered by significant toxicity. Although it has a greater affinity for ergosterol, its affinity with cholesterol in the mammalial cell membrane is what; more likely, plays a role in its toxicity. Formulations using a lipid carrier have significantly improved tolerability and reduced the side effects. Three lipid-based products are currently available: AMB colloidal dispersion (ABCD), liposomal AMB (L-AMB) and AMB lipid complex (ABLC). These three commercial lipid formulations are distinct regarding their phospholipd content, particle size and shape, electrostatic charge and bilayer rigidity (Chapman *et al*., 2011). In general, all three lipid formulations are indicated for the treatment of systemic fungal infections in patients refractory to or intolerant to therapy with AMB-d. For the empiric therapy of presumed fungal infection in febrile neutropenic patients L-AMB has also been approved. In routine clinical practice, however, lipid formulations are frequently used as primary therapy for patients with baseline renal insufficiency and in patients at high risk of renal failure, including transplant recipients and patients receiving concurrent treatment with other nephrotoxic agents (Drew, 2010a).

AMB is active against the majority of the most common yeasts, moulds and dimorphic fungi causing human infection including: *Candida* spp., *Cryptococcus neoformans*, *Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides* spp., *Paracoccidioides brasiliensis, Sporothrix schenckii, Aspergillus* spp. and the zygomycetes. In sharp contrast, relatively few organisms manifest intrinsic resistance

to AMB is the case of *Scedosporium apiospermum*, *Candida lusitaniae, Candida guilliermondii, Scopulariopsis* spp., *Aspergillus terreus* and *Fusarium* spp. (Walsh *et al*., 2004; Pfaller and Diekema, 2004; Guarro, 2011). Resistance to AMB is uncommonly acquired through selective laboratory techniques or after clinical usage. However, resistant isolates of *C. albicans, C. glabrata, C. tropicalis,* and *C. neoformans* have been recently isolated from patients with AIDS (Pfaller *et al*., 2007; Drew 2010a).

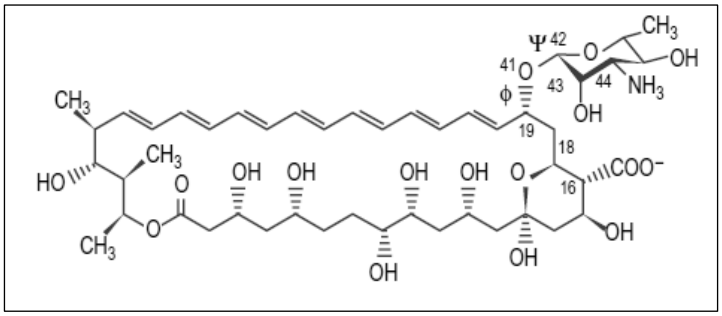

Figure 2. Structure of Amphotericin B.

1.3.2 Fluorinated pyrimidine analog

Flucytosine (5-fluorocytosine; 5-flucytosine; 5-FC) is a fluorinated pyrimidine analog (figure 3) and is one of the oldest antifungal agents in use. Currently, it is the only member of this group of antifungal drugs. It was initially synthesized in 1957, but was not discovered to possess significant antifungal properties until 1964, when activity against *Cryptococcus neoformans* and *Candida* species was shown (Larsen, 2011). To exert its effect, flucytosine is taken up by the transport enzyme cytosine permease, where rapidly undergoes intracellular conversion to 5-fluorouracil via cytosine deaminase. After this (intracellular conversion into 5-fluorouracil), it is processed into 5-fluorouridine triphosphate, which is incorporated into fungal RNA. This results in miscoding during translation from RNA into amino acid sequencing, causing structural abnormalities during protein synthesis. The second mechanism of action is characterized by the conversion of 5-fluorouracil into 5-fluorodeoxyuridine

monophosphate, which inhibits thymidylate synthetase and subsequently DNA biosynthesis (Gubbins and Anaissie, 2009)

The antifungal spectrum of flucytosine is extremely narrow and is limited to *Candida* species and *C. neoformans*, although there are some anecdotal recommendations for aspergillosis and chromoblastomycosis. Because resistance to flucytosine may occur at multiple steps in its mode of action, including transport into the cell and deamination to the active compound, flucytosine is only used in combination with other agents, including AMB and fluconazole (Drew, 2010b).

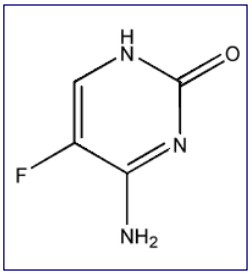

Figure 3. Structure of 5-fluocytocine

1.3.3 Azoles

The introduction of the azoles class as antifungal drugs as well as the licensing of miconazole in 1979 marked the beginning of a new era in the systemic fungal diseases' therapy (Andes, 2011). The azoles are characterized by a core 5-member azole ring and it can be divided in two classes: the imidazoles, which contain 2 nitrogen atoms within the azole ring, and the triazoles wich, contain 3 nitrogen atoms within the same ring (figure 4). The imidazoles, including ketoconazole, were the initial azoles available. Ketoconazole was followed by the development of the first triazole antifungals, fluconazole and intraconazole. More recently VRC and PSC were developed. Additional triazole antifungals remain in pre-clinical development

(ravuconazole, isavuconazole and albaconazole) (Gubbins and Anaissie 2009; Lattif and Swindell, 2010)

The azoles exert their effects within the fungal cell membrane. The primary antifungal effect of the azoles occurs via inhibition of a fungal cytochrome P-450 enzyme involved in the synthesis of ergosterol, the major sterol in the fungal cell membrane. On a molecular level, binding of the free azole nitrogen with the heme moiety of fungal C-14α demethylase inhibits demethylation of lanosterol: Thereby, depriving the cell of ergosterol and allowing accumulation of various 14α methylsterols. The net result is the disruption of the normal structure and function of the cell membrane and secondarily the acumulation of toxic methylsterols inhibits the cell growth and morphogenesis (Johnson and Kauffman, 2003).

The azoles have a broad spectrum of activity against yeasts and moulds (Gubbins and Anaissie 2009). Its activity varies depending on the different antifungals compounds i.e, **fluconazole** (FLC) is generally considered fungistatic. Its activity is relatively narrow since it is essentially limited to yeasts and some of the endemic fungi. It has activity against most *Candida* species, with exception of *C. glabrata* and *C. krusei* (Pfaller *et al*., 2007b).**Itraconazole** (ITR) offers a broader spectrum of activity compared to fluconazole. In fact its *in vitro* activity is generally considered fungicidal against filamentous fungi, such as *Aspergillus* species and dimorphic fungi (Sabatelli *et al*., 2006). **Voriconazole** (VRC) has a broad spectrum of activity that includes the most frequently isolated yeasts and moulds causing opportunistic diseases. Additionally, this agent has activity against common dermatophytes and pathogens causing endemic mycoses. *In vitro* data have shown that VRC is also effective against emerging fungal pathogens including *Scedosporium apiospermum*, *Trichosporon* spp., *Acremonium* spp. and *Fusarium* spp . Like fluconazole, VRC, has fungistatic activity against *Candida* spp.; however, it

offers better activity against most isolates than its predecessor. VRC also retains activity against some fluconazole-resistant strains of *Candida* that may represent a therapeutic niche for this agent when results of susceptibility testing are available. Fungicidal activity is seen against filamentous fungi including *Aspergillus* spp (Pfaller *et al*., 2002; Pfaller *et al*., 2003; Theuretzbacher, 2006; Gubbins and Anaissie, 2009).

Posaconazole (PSC) provides a similar spectrum of activity to VRC. It is more active against all *Candida* spp. and *C*. *neoformans* than itraconazole and fluconazole. Like VRC, PSC demonstrates *in vitro* fungicidal activity against *Aspergillus* spp and *C*. *neoformans* being highly active against *A*. *fumigatus*, *A*. *flavus*, and *A*. *terreus*. In addition, it has a very potent activity against the dimorphic fungi including *C*. *immitis*, *H*. *capsulatum*, *B*. *dermatitidis* and *S*. *schenckii*. It also demonstrates variable activity against many amphotericin-resistant molds, including certain strains of *S. apiospermum* and *S*. *boydii* (Lackner *et al*., 2012), but is not active against *Fusarium* spp. PSC also has variable activity against the agents of zygomycosis (Cuenca-Estrella *et al*.,1999; Paphitou *et al*.,2002a; Paphitou *et al*.,2002b; Meletiadis *et al*., 2002; Carrillo *et al*., 2001; Espinel-Ingroff *et al*.,2001; Dannaoui *et al*.,2003; Sun *et al*., 2002; Li *et al*., 2010; Andes *et al*., 2011).

1.3.4 Echinocandins

Echinocandins are the most recent addition to the antifungal armamentarium, they are large lipoprotein molecules that contain an amphiphilic cyclic hexapeptide (Denning, 2003). Echinocandins have a unique N-linked acyl lipid side chain, which confers different physicochemical properties to each agent (figure 5).

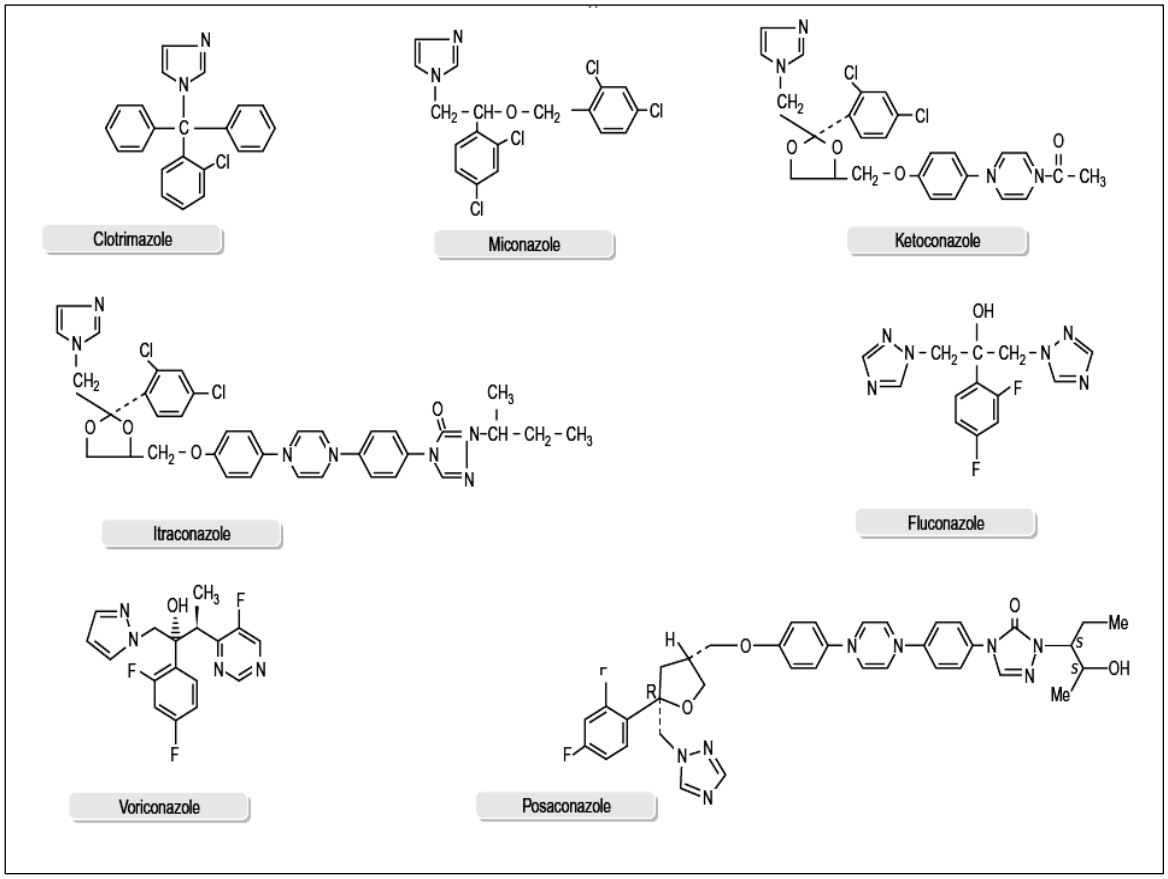

Figure 4. Structure of azoles antifungals

The first compound of this class undergoing major preclinical evaluation was cilofungin (LY 121019), a derivative semisynthetic echinocandin B with limited activity against *Candida* spp. However, clinical development was abandoned in early stages due to toxicity concerns associated with the intravenous polyethylene glycol formulation vehicle. Over the past decade, second generation semisynthetic echinocandins with extended antifungal spectrum against *Candida* and *Aspergillus* spp., excellent safety profiles and favorable pharmacokinetic characteristics have been developed. These include anidulafungin (AFG), caspofungin (CSP) and micafungin (MFG). while CSP was derived from *Glarea lozoyensis*; MFG and AFG have/had as origin *Coleophoma empetri* and *Aspergillus nidulans*, respectively (Johnson and Mohr, 2010; Groll *et al*., 2011).

The echinocandins act by noncompetitive inhibition of the synthesis of 1,3-β D-glucan,an essential component of fungal cell's wall . These antifungal compounds disrupt cell wall synthesis by inhibiting a novel target, 1,3-βD-glucan synthase, which blocks β1,3-d-glucan synthesis. This enzyme is present in most fungal pathogens but is not present in mammalian cells and its inhibition ultimately produces osmotic lysis of the cell (Denning, 2003).

The echinocandins possess a narrow antifungal spectrum that is restricted to *Candida* spp. and *Aspergillus* spp. and there is little difference among the individual drugs. All the echinocandins exert fungicidal activity against *Candida* spp.; however, in *Aspergillus* spp. these compounds do not usually cause complete inhibition of growth but instead induce abnormal morphologic hyphal growth. Therefore, these agents are considered to be fungistatic against *Aspergillus* spp and has been demonstrated that are very active against *A*. *flavus*, *A*. *fumigatus* and *A*. *terreus* (Johnson and Mohr, 2010; Groll *et al*., 2011). The echinocandins are highly active against *C*. *albicans*, *C*. *glabrata*, *C*. *tropicalis*, *C*. *dubliniensis*, and *C*. *kruse in In vitro* studies (Pfaller *et al*., 2011). They are slightly less active against *C*. *parapsilosis*, *C*. *guilliermondii*, and *C*. *lusitaniae* and MIC values are typically higher for these pathogens than other *Candida.*

1.3.5 Allylamines

These agents are highly selective for the fungal enzyme and have a minimal effect on mammalian cholesterol synthesis. Naftifine, the original member of the allylamine series, possesses only topical activity, whereas the terbinafine is active both topically and orally (Birnbaum, 1990; Gupta *et al*., 2008).These antifungals inhibit squalene epoxidase, the enzyme which catalyzes the conversion of squalene

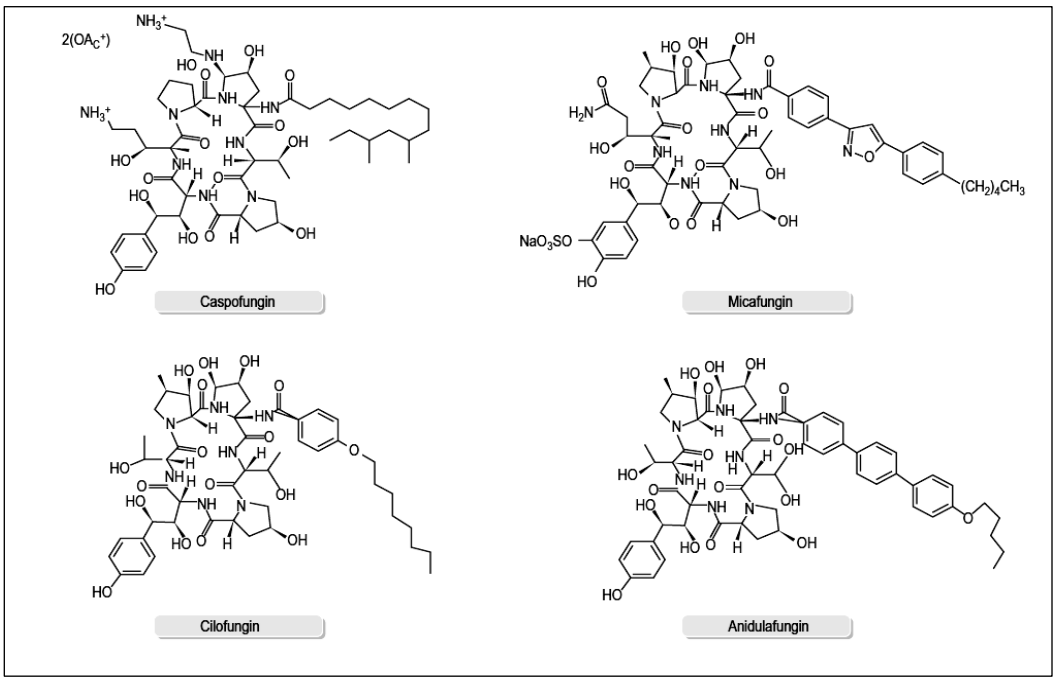

Figure 5. Structure of Echinocandin antifungals

to squalene-2,3 epoxide, a precursor of lanosterol, which in turn is a direct precursor of ergosterol. A deficiency of ergosterol is detrimental to the integrity of the cell membrane resulting in a fungistatic effect similar to that seen with the azole antifungal compounds. In addition to this action, terbinafine also causes excessive intracellular accumulation of squalene, which is believed to exert a further toxic effect on susceptible fungal cells, thereby exerting a fungicidal effect (Pappas, 2011). Based on *in vitro* studies, terbinafine demonstrates excellent fungicidal activity against many dermatophytes including *Trichophyton rubrum*, *T*. *mentagrophytes*, *T*. *tonsurans*, *Microsporum canis* and *Epidermophyton floccosum*. Other susceptible moulds include dematiaceous fungi such as *Fonsecaea* spp. and *Cladophialophora* spp. However, terbinafine demonstrates variable and somewhat poor *in vitro* activity against yeast. Initial animal studies showed no activity *in vivo* against systemic pathogens as a consequence the drug was abandoned for these indications (Gubbins and Anaissie, 2009).

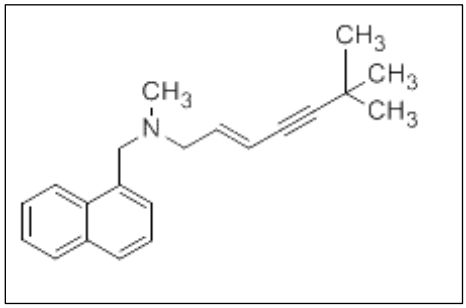

Figure 6. Structure of terbinafine

1.3.6 Other antifungals

There are, currently, several antifungal drugs under development. Some of them act on target fungal DNA or RNA (Gubbins and Anaissiei, 2009). One of them is the Yatakemycin, which is a natural product isolated from *Streptomyces* spp. that acts via sequence-specific DNA alkylation (Igarashi *et al*., 2003). Another is the Icofungipen, a synthetic derivative of the naturally β-amino acid cispentacin, which blocks isoleucyl-tRNA synthetase, resulting in the inhibition of protein synthesis and the growth of fungal cells (Petraitiene *et al*., 2005). On the other hand, Polyoxins and nikkomycins are chitin synthase inhibitors which act on the chitin of the fungal cell wall.Another target that has been studied for many years is elongation factor 2 (EF2) with the sordarins as the most studied class of compounds directed at this target. These derivatives are specific inhibitors of fungal translation factor EF2, which catalyzes the translocation of tRNA and mRNA after peptide bond formation (Debono and Gordee, 1994).

1.4 Antifungal combination

The introduction of systemic antifungal therapy in the 1950s represented a major advance in what was, in many cases, an untreatable disease. However, even with the introduction of newer therapies in the following years, the rate of cures the

cure rates for serious invasive fungal diseases remain low. With the introduction of new classes of antifungal drugs in recent years, is normal to hypothesize that combinations of antifungal drugs may be more effective than the use of the same drugs alone (Clemons and Stevens, 2006). The idea to combine antimicrobial drugs to treat infectious diseasesThe concept of using combinations of antimicrobial drugs in the treatment of infectious diseases, specifically in antifungal therapy, is not new. Since two decades ago the first combinations with 5-fluocitosine plus AMB were used for cryptococcosis (Baddley and Pappas, 2006).

There are a number of scenarios in which combination antifungal therapy may provide benefits. These include a desire to (1) increase antifungal killing activity of one or more antifungal agents;(2) broaden the spectrum of antifungal activity to cover more pathogens or potentially resistant pathogens;(3) reduce the emergence of resistant pathogens; (4) potentially minimize drug-associated toxicities by reducing dosages of antifungal agents in combinations and (5) deliver antifungal agents to multiple body sites (Enoch *et al*., 2006; Dodds and Johnson, 2011).

Get a synergistic effect combining antifungal is used as justification for combination therapy. This effect is defined as increasing activity of one or more agents beyond that expected when using either agent alone. In addition the drugs can also interact with each other in an indifferent or antagonistic mode. Indifferent interaction is defined as the one where the effect of the combination is equal to the effect of each compound alone and antagonism occurs when the combination has a smaller effect than that produced by each drug alone (Dannaoui *et al*., 2004, Johnson *et al*., 2004; Dodds and Johnson, 2011). From experimental *in vitro* and *in vivo* studies we can identify the effect of the drugs given alone or in combination.

Antifungal combinations are successfully and increasingly used in clinical practice, like in combined therapies of fluconazole with AMB in patients with

candidiasis, azoles plus 5 - fluorocytosine for cryptococcosis and terbinafine plus VRC in deep infections caused by *Scedosporium prolificans*, and the combination of VRC with CSP for invasive aspergillosis, etc(Enoch *et al*.,2006; Safdar, 2007, Ruiz-Camps and Cuenca-Estrella, 2009). It is likely that combination therapy, as an alternative to monotherapy, will also be used in the future treatment of multiresistant fungi.

1.5 Antifungal susceptibility testing

Antifungal susceptibility testing is a very dynamic field of Medical Mycology. Standarization of *in vitro* susceptibility test by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) and current availability of reference methods constituted the major remarkable steps in the field (Arikan, 2007).

1.5.1 *In vitro* testing

Although antifungal susceptibility testing remains less well developed and used than antibacterial testing, scientific support to its validity has benefited greatly, by extrapolation, from antibacterial testing (Lattif and Swindell, 2010). Until the early 1990s, testing methods were not standardized and therefore, intra-interlaboratory reproducibility was poor. The most common technique for the study of the *in vitro* susceptibility is the broth microdilution method. This technique can measure antifungal activity, expressed as the minimum inhibitory concentration (MIC) of an antifungal, which indicates the minimal concentration of drug able to inhibit the fungal growth. Thus is possible to evaluate the *in vitro* susceptibility of a fungus to one or more antifungals and correlate the results with the *in vivo* efficacy for a possible guidance on clinical effectiveness. This method is also useful to detect the

emergence of resistant strains and to determine the potential therapeutic value of a new antifungal agent (Espinel-Ingroff, 1994). Unfortunately, the process required to standardize these methodologies has been slow. Numerous studies have attested that the *in vitro* results have been influenced by a number of technical factors, including concentration of the fungal inocula, the composition and pH of the medium; the incubation temperature and the length of incubation (Doern *et al*., 1986; Espinel-Ingroff *and* Shadomy,1989; Rex *et al*., 2001). On the other hand, it has also influenced that fungal infections are less common than bacterial infections, and this implies that there is a smaller number of data to establish possible correlations between the efficacy of *in vitro* and *in vivo*. It is for this reason that methods to evaluate the *in vitro* susceptibility to the different antifungal drugs were not standardized in the case of yeasts until 1997 and in the case of filamentous fungi to 2002.

The CLSI subcommittee for Antifungal Testing has developed standardized both: broth microdilution (BMD) and disk diffusion methods for *in vitro* susceptibility testing of yeasts and moulds (CLSI 2008, CLSI 2009). These methods are reproducible and accurate, and also provide clinically useful information that is comparable to that of antibacterial testing.

Establishing a clinical correlation between *in vitro* susceptibility tests and clinical outcome has been difficult (Arendrup *et al*., 2008). Antifungal susceptibility testing can be said to predict the outcome of treatment consistent with the “90–60” Rule (Arikan, 2007). According to this rule, infections due to susceptible isolates respond to therapy ~90% of the time, whereas infections due to resistant isolates respond to therapy ~60% of the time. Thus, low MICs are not entirely predictive of clinical success, and high MICs help to predict which patients are less likely to have a favourable response to a given antifungal agent. The 90–60 rule reflects the fact that

the *in vitro* susceptibility of an infecting organism to the antifungal agent is only one of several factors that may influence the likely success of an infection's therapy. Despite considerable progress, it remains to be seen how useful could be antifungal susceptibility testing in guiding therapeutic decision making. Future efforts will be directed toward further validation of interpretive breakpoints for established antifungal agents and developing them for newly introduced systemically active agents.

1.5.2 *In vivo* testing

Animal models of fungal infections play an important role in medical mycology. Nowadays many different types of animal models of fungal infection have been developed, being the murine model the most frequently used, for studies of pathogenesis, virulence, immunology, diagnosis and antifungal therapy (Andriole *et al*., 1992; Odds *et al*., 1998; Polak, 1998; Graybiil, 2000; Capilla *et al*., 2007). Particularly important, for the use of animal models, is the ability to control variables, which allows a well defined model system to be used to address various issues of efficacy (Clemons and Stevens, 2006). A major benefit is that of being able to examine novel indications and therapies in a timely manner using sufficient numbers of animals to generate statistically valid and objective results. Thus, therapeutics can be tried in a variety of scenarios in animal models prior to a clinical trial or where such a trial is impractical (Guarro, 2011).

One of the main uses of animal models is the demonstration of therapeutic efficacy of antifungal agents since tests conducted in experimental animals are crucial to the development of new drugs (Andriole, 1992; Polak, 1998). The initial standard approach to discovery and development of antifungal is to screen activity of a large number of various compounds (e.g., chemical libraries) against reference organisms. This step is known as primary screening. When a potential candidate is

identified, antifungal activity against a large panel of clinical isolates is undertaken. Candidate compounds, which demonstrate an appreciable antifungal activity in these tests, are selected and their properties characterized further using many different *in vitro* and *in vivo* test systems (e.g., pharmacokinetic properties). Hence, no matter how sophisticated drug screening and development may be, an essential step in the discovery and development of new antifungal drugs before testing in humans, is the evaluation of the drug for its efficacy and toxicity in animal models (Polak, 1998). In this regard, showing that a candidate compound is active in *in vitro* does not necessarily guarantee that it is active in *in vivo*. More importantly, some compounds that possess outstanding activity *in vitro* turn up to be very toxic when introduced into animals. Therefore, evaluation of candidate drugs in animal models is a critical step in predicting the efficacy and toxicity of antifungal agents in humans (Clemons and Stevens, 2006, Capilla *et al*., 2007).

Critical to the determination of drug efficacy in an animal model are the parameters used to evaluate the activity of the treatment. Survival and fungal burden in the tissues measured by the number of colony-forming units or surrogates of fungal mass are objective and the two primary outcome measures used by many investigators. The use of both is necessary if there are few deaths in an experiment, but at the same time may be unnecessary if there are few survivors. In addition, more evaluations such as clinical appearance, histology studies and the serum determinations of antibody or antigens as 1,3 β-d glucan or galactomannans, among others, have also been used (Andriole, 1992, Clemons and Stevens, 2006).

1.5.3 Correlation *in vitro- in vivo*

Nowadays to predict the therapeutic success based on the MIC of a fungal strain is not always possible. The studies conducted until now have not always

shown a correlation between the *in vitro* data with the *in vivo o*utcome.There are so many factors that play significant roles in the response to an antifungal drugs such as host immune function, underlying diseases, intravenous catheter removal, adjunctive surgical interventions, and pharmacokinetic parameters (Rex 2002). Although *in vitro* susceptibility testing is often used to select antifungal agents likely to be clinically active for a give infection, perhaps its more important function is the detection of *in vitro* resistance, which will indicate the less active and effective drugs (Pfaller and Diekema, 2012). Improvements in the ability of antifungal susceptibility testing methods to detect emerging resistance patterns, coupled with molecular characterization of resistance mechanism have provide useful adjuncts to optimize the effectiveness of antifungal therapy. In adittion, are aids in drug development studies and as a means of tracking the development of antifungal resistance in epidemiological studies. Clinical interpretative breakpoints (CBPs) for *in vitro* antimicrobial susceptible testing may be used to indicate those clinical isolates that are likely to respond to treatment with a given antimicrobial agent administered using the approved dosing regimen for that agent. Conversely epidemiological cutoff values (ECVs) can be considered to represent the most sensistive measure of the emergence of strains with decreased susceptibility to a given agent. An ECV is a MIC threshold value that allows the discrimination of wild type (WT) strains (those without mutational or acquired resistance mechanism) from non-WT strains (those having mutational or acquired resistance mechanism). Although the clinical relevance of the epidemiological cutoffs remains uncertain, its use could be important for the detection and study in the laboratory of resistance to certain antifungal.

1.6 Histopathology

Mycological evidence begins with the specimen. Thus, specimens obtained from normally sterile but clinically abnormal sites were rated to be more reliable than those obtained from adjacent normal sites or sites normally colonized with resident commensal flora (Pirofski and Casadevall, 2002; Ascioglu *et al*., 2002; Schnadig and Woods, 2009). These specimens were considered necessary to provide invasive fungal infections (IFIs). Hence, mycological evidence acquired by means of either direct examination or culture of specimens from sites that may be colonized (e.g., sputum, bronchoalveolar lavage fluid, or sinus aspirate) were thought only to support the diagnosis, not to prove it. Similarly, with the sole exception of *Cryptococcus neoformans,* indirect tests to detect antigen were considered to be suggestive but not conclusive. Thus, the nature and quality of the specimen and the use of direct and indirect mycological techniques were incorporated into each of the criteria (Ascioglu *et al*., 2002).

The histopathological study of tissue biopsies for the identification of fungal cells is a very important diagnostic tool (Liu *et al*., 1998; Reller *et al*., 2001; Guarner and Brandt, 2011). Despite that, the conventional isolation of the etiological agent by culture followed by its identification is the best way to identify the pathogen, even though to distinguish infection and colonization is a frequent problem that has important treatment implications for the patients. In general, the histopathology of fungal infections is particularly useful when cultures cannot be made or the pathogen is slow growing or no-culturable as in the case of *Lacazia loboi and Pneumocystis spp* (Reller *et al*., 2001). The major advantages of histopathology are: its quick in comparison with the culture, its low cost and the ability to provide presumptive identification of the infecting fungus as well as demonstrating the host tissue reaction and that shows the progress of the infection from acute to chronic. However,

achieving a successful histopathologic diagnosis begins with the selection of the tissue sample to be examined and the proper staining. To visualize the fungi in tissue sections there are several available stains, although some of them are special for fungi, others are more general and allow observing the tissue reactions. In this sense, the haematoxylin-eosin (H&E) stain is the most common for the histopathologic evaluation of tissue section. Although is very useful to observed the host´s response is not a special fungal stain. On the other hand, periodic acid-Shiff (PAS) and Grocott-Gomori methenamine silver stain (GMS) are the most efficient techniques to observe fungi. Among these stains GMS is the best to visualize fungi since it stains old and non-viable fungal cell. Other stains utilized are Mayer´s mucicarmine for Mucin, and Alcian blue, which stain the mucopolysaccharide capsule of *C. neoformans* and Fontana-Masson for the melanine pigment of dematiaceous fungi (Gupta *et al*., 2009; Guarner and Brandt 2011).

Taking into account the type of tissue's host response to fungi, which depends on the host's immune status and the manner in which the fungus interacts with the host, we can identify five main histopathologic types of host responses to fungi (Schnadig and Woods, 2009).

1. **Non-invasive colonization of a preexisting cavity.** Non-invasive fungal colonization of a cavity is referred to as a fungus ball. Acute and chronic inflammation may be seen within the cavity wall and occasionally superficial mucosal erosion can occur; there is no fungal invasion into adjacent tissue. Common sites for fungus balls are the paranasal sinuses, external auditive conducte and old pulmonary cavities such as those seen in chronic tuberculosis (aspergiloma).

2. **Allergic mucin-producing, non-invasive fungal disease.** These represent allergic hypersensitivity reactions associated with elevated IgE levels, fungus-specific precipitins and production of allergic mucin (AM). AM is composed of a mixture of

mucin, cellular debris, eosinophils and Charcot–Leyden crystals and may be found in fungal and non-fungal allergic disease, including asthma.

3. **Predominantly neutrophilic inflammatory response.** Patients with mild neutrophilic impairment may develop localized infections and severely neutropenic patients are at risk for disseminated infection. Invasive infections are characterized by neutrophilic exudate and necrosis with liquefaction. Coagulative necrosis, without an associated inflammatory response, is seen in severely neutropenic patients. Fungal vascular invasion is typically found in severe infections

4. **Granulomata versus diffuse macrophage infiltration.** In patients with localized granuloma formation, extensive necrotizing granuloma formation and diffuse macrophage infiltration, the following may occur: small localized granulomata; extensive granuloma formation often with caseation, cavitation and fibrosis; or marked macrophage response without epithelioid transformation. Macrophages appear virtually engorged with intracytoplasmic organisms. The latter is found in AIDS and other severely T cell immunosuppressive conditions and should not be called granulomatous. Neutrophils are not typically seen in histologic or cytologic material from these types of infections unless there is bacterial superinfection or extensive necrosis.

5. **Mixed granulomatous and purulent inflammation.** The host response is a mixture of epithelioid macrophages and neutrophils. In localized controlled infections the granulomatous reaction dominates and organisms are very scant. In more fulminant infections, one sees a predominance of neutrophils and organisms are readily seen. In advanced AIDS, the granulomatous response is essentially one sees abundant organisms, variable numbers of neutrophils and extensive necrosis. Most dimorphic fungi will produce this spectrum of disease. Mixed purulent and

granulomatous inflammation is seen in infections with *Sporothrix schenckii* and dematiaceous fungal infections.

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II. Interest and Objectives

2.1 Interest and objectives

Fungal infections' therapies have improved greatly over the last decade, but invasive infections still represent a serious threat, mainly for immunocompromised and immunodeficient patients. Apart from the most common opportunistic fungi, *Aspergillus fumigatus* or *Candida albicans*, many other species have emerged in recent years as agents of mycoses whose diagnosis and treatment represent important challenges in the clinical practice.

The advent of molecular techniques, mainly DNA sequencing, has led to discover new pathogenic fungi, most of them being cryptic species belonging to complexes of species that have replaced traditional single species. This has occurred in several genera like *Sporothrix, Acremonium* and *Scedosporium*, among others. The correct identification of these species is crucial for a better management of patients since, in many occasions they show different virulences and responses to antifungals. For that reason, their emergence, as serious agents of disease among specific patient cohorts, present new challenges for the delivery of a safe and effective antifungal therapy. The release, in recent years, of new antifungal has allowed the development of new therapeutic options. However, despite these advances, an important number of fungi remain as serious threats to the health of susceptible patients and the (studies) outcomes are far from optimal, requiring a better knowledge of their pathogenic mechanisms and the development of new therapeutic strategies. Based on this, the **main objective of this thesis has been to contribute to the development of new experimental treatments for invasive fungal infections caused by emerging opportunistic agents.** Therefore, some studies of virulence, *in vitro* antifungal susceptibility and on the *in vivo* efficacy of some of antifungal agents such as voriconazole, posaconazole, caspofungin, anidulafungin, micafungin and amphotericin B and combinations of all of them have

been carried out. The fungi selected to be tested have been the emerging yeast *Candida glabrata* and new described species of filamentous fungi belonging to the genera *Pseudallescheria*/*Scedosporium* complex*, Acremonium/Sarocladium* and *Sporothrix.*

The *Sporothrix* infections, called sporotrichosis, are the most common and cosmopolitan subcutaneous mycosis. Despite that the lymphocutaneous form is the most frequent clinical manifestation of this disease; systemic infections have also been reported, particularly in immunosuppressed patients. Until recently, sporotrichosis was considered to be caused by the single species *Sporothrix schenckii*; however, molecular studies have demonstrated that *S. schenckii sensu lato* is a complex of cryptic species, being *S. brasiliensis* and *S. schenckii sensu stricto* the most common species of the complex showing particular pathogenic behaviour as well as different antifungal susceptibility patterns. By contrast, *Sporothrix luriei* is a very rare species phylogenetically related to the species of the *S. schenckii* complex whose habitat and pathogenic behaviour are practically unknown.

Currently, treatment options for disseminated sporotrichosis are limited to amphotericin B and there is little information on the effectiveness of other antifungal drugs. According to the mentioned above, one of the objectives of the present thesis has been to determine the virulence of *S. luriei* and to evaluate the efficacy of new experimental therapies against the disseminated sporotrichosis.

Based on that, the specific objectives of our studies were to evaluate the:

- Virulence of *Sporothrix luriei* in a murine model.
- *In vitro* activity of posaconazole and voriconazole against *S. schenckii* sensu stricto and *S. brasiliensis.*

- *In vivo* efficacy of posaconazole as an alternative therapeutic option against *S. schenckii* sensu stricto and *S. brasiliensis* in a murine model of disseminated sporotrichosis
- To assess the correlation between the *in vitro* susceptibility to voriconazole and its *in vivo* efficacy against *S. schenckii* sensu stricto and *S. brasiliensis* in a murine model of disseminated infection.

The *Acremonium* infections have dramatically increased in recent years. Such infections show a variety of clinical manifestations, being disseminated infections affecting patients with severe immunosuppression, often associated with a high mortality rate, the most serious. The optimal treatment for invasive infections by *Acremonium* spp. has not been established yet. Only scarce *in vitro* data on the antifungal susceptibility of these fungi have been reported as well as studies in animal models, which could be useful for testing potential therapies. Therefore, another objective of this thesis was to develop a standardized animal model of disseminated infection by *Acremonium* spp. and evaluate the effectiveness of new treatment strategies. Based on that, the specific objectives of the studies were:

- To evaluate the *in vitro* susceptibility of *Acremonium* against different antifungal drugs and to determine the *in vitro* activity of different antifungal combinations.
- To develop a murine model of disseminated infection by A*cremonium* spp. in order to determine their virulence and differences between species.
- To assess the effectiveness of different antifungal drugs such as amphotericin B, andifulafungin, posaconazole and voriconazole in that murine model.

- To determine, using the mentioned animal model, the *in vivo* efficacy of dual antifungal combinations against disseminated infection by *Acremonium.*

The *Pseudallescheria/Scedosporium* species are relatively common agents of a wide diversity of opportunistic infection. These infections are difficult to treat due to their therapy-refractory profile. *Pseudallescheria boydii* and *Pseudallescheria apiosperma* are the most prevalent *s*pecies of the genus. Both species show similar antifungal susceptibility profiles, being voriconazole the antifungal drug with better *in vitro* activity against these fungi, followed by micafungin and posaconazole. Taking into account that these antifungals show different targets in the fungal cell, micafungin inhibiting the 1,3 β-D glucan of the cell wall and azoles in the inhibiting the ergosterol synthesis in the membrane, to test the combination of such drugs could be of interest in order to evaluate possible new therapies. Based on that, the specific objectives of our studies were:

- To determine the *in vitro* interaction between micafungin and posaconazole or voriconazole by using two *in vitro* methods, E-test®, and checkerboard broth microdilution method.
- To evaluate the *in vivo* efficacy of the combination micafungin plus posaconazole or voriconazole in a murine model of disseminated infection against *Pseudallescheria boydii* and *Pseudallescheria apiosperma.*

Candida glabrata has become an important agent of invasive candidiasis, being one of the most common *Candida* species after *C. albicans.* Currently, the echinocandins such as caspofungin are the recommended drugs for its treatment

due to the reduced susceptibility of thisfungus to the azoles, especially to fluconazole. However, emergence of echinocandins resistance has been also observed in the last years. This resistance has been associated with the presence of mutations in two hot spot (HS) regions of the *FKS* genes, which encode the major subunit of the 1,3 β-D-glucan synthase complex and is the target for echinocandins. A critical issue associated to the progress in the therapeutic control of *C. glabrata* infections is to explore the molecular mechanisms involved in the resistance and failure of the therapies used as well as the reassessment of breakpoints to better predict therapeutic success. Since clinical data are limited, one of the objectives of this thesis was to determine the efficacy of caspofungin in an experimental model of disseminated infection by *C. glabrata* to investigate the impact of these revised breakpoints on the detection of resistant isolates and in predicting the response to echinocandins therapy.

The specific objectives were:

To determine the caspofungin MICs against a set of clinical isolates of *C. glabrata* strains by broth microdilution method and try to correlate them with the presence of *FKS* mutations.

- To determine whether MICs of caspofungin and the presence or absence of *FKS* mutations *in Candida glabrata* are predictive of *in vivo* outcome by using a murine model of invasive candidiasis by *C. glabrata.*

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III. Material and Methods

3.1 *In vitro* studies

3.1.1 Strains

To evaluate the *in vitro* susceptibility to different antifungal drugs, 116 strains belonging to 12 opportunistic fungal species of clinical interest were assayed (Table1).

Table 1. Number of Isolates assayed *in vitro* and their origin.

Species (N° total of isolates)	Collection	N° of strains
***Candida glabrata* (36)**	**FMR**	**10**
	JMI	**9**
	UTHSC	**17**
***Sporothrix schenckii sensu stricto* (10)**	**FMR**	**10**
***Sporothrix brasiliensis* (5)**	**FMR**	**5**
***Sporothrix luriei* (1)**	**CBS**	**1**
***Sarocladium (Acremonium) kiliense* (12)**	**UTHSC**	**12**
***Acremonium implicatum*(2)**	**UTHSC**	**2**
***Acremonium sclerotigenum-egyptiacum* complex (2)**	**UTHSC**	**2**
***Pseudallescheria apiosperma* (26)**	**FMR**	**21**
	CBS	**3**
	IHEM	**2**
***Pseudallescheria boydii* (17)**	**CBS**	**3**
	FMR	**11**
	IHEM	**4**
***Scedosporium dehoogii* (2)**	**FMR**	**2**
***Scedosporium aurantiacum* (1)**	**FMR**	**1**
***Scedosporium prolificans* (1)**	**IHEM**	**1**

FMR, Facultat de Medicina de Reus, Universitat Rovira i Virgili, Reus, Spain.

CBS, Fungal Biodiversity Centre, Utrecht, The Netherlands.

IHEM, Scientific Institute of Public Health, Louis Pasteur Institute, Belgium.

JMI, JMI Laboratories, North Liberty, Iowa, USA.

UTHSC, Fungus testing Laboratory, University of Texas Health Science Center.

3.1.2 Conservation of strains

All strains were conserved as submerged cultures in liquid paraffin. Working strains were subcultured into potato dextrose agar plates (PDA; Pronadisa, Madrid, Spain)

or Sabouraud agar plates (SAB, Pronadisa, Madrid, Spain) until evident growth or sporulation. Then they were conserved in slant tubes of PDA or SAB at room temperature.

3.1.3 Antifungal drugs

A total of 8 antifungal drugs (Table 2) were used in order to determine the MIC against the fungal species included in the *in vitro* studies (Table 1)

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

Antifungal drugs	Purity (%)	Conservation	Dissolvent	Origen
Amphotericin B (AMB)	**99.8**	**RT**	**DMSO**	Sigma Chemical Co. St. Louis, EUA
Caspofungin (CSP)	**100**	**-20°C**	**Water**	Merk & Co ., Inc.,Rahway, EUA
Anidulafungin (AFG)	**100**	**-20°C**	**DMSO**	Pfizer Inc., Madrid, Spain
Micafungin (MFG)	**100**	**-20°C**	**Water**	Fujisawa Pharmaceutical Co. Ltd., Osaka, Japón
Voriconazole (VRC)	**100**	**RT**	**DMSO**	Pfizer Inc., Madrid, Spain
Posaconazole (PSC)	**100**	**RT**	**DMSO**	Schering-Plough Res. Inst., Kenilworth, EUA
Itraconazole (ITR)	**100**	**RT**	**DMSO**	Janssen Pharmaceutical, Madrid, Spain.
Terbinafine (TRB)	**100**	**-20°C**	**DMSO**	Novartis Pharmaceutical, Barcelona, Spain.

RT: Room temperature. DMSO: Dimethyl sulfoxide.

3.1.4 Antifungal susceptibility test

To evaluate the activity of the different antifungal drugs alone or in combination against the fungal species listed on table 1, E-test® and broth microdilution method were performed.

3.1.4.1 Broth microdilution methods

3.1.4.1.1 Single antifungal susceptibility test

Antifungal susceptibility testing was performed by a broth microdilution technique according to the guidelines of the Clinical Laboratory Standards Institute (CLSI). For filamentous fungi we used the document M38-A2 (CLSI-2008) and for yeast the document M27-A3 (CLSI-2009). To prepare the stock solutions of antifungal, the drugs were weighed and dissolved in dimethyl sulfoxide (DMSO) or in distilled water, depending on their solubility (Table 2). Those water in-soluble drugs were dissolved at concentrations at least 100 times higher than the highest desired test concentration in DMSO. Then a series of 2-fold dilutions at 100 times the final concentration were prepared from the antifungal stock solution. These intermediate solutions of antifungal drugs were prepared in DMSO following the scheme of figure 1 and then were diluted 1:50 in RPMI medium. This procedure avoids dilutions artifacts that results from precipitation of compounds with low solubility in aqueous media (CLSI-2008, CLSI 2009).

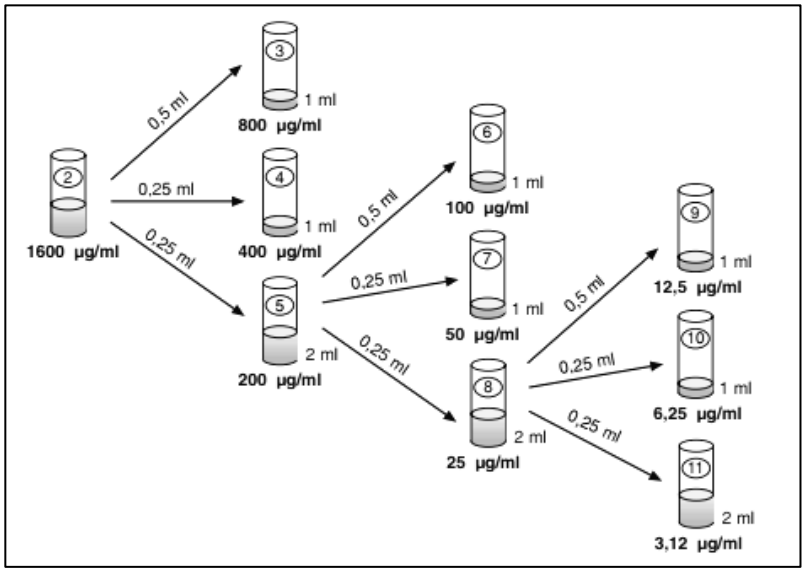

Figure 1. Scheme for the dilution of water in-soluble antifungal drugs.

Those drugs water-soluble were prepared in a twofold concentration in sterilized distilled water (figure 2) then each dilution was diluted 1:5 in RPMI. The RPMI medium was prepared with RPMI-1640 (with glutamine, without bicarbonate and with phenol red as a ph indicator) buffered to a pH of 7.0±0.1 at 25°C with MOPS (3-[N-morpholino] propanesulfonic acid) at a final concentration of 0.165 mol/L.

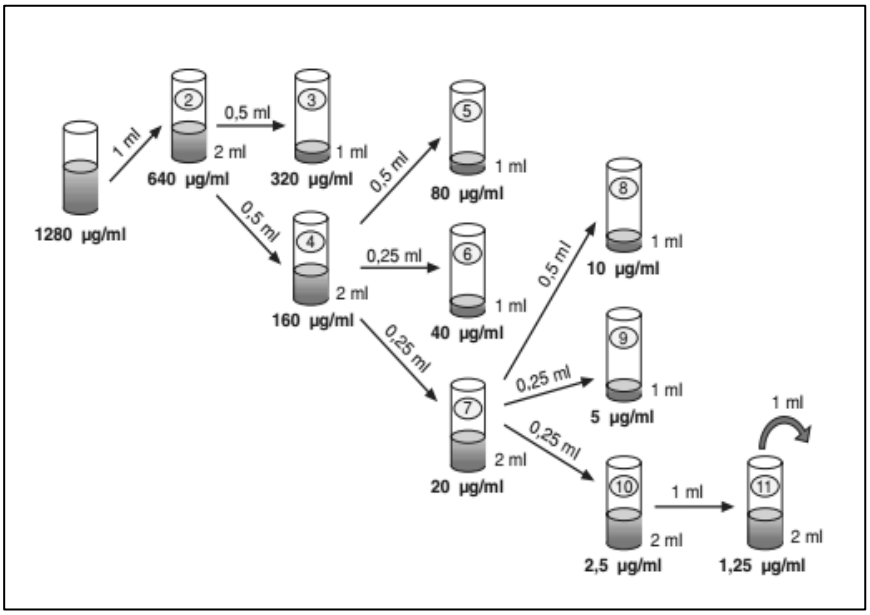

Figure 2. Scheme for the dilution of water soluble antifungal drugs.

One hundred microlitres of each drug dilution were dispensed onto rows of multiwell microdilution plates (96 wells). Drug-free wells containing RPMI + 2% DMSO in the medium served as the growth control (positive control) and control of sterility of medium (negative control) (figure 3). Last row of each plate was reserved in aim to include a quality control strain (QC).

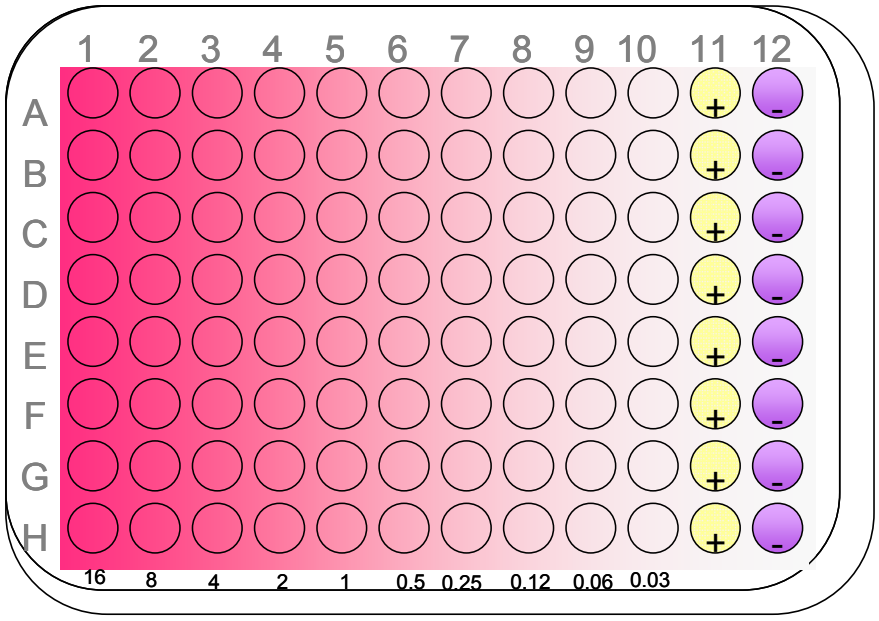

Figure 3. Schematic distribution of the different antifungal concentrations in a range 0.03 to 16µg/ml. Columns 11 and 12 are used as positive (-) and negative (+)controls, respectively. Row H include the quality control strain.

3.1.4.1.2 Antifungal combinations assays

Due to poor *in vitro* activity of the assayed antifungal drugs against *Pseudallescheria* spp. and *Sarocladium kiliense, in vitro* combinations of antifungal drugs were assayed. To evaluate the *in vitro* activity of double combinations, a two-dimensional checkerboard based on the document from the CLSI M38-A28 (CLSI, 2008) and modified by Dannaoui et al. (Dannaoui et al., 2004) was used (figure 4).

In brief, 96-well microdilution plates were used; the final volume of drugs in each well was 100µl. In total 9 different combinations were assayed (Table 3), which were placed on rows or columns (figure 4).

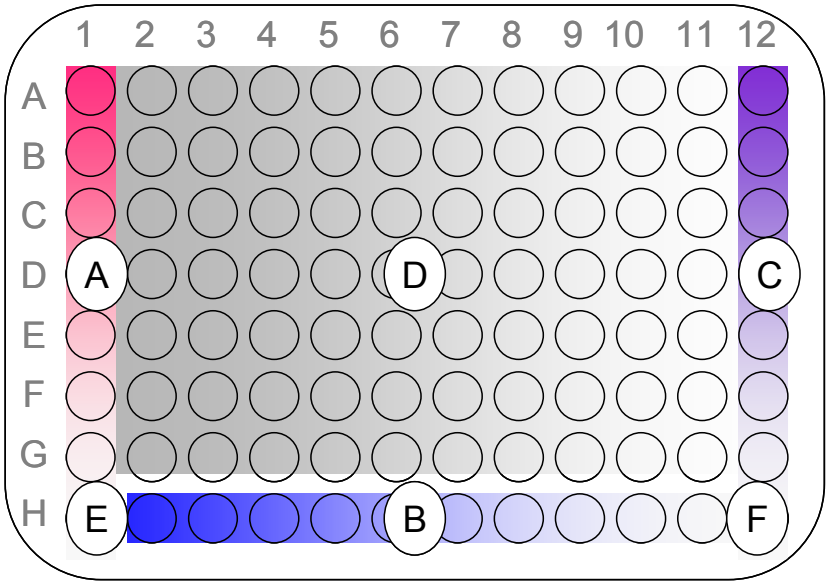

Figure 4. Schematic distribution of antifungal compounds (A and B) in microplates. Six zones are defined: A and B, zones to determine the minimal inhibitory concentration (MIC) of each antifungal alone; C, defined as the zone of positive control without antifungal; D, zone of antifungal combination; E and F, defined as the zone of negative control.

Table 3. Antifungal drugs used for the synergism studies and their combinations.

Organism	Combination	Range of concentration (µg/ml)
***Pseudallescheria* spp.**	MFG plus VRC MFG plus PSC	MFG (64 - 0.003) VRC (32 - 0.03) PSC (64 - 0.03)
Sarocladium kiliense	AMB plus AFG AMB plus PSC AMB plus VRC VRC plus AFG VRC plus TER PSC plus AFG PSC plus TER	AMB (8.0 - 0.12) AFG (8.0 - 0.12) PSC (16 - 0.03) VRC (16 - 0.03) TER (4.0 - 0.06)

3.1.4.2 E-test®

3.1.4.2.1 Single antifungal susceptibility test

In parallel to the *in vitro* susceptibility studies done by microdilution method, in those studies including *Pseudallescheria*/*Scedosporium* spp., we also determined the susceptibility to posaconazole, micafungin and voriconazole by E-test®. E-test® consists on a plastic strip containing a stable gradient of an antifungal compound. Strips of PSC, MCF and VRC were used following the manufacturer´s instructions. Briefly, agar medium containing RPMI-1640 with 2% glucose was inoculated dipping sterile swab into the inoculum suspension adjusted as previously described at $1x10^6$-$5x10^6$ CFU/ml and then evenly streaking it in three directions over the entire surface of the culture medium. The agar surface was allowed to dry for 15 min, and the strips were placed onto the inoculated agar. The plates were incubated at 30°C, and the MICs were determined after incubation for 48 h to 72 h.

3.1.4.2.2 Antifungal combination assays

Once the MICs for individual antifungal drugs against the *Scedosporium* spp. isolates were determined, the *in vitro* activity of posaconazole plus micafungin and voriconazole plus micafungin combinations (table 3) was determined by placing E-test® strips of the two antifungal drugs on the agar at a 90° angle with the intersection at the respective MICs for the isolates (figure 5). The agar plates were incubated at 30°C for 48 to 72 h, and the MIC of each antimicrobial in combination was read.

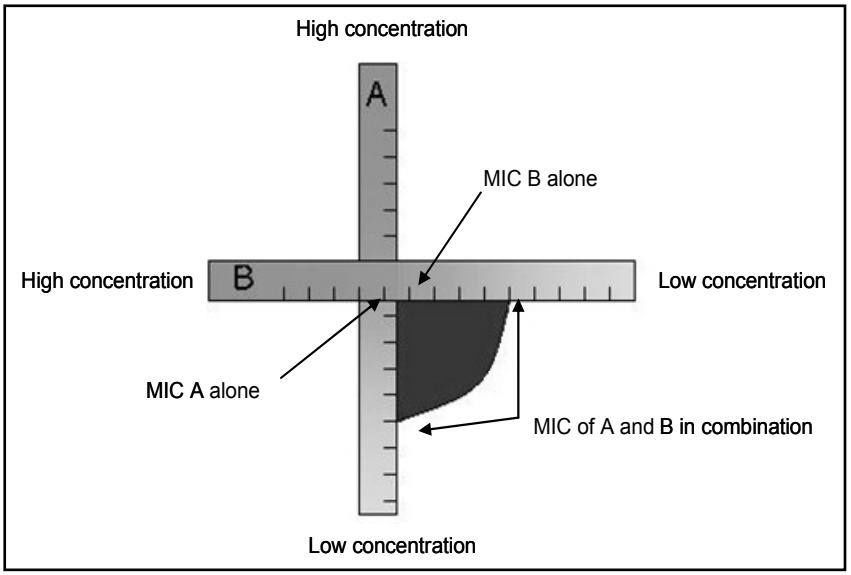

Figure 5. Placement of E-test® strips for the E-test methods for synergy

3.1.4.3 Inocula preparation, inoculation and incubation

For the preparation of inocula the isolates were grown on PDA for 7 days at 30 °C until sporulation, in the case of filamentous fungi, while the yeast was on SBD for 48 hours at 35°C. For filamentous fungi the inocula were prepared by flooding with 5 ml of saline solution onto the surface of the agar plate, scraping the sporulating mycelium and filtering the obtained suspension twice, through several layers of sterile gauze in order to remove clumps of medium and hyphal fragments. The resulting suspension was mixed with a vortex and the conidia were adjusted by Neubauer chamber count and serial dilutions in saline to 1-5x10^6CFU/ml according to CLSI M38-A2 (CLSI2008). The inocula of yeast were prepared according to CLSI M27-A3 (CLSI 2009). Five colonies were picked from the agar, suspended in saline solution and size of inocula adjusted to 1-5x10^6 CFU/ml as previously described (CLSI 2009). With the aim to evaluate the viability of the adjusted inocula 100 µL of

each conidial suspension were transferred to PDA or SAB plates and incubated at 35ºC for 48-72 h in the case of filamentous fungi and 24-48 h in the case of yeasts.

From each inoculums 100µl of conidial suspension diluted in RPMI was inoculated in microtiter plates and those were incubated depending on the optimal growth conditions for each species. In the case of *Sporothrix* spp., *Pseudallescheria/Scedosporium* spp. and *Sarocladium* sp., the time of incubation was 48-72 h and for *Candida glabrata* 24-48 h, all of them incubated at 35ºC in darkness.

The fungal growth was visually assessed with the aid of a reading mirror. As quality control *Paecillomyces variotii* **MYA-3630**, *Aspergillus fumigatus* **MYA-3626** or *Candida parapsilosis* **ATCC 22019** were used.

3.1.4.4 Reading and interpretation of the results.

3.1.4.4.1 Microdilution method

Filamentous fungi: for azoles, 5-fluorocytosine and Amphothericin B MIC was defined as the lowest concentration of drug able to produce a 100% of inhibition with respect to the control. In the case of echinocandins against filamentous fungi the minimal effective concentration (MEC) was determined. The MEC is defined as the lowest concentration of drug that leads to the abnormal growth of the fungus (small, rounded, and/or compact hyphal forms) in comparison to the control hyphal growth.

Yeast: for Amphothericin B the MIC was the lowest concentration of drug able to produce 100% of inhibition. For echinocandins, azoles and 5-fluorocitosine the MIC was considered as the lowest concentration able to produce a 50% reduction on fungal growth in comparison to the control well.

3.1.4.4.2 *E-test*®

To evaluate *in vitro* activity for each strain by *E-test*®, the MIC for azoles and the MEC for echinocandins was determined as the lowest drug concentration at which the border of the elliptical inhibition intercepted the scale on the antifungal strip (figure 6).

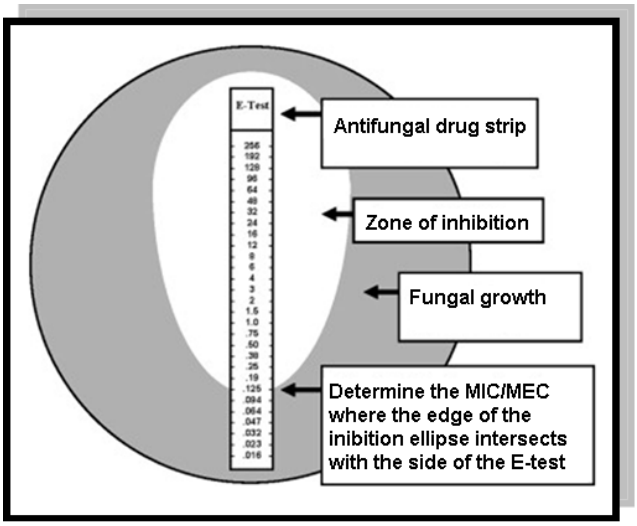

Figure 6. The E-test strip containing an antifungal gradient is placed onto the inoculated agar plate. After incubation, the MIC corresponds to the place where the edge of the inhibition ellipse intersects with the side of the E-test.

3.1.4.4.3 Double combinations by microdilution method and E-test®

To evaluate the interaction of *in vitro* combination by the microdilution or E-test method (figure 5), the fractional inhibitory concentration index (FICI) was calculated for each combination as follows:

$$\sum FIC = C_A^{comb}/MIC_A^{alone} + C_B^{comb}/MIC_B^{alone}$$

Where MIC_A^{alone} and MIC_B^{alone} are the MICs of drug A and B when acting alone and C_A^{comb} and C_B^{comb} are the concentrations of drugs A and B at the combinations, respectively. Drug interactions were classified as synergistic (when FICI <0.5), no interaction (FICI >0.5 but ≤4), or antagonistic (FICI >4) (Dannaoui et al., 2004; Johnson et al., 2004).

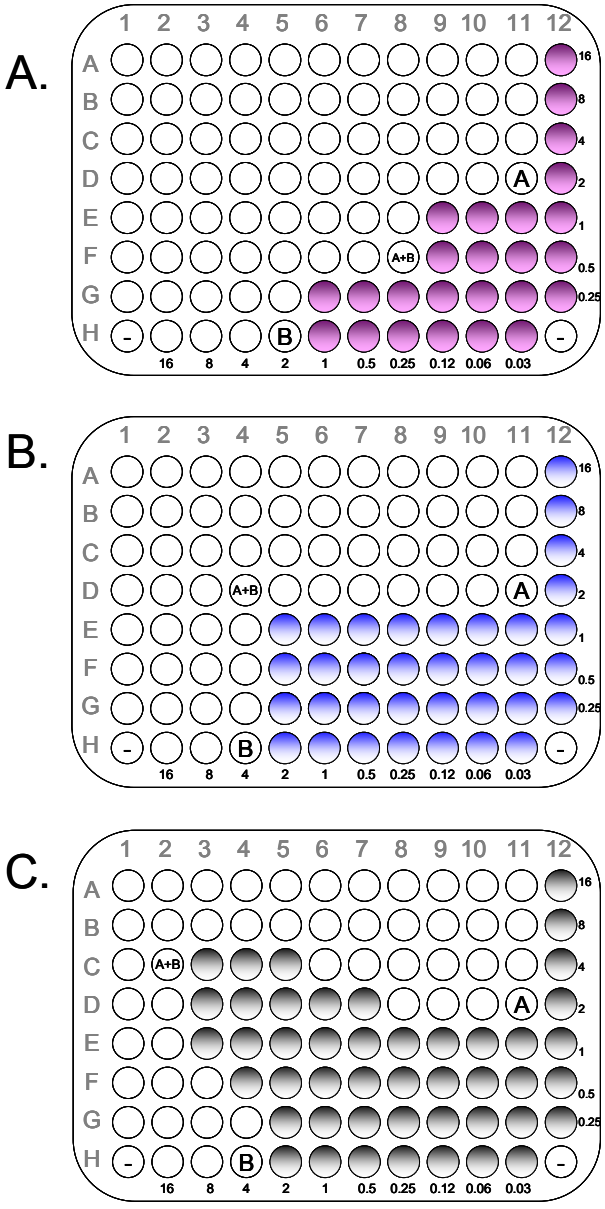

Figure.7. Checkerboard technique: interpretation of the results. (A) synergism; (B) indifference; (C) antagonism.

3.2 *In vivo* studies

The *in vivo* studies consisted in the development of animal models of systemic infection with the species detailed on Table 4 to perform virulence studies and to assess the efficacy of experimental antifungal treatments.

3.2.1 Strains

For the *in vivo* studies 36 strains from different species were evaluated (Table 4).

Table 4 . Isolates used in the animal model. Species and their origins

Candida glabrata	
FMR 8489	Urine ; Spain
FMR 8498	Clinical ; Spain
FMR 11381	Clinical; USA
UTHSC 08-134	Clinical; USA
UTHSC 11-149	Clinical; USA
UTHSC 11-68	Clinical; USA
UTHSC 07-3662	Clinical; USA
UTHSC 10-461	Clinical; USA
JMI-206	Clinical; USA
JMI-211	Clinical; USA
JMI-297	Clinical; USA
JMI-760	Clinical; USA
JMI-2092	Clinical; USA
JMI-10956	Clinical; USA
JMI-14378	Clinical; USA
JMI-127	Clinical; USA
JMI-729	Clinical; USA
Sporothrix schenckii	
FMR 8604	Cutaneous lymphatic sporotrichosis; Perú
FMR 8606	Cutaneous lymphatic sporotrichosis; Perú
FMR 8609	Cutaneous lymphatic sporotrichosis; Perú
FMR 9010	Unknown; USA
FMR 9018	Unknown; USA

Table 4 (continued) . Isolates used in the animal model. Species and their origins	
Sporothrix brasiliensis	
FMR 8314	Skin lesion; Brazil
FMR 8319	Skin lesion; Brazil
FMR 8326	Skin lesion; Brazil
Sporothrix luriei	
CBS 937.72	Clinical, India
Sarocladium (Acremonium)kiliense	
UTHSC 03-3197	Vitreous fluid; Florida, USA
UTHSC 07-550	Blood; Arkansas, USA
Acremonium implicatum	
UTHSC 07-3260	Bone ; Illinois, USA
UTHSC 07-3667	Bronch wash; Minnesota, USA
Acremonium sclerotigenum-egyptiacum complex	
UTHSC 01-194	Blood; Washington, USA
UTHSC 05-2270	Blood; Utah, USA
Pseudallescheria apiosperma	
FMR 13015	Clinical; Netherlands
CBS 117419	Mycetoma; New Zeland
Pseudallescheria boydii	
FMR 12741	Maxillary senus; India
FMR 13004	Clinical; China

3.2.2 Animals

Male OF-1 mice (Charles River, Criffa S. A., Barcelona), weighing 30 g were used (figure 8A). All animals' care procedure were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. The animals were housed in standard boxes with corncob bedding and had free access to food and water. They were supervised twice a day to monitor the clinical course of the disease/infection. To do so, the clinical parameters used to assess the general health status were: body weight, behaviour, fur coat and posture, appearance of eyes and nose.

Euthanasia due to animal suffering was by CO_2 inhalation. This same procedure was used to end each study.

3.2.3 Immunosuppression

In order to mimic the clinical situation of patients suffering scedosporiosis, acremoniosis, or candidiasis, neutropenia was induced to mice by single administration of cyclophosphamide at 200mg/kg given intraperitoneally plus 5-fluorouracil at 150 mg/kg intravenously both administered the day before to infection (Ortoneda et al., 06).

3.2.4 Infection

The inocula of *Candida glabrata* or *Pseudallescheria/Scedosporium* spp. were prepared as in **3.1.4.3.** While, in those studies that included *Sarocladium/Acremonium* spp. and *Sporothrix* spp. the inocula were prepared as follows: the strains were subculture on PDA and incubated at room temperature for 7-10 days. Then, the surface of the agar plates was flooded with saline solution, scraping the sporulating mycelium. The resulting solution was transferred to 100 ml of potato dextrose broth (PDB) (Arrillaga *et al*., 2009) and incubated in an orbital shaker at 150 rpm at 25°-30º C for 5 days. Then, cultures were filtered twice through sterile gauze, and centrifuged at 7000 rpm. The pellet was washed once with saline solutions and the conidia concentrations were adjusted to desired concentrations by haemocytometer counting. To verify the viability and size of inocula 10-fold dilutions were placed in PDA for CFU determinations. Systemic infections were performed by intravenous injection in the lateral tail vein of the mice of a conidial or yeast suspension. After, infection animals were checked twice a day . At the end of the

experimental time or when animals showed signs of discomfort, mice were euthanatized by anoxia in a CO_2 chamber.

3.2.5 Antifungal treatments

Antifungal agents used in the experimental treatments are shown in Table 5. Treatments started 24 hours after infection (day +1) and were administered daily for 7, 10 or 18 days depending on the study performed. To prevent bacterial infections due to the state of immunosuppression of the animals, during the first week after the infection, ceftazidime at 5 mg/kg was administered.

Table 5. Antifungal drugs evaluated *in vivo*

Drugs	Origin	Solvent
Amphotericin B	Amphotericin B desoxicolato, Farmacia Xalabarder, Barcelona, Spain	5% dextrose solution
Anidulafungin	Ecalta® Pfizer, Ltd., UK	Saline solution
Caspofungin	Cancidas® Merck & Co. Ltd., Herfordshire, Reino Unido	Saline solution
Micafungin	Mycamine® Astellas Pharma, US	Saline solution
Voriconazole	Vfend® Pfizer, Ltd., UK.	Saline solution
Posaconazole	Noxafil® Schering-Plough, Madrid, Spain	Saline solution
Ceftazidime	Combino Pharm S. L., S. Joan Despí, España	Saline solution
Cyclophosphamide	Genoxal® Baxter S. L., Valencia, España	Bidistilled water for injection
5-Fluoruoracil	Ferrer Farma, S. A., Barcelona, España	
Sevofluorane	Sevorane® Abbott Lab., Madrid, España	

3.2.6 Treatment, doses and drug administration

Antifungal therapies were administered via different routes according to the instructions set by the manufacturer as shown on Table 6.

- **Intravenous:** The puncture was performed through one of the lateral tail veins of the mice. It was used to administrate amphotericin B (figure 8B).
- **Intraperitoneal:** The puncture was made in the lower and lateral abdomen of the immobilized animal in Trendelenburg position. It was used for the administration of echinocandins treatment (figure 8C).
- **Oral:** The administration of fluids was given directly into the lower esophagus or stomach using a feeding needle introduced into the mouth and threaded down the esophagus. It was used for administration of posaconazole and voriconazole (figure 8D).
- **Subcutaneous:** The puncture was done above the shoulders, into the loose skin over the neck. It was used for the administration of ceftazidime (figure 8E).
- **Inhalation:** Used for the administration of sevofluorane. It was used to obtain blood by cardiac puncture. The mice were placed on a chamber with cotton embedded with the anaesthetic. The reflexes were tested; depth anaesthesia is reached if the toe pinch reflex is lost.

Table 6. Drugs, doses and its administration in the mice.

Drugs	Dosing (mg/kg/day)	Routes	Volume (ml)
Amphotericin B	0.8	Intravenous	0.1
Anidulafungin	10	Intraperitoneal	0.1
Caspofungin	1	Intraperitoneal	0.1
Micafungin	10	Intraperitonea	0.1
Voriconazole	20, 40	Oral	0.2
Posaconazole	2, 4, 5, 10, 20	Oral	0.2
Ceftazidime	5	Subcutaneous	0.1
Cyclophosphamide	200	Intraperitoneal	0.3
5-fluoruoracil	150	Intravenous	0.1
Sevofluorane		Inhalatory	-

3.2.7 Fungal load determination

To determine the fungal load, mice were sacrificed by CO_2 inhalation. Then, the target organs (depending on the infecting species) were aseptically removed, weighed and mechanically homogenized in 1 ml sterile saline. Serial 10-fold dilutions were done and the homogenates were plated on SBD or PDA and incubated: for 48-96 h at 30-35ºC depending on the fungus studied. The numbers of CFU/g of tissue were calculated as follows.

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

***, volume of saline used for the organs homogenization.**

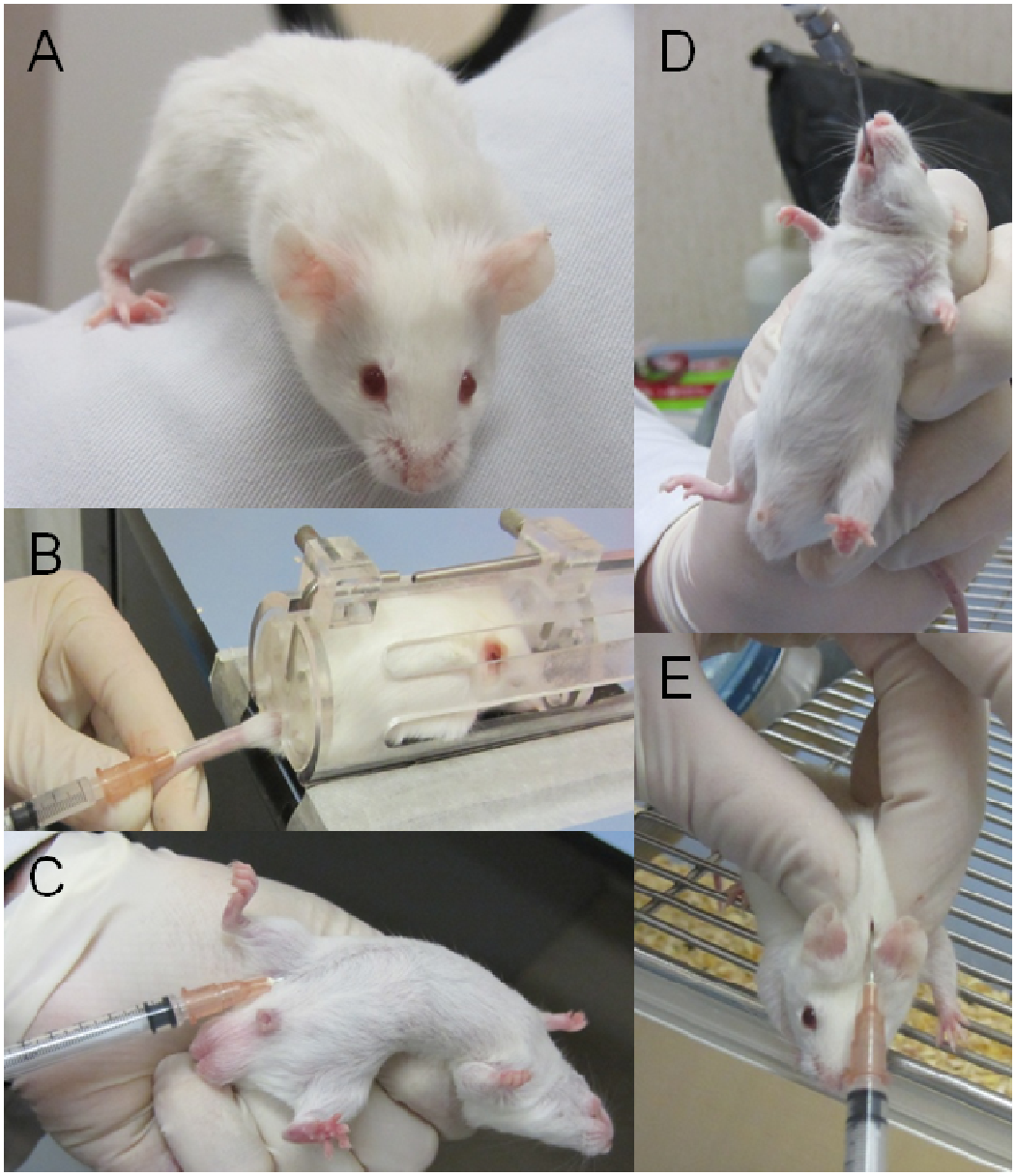

Figure 8. Animal handling and route of administration of drugs. Male OF-1 mice(A); intravenous (B); intraperitoneal (C); oral (D); and subcutaneous (E) routes.

3.2.8 Bioassay

The bioassay for determining voriconazole levels in serum and tissue was performed in the study of disseminated sporotrichosis in order to correlate the levels of voriconazole with the MIC strains and the efficacy of the treatment. For that purpose first a standard curve of the antifungal tested, was prepared. This standard curve was prepared from a known range (from 0.125 to 15 µg/ml) of serial two-fold dilutions of drug. *Candida parapsilosis* ATCC 22019 from SAB plate cultivated 24 h before and incubated at 35°C was used as indicator microorganism. For determining the standard curve of the antifungal the procedure followed was:

Twenty ml of the sterilized Yeast Nitrogen Base (YNB) were dispensed into a sterile plastic tube. When the YNB reached a temperature of approximately 50° C, the inoculum of *Candida parapsilosis* at $2x10^6$ conidia/ml was added. After homogenizing the mixture was dispensed into a sterile Petri dish. The plates were allowed to cool.

When the YNB was solidified wells of 4 mm were done and filled with 20 µl of each concentration of antifungal previously prepared. All assays were performed by duplicate. The plates were incubated at 35 ° C. The diameters of the zones of growth inhibition were measured at 24 hours and then the standard curve and its correlation coefficient werecalculated.

To calculate the concentration of drugs in the serum from animals treated with antifungal, the serums were processed in the same manner that was obtained the standard curve, thereby obtaining the antifungal concentration per ml of serum.

In the case of determination of drugs levels in tissue the organs (liver and spleen) were removed aseptically, weighed and homogenized in 1 ml of ethanol. After

incubating overnight at room temperature, the homogenate was centrifuged and the supernatant collected. Using a thermoblock, supernatant was evaporated, and the precipitate reconstituted with a volume, equal to the previously evaporated, of serum from untreated mice. The samples were dispensed into YNB containing 4 mm wells. After incubation at 35ºC the diameters of the zones of growth inhibition were measured and compared to the standard curve obtaining the antifungal concentration per gram of tissue.

3.2.9 Detection and quantification of the 1,3 β-D-Glucan

The 1,3 β-D-Glucan is a fungal wall component consisting on monomers of glucose linked each other by β-1, 3 and β-1, 6 bonds. Its detection was used as additional marker of the infection in some studies performed in the present thesis in order to report the utility of this assay for *Acremonium* spp.

The detection and quantification of 1,3 β-D-Glucan was done by using a protease zymogen-based colorimetric method commercially available as Fungitell which measures levels of 1,3 β-D-Glucan in serum. The procedure was done by following the manufacturers' instructions.

3.2.10 Histopathology

The histopathology studies were done using the organs of interest depending on the study developed. In *Candida glabrata* infections the target organ of the infection is the kidney, for *Sporothrix* infection were liver and spleen; for *Pseudallescheria/Scedosporium* infections were kidneys and brain. In the case of *Sarocladium/Acremonium* infection since no experimental data about the target organs had been reported yet.

The protocols to prepare the organs for histopathology studies are:

- **Fixation**: The organs were fixed in 10% formalin, these should swim in surrounded by formalin.
- **Dehydration**: Tissues are dehydrated by using increasing strength of alcohol; e.g. 50%, 70%, 90% and 100%.
- **Clearing**: During dehydration, water in tissue has been replaced by alcohol. In The next step alcohol should be replaced by paraffin wax. As paraffin wax is not alcohol soluble, we replace alcohol with a substance in which wax is soluble. Xylene is commonly used.
- **Impregnation with Wax**: In general, tissue samples are placed in molds together with liquid embedding material which is then hardened. The result of this stage in the preparation of histology slides is hardened blocks containing the original biological samples together with other substances used so far in the preparation process.
- **Sectioning**: Serial cuts of waxed pieces of 2-3μm using a microtome was done.
- **Dewaxing and hydration of the samples**. Before staining, the samples were deparaffinized and rehydrated to obtain histological preparations of quality. The samples were covered with xylene for 10 minutes. Then they were introduced in decreasing concentrations of isopropyl alcohol, first in alcohol at 100% and then in alcohol at 80%, respectively, for a period of 5 minutes in both cases.

- **Staining** .

  - **Hematoxylin-eosin (H-E).** In the samples stained with (H-E) the cytoplasm, hyaline substances, amyloids and collagen showed a pink colour. The structures of fungal cell, nuclei and mucoid substances stained deep purple.

  - **Periodic acid-Schiff (PAS)**. The cell nuclei were stained purple colour, while the mucopolysaccharides and fungal structures showed a red color.

  - **Grocott methenamine silver (GMS)**. Tissues taken a green colour, while fungal cells were stained black.

3.3 Molecular studies

In order to detect mutations in the Hot spot 1 in *FKS* genes, strains of *Candida glabrata* used for animal modelling (Table 4) were analysed.

3.3.1 DNA extraction

From 24h-old on cultures five or six *C. glabrata* colonies were transferred to a 2 mL Eppendorf tube containing 300 µL CTAB (cetyltrimethylammonium bromide) buffer [CTAB 2% (w/v), NaCl 1.4 M, Tris-HCl 100 mM, pH 8.0; EDTA 20 mM, b-mercaptoethanol 0.2 % (v/v)] and about 80 mg of a silica mixture (silica gel H, Merck 7736, Darmstadt, Germany / Kieselguhr Celite 545, Machery, Düren, Germany, 2:1, w/w). Cells were disrupted manually with a sterile pestle for approximately 5 min. Subsequently 200 µL CTAB buffer was added and the mixture vortexed and incubated for 10 min at 65 °C. After the addition of 500 µL chloroform, the solution was mixed and centrifuged for 5 min at 10000 rpm and the supernatant transferred to

a new tube with 2 vols of ice cold 96 % ethanol. DNA was allowed to precipitate for 30 min at −20°C and then centrifuged again for 5 min at 20 500 g. Then the pellet was washed with cold 70 % ethanol. After drying at room temperature it was re-suspended in 97.5 µL TE-buffer plus 2.5 µL RNAse 20 U.mL−1 and incubated for 5 min at 37 °C, before storaging it at −20 °C (Lackner et al., 2012b).

3.3.2 Amplification and Sequencing

The Amplification and sequencing of the Hot spot (HS) 1 in the FKS1 and FKS2 gene were performed with the primer pairs described by Castanheira *et al*. (Castaneira et al., 2010) (Table 7).

HS-1 regions from FKS1 and FKS2 gene were amplified in a Gene Touch Thermal Cycler (Biozym Scientific GmbH) using an initial denaturising step at 95°C for 3 min, followed by 45 cycles consisting of 94°C for 30 seconds, 57°C (*fks* 1) or 54°C (*fks* 2) for 10 seconds and 72°C for 30 s, with a delay at 72°C for 5 min and cooling at 4°C. The PCR amplicons were purified using USB® ExoSAP-IT® PCR Product Cleanup as first step of cleaning and then Big Dye Terminator (Applied Biosystems®) for the second clean up, following the manufacturer´s instruction and then sequenced with a 3500 Genetic analyzer (Applied Biosystems). The sequence quality, alignment and mutations were verified using the BioNumerics Software version 6.6. Nucleic acid to protein translation was performed using EBI Transeq. The protein alignments were made using ClustalW2 tool.

Table 7. Primers used in this study		
Primer	**Gene**	**Sequences 5´→ 3´**
Fks1 HS1	***fks1***	**F: CCA TTG GGT GGT CTG TTC ACG**
		R: GAT TGG GCA AAG AAA GAA ATA CGA C
Fks2 HS1	***fks2***	**F: GCT TCT CAG ACT TTC ACC G**
		R: CAG AAT AGT GTG GAG TCA AGA CG

F, forward; R, reverse

3.4 Statistical analyzes

Statistical analyzes were done using SPSS statistical software for windows version 15 and GraphPad Prism version 4.0 for windows, Graphpad software, San Diego

3.4.1 Kaplan Meier

This method calculates the Mean Survival Time (MST) to one group of mice. It takes into account the animals left alive at the end of the study and also the days in which the animals that not survive at the end of the observation period. Then, the log-rank test is used to compare the MST of the different groups with each other or in the control group and establish which groups are statistically different and which are considered equal.

3.4.2 Mann- Withney test

This nonparametric test for comparing the data obtained in the colony counts per gram of organ. No comparing means, it sorts the data of the two groups which are being compare dand determines a range. In this test, therefore there is no of data with a normal distribution and can work with the original data or with logarithms as

both get the same results. Kruskal-Wallis nonparametric test is equivalent to prior to multiple groups.

3.4.3 Ancova

This method consists in a measure of how much two variables change together and how strong is the relationship between them. It was used to analize the betaglucan data.

3.4.4 Bonferroni correction

It is a method used to counteract the problem of multiple comparisons. It is considered the simplest and most conservative method to control the familywise error rate. It was used in the analysis of colony counts from organs and betaglucan level comparisons.

IV. Results

4.1. Virulence of *Sporothrix luriei* in a murine model of disseminated infection

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Virulence of *Sporothrix luriei* in a Murine Model of Disseminated Infection

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Abstract *Sporothrix luriei* is a rare fungus causing sporotrichosis in humans. The virulence of this fungus was evaluated in a murine model of disseminated infection. Mice were challenged intravenously with two different inocula (2×10^5 and 2×10^7 CFU/animals) but only the highest one was able to kill the animals. Infected mice died between days 12 and 16, liver and spleen being the most affected organs. In the infected tissues, a massive infiltration of fungal cells and phagocytes were observed, but not the typical "eyeglass" cells described in infected human tissue.

Keywords Animal models · *S. luriei* · Virulence · Histopathology

Introduction

Sporotrichosis is a granulomatous disease caused by species of the genera *Sporothrix* [1, 2]. These infections are mainly reported from tropical and temperate zones of South America, mostly acquired by traumatic inoculation of the fungus from soil, wood or plants. Major clinical manifestations include subacute or chronic lymphocutaneous forms. The most severe complication of the disease is a disseminated infection that especially occurs in immunosuppressed patients [3–6].

Until recently, sporotrichosis was considered to be caused by a single species, *Sporothrix schenckii*; however, some molecular studies have demonstrated that *S. schenckii sensu lato* is a complex of cryptic species [2, 7, 8]. *S. brasiliensis* and *S. schenckii sensu stricto* are the most common *species of the complex* with a different geographical distribution, degree of virulence and antifungal susceptibility. *Sporothrix luriei* is a separate species, phylogenetically related to the species of the *S. schenckii* complex [9] and reported rarely in human infection.

The first case of human infection caused by *S. luriei* was reported in a South African male by Ajello and Kaplan in 1956 [10]. So far, *S. luriei* has been reported as the causal agent in four cases of human infection, one of them being fatal [11]. Such infections were histopathologically characterized by the presence of thick-walled cells and typical large cells with an "eyeglass" configuration in tissue [10–13]. Although it is likely that these species were involved in more cases than those reported, principally because diagnosis of sporotrichosis is usually based on clinical manifestations and the fungus is not always isolated.

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In addition, the morphological similarity of the different species of *Sporothrix* complicates the diagnosis. Recently, molecular tools have been used for a more accurate identification [7, 14]. The scarcity of the infections caused by this fungus in comparison with other species of *Sporothrix* and the meaning of the presence of the "eyeglass" cells are intriguing aspects in this illness. The goal of the present study was to evaluate the virulence of *S. luriei* in a murine model of disseminated infection and to compare it with other species of *Sporothrix* from our previous findings in order to asses the potential pathogenesis of this species.

Materials and Methods

Strain and Inocula Preparation

One strain of *S. luriei* (CBS937.72), which was isolated in the first reported clinical case [10], was included. The strain was stored by lyophilization, subcultured on PDA plates and incubated at 30°C for 8–10 days. In aim to restore the virulence of the strain, which may be diminished after long period storage, a conidial suspension was intravenously administered to two mice. Seven days later, kidneys and liver were removed, homogenized and placed on PDA for strain recovery. Subcultures were used as stock to prepare the experimental inocula. The inocula were prepared by flooding the plate surface with saline solution. The resultant suspension was added into 100 ml of potato dextrose broth (PDB) and incubated in an orbital shaker (150 rpm) at 30°C for 4 days. Then, the cultures were filtered twice through sterile gauze and centrifuged at 7,000 rpm for 20 min. Conidia suspensions were adjusted to the desired concentration by haemocytometer counting. To verify the viability and size of inocula, 10-fold dilutions were placed in PDA for CFU determinations.

Animals

Six-week-old OF-1 male mice (Charles River, Criffa SA, Barcelona, Spain), weighing 28–30 g, were used. Animals were housed five per cage and provided with food and water ad libitum. After infection, animals were checked twice a day and mobility, fur aspect, orientation, skin lesions and food ingest were used for discomfort evaluation. Conditions were approved by the animal Welfare Committee of the Universitat Rovira i Virgili.

Mortality

For the survival study, two groups of 8 animals were inoculated intravenously (i.v.) via the lateral tail vein with 2×10^5 CFU/animal (low inoculum) or 2×10^7 CFU/animal (high inoculum). Animals were checked twice a day for 30 days.

Tissue Burden

Fungal load only was determined in those organs from animals infected with the inoculum that was able to cause death of animals. Two groups of animals (five per group) were infected i.v. with the high inoculum. The first group was killed on day 7 and the remaining group on day 15 post-infection; mice were killed by CO_2 anoxia, and heart, brain, kidneys, spleen, and liver were removed aseptically. Half of the organs were homogenized in 1 ml of sterile 0.9% saline, 10-fold diluted, placed on PDA plates and incubated at 30°C for 4 days for CFU determination.

Histopathology Study

In order to evaluate the histopathological changes in different tissues, two groups of 15 animals were infected i.v. with high or low inoculum. Macroscopically changes as size, weight, color, presence or absence of hemorrhage or abscesses were assessed. On days 7, 15 and 30 after challenge, five animals per group were killed as previously described. Half of each of the above-mentioned organs was fixed with 10% buffered formalin. Samples were dehydrated, paraffin-embedded, sliced into 2 μm sections and stained with hematoxylin and eosin (H-E), Periodic acid-Schiff (PAS) and Grocott methenamine silver. Sections were examined by light microscopy for histopathological changes.

Statistical Analysis

Organ burden data were $\log_{10}$-transformed and compared by the two-tailed Mann–Whitney *U*-test, using Graph Pad Prism 5 for Windows. Survival was compared using a two-tailed log-rank test. *P* values < 0.05 were considered significant.

Results

Viability of Inocula

For the low inoculum, the viability was 1.3×10^6 CFU/ml and for high inoculum 11×10^8 CFU/ml.

Mortality

The mortality rate of mice infected with *S. luriei* correlated with inocula size (Fig. 1). All the animals challenged with the low inoculum (2×10^5 CFU/animal) survived to the end of experiment (30 days), while the high inoculum (2×10^7 CFU/animal) caused 100% mortality in 12–16 days.

Tissue Burden

Mice infected with the high inoculum showed fungal loads in all studied organs on day 7 post-infection. The mean CFU/g was high in all the studied organs, ranging from 5.56 ± 0.35 to 6.34 ± 0.27 CFU/g. The most affected organs were liver and heart followed by brain, kidneys, lungs and spleen (Fig. 2). Fungal load increased significantly in all organs at day 15 post-infection in comparison with those obtained on day 7 post-infection ($P < 0.007$). Furthermore, in these cases, there was a significant increase in burden, with liver being the most infected organ (8.06 ± 0.9 CFU/g), followed by brain, lungs, kidneys, heart and spleen.

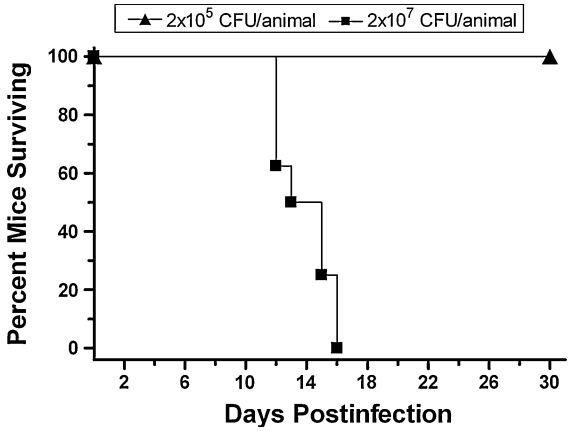

Fig. 1 Cumulative mortality of mice infected with *S. luriei* (FMR 9290). Mice were infected with 2×10^5 CFU/animal or 2×10^7 CFU/animal

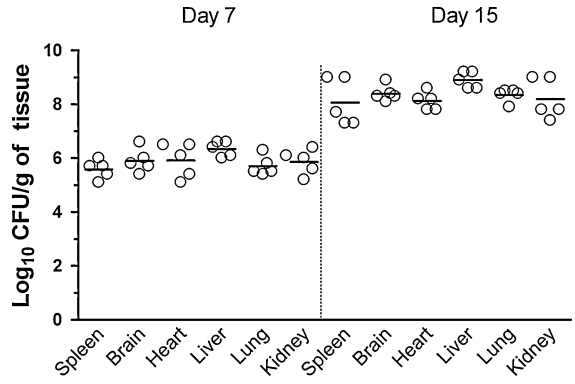

Fig. 2 Quantitative fungal recovery from different organs of mice infected with *S. luriei* (FMR 9290) 2×10^7 CFU/g of tissue

Histopathology

All animals, regardless the inoculum size, developed cutaneous lesions on the tail in 9–12 days post-infection, which progressed to ulcerative lesions with a visible swelling.

At 7 days post-infection, animals infected with the low inoculum showed a marked splenomegaly and hepatomegaly with a few nodules approximately 1 mm in diameter in both organs. Microscopically, microgranulomatous lesions with a necrotic center and few free fungal cells were noticed in the interstitial spaces of tissue. However, abundant yeast-like elements were widely observed in the cytoplasm of the Kupffer cells probably as a result of intense phagocytosis. Neither lesions nor histological changes were found in other organs. After 15 days of infection, reduction in the size and number of nodules in liver and spleen was observed corresponding to a decreasing number of fungal elements in tissue. Remission of lesions and scarce fungal cells was observed 30 days after infection in the surviving animals.

Animals infected with the high inoculum showed intense splenomegaly and hepatomegaly and numerous nodules in all studied organs. Microscopically, large necrotic areas were found in liver, spleen, kidneys and heart, 7 days post-infection. In addition, tissue areas were substituted by fungal elements consisting of mature, round and immature, oval or cigar-shaped cells, which were especially abundant in liver. Although tissue damage was evident in liver, inflammatory cells were not observed. In brain and lungs, only a few yeast cells were observed (data no

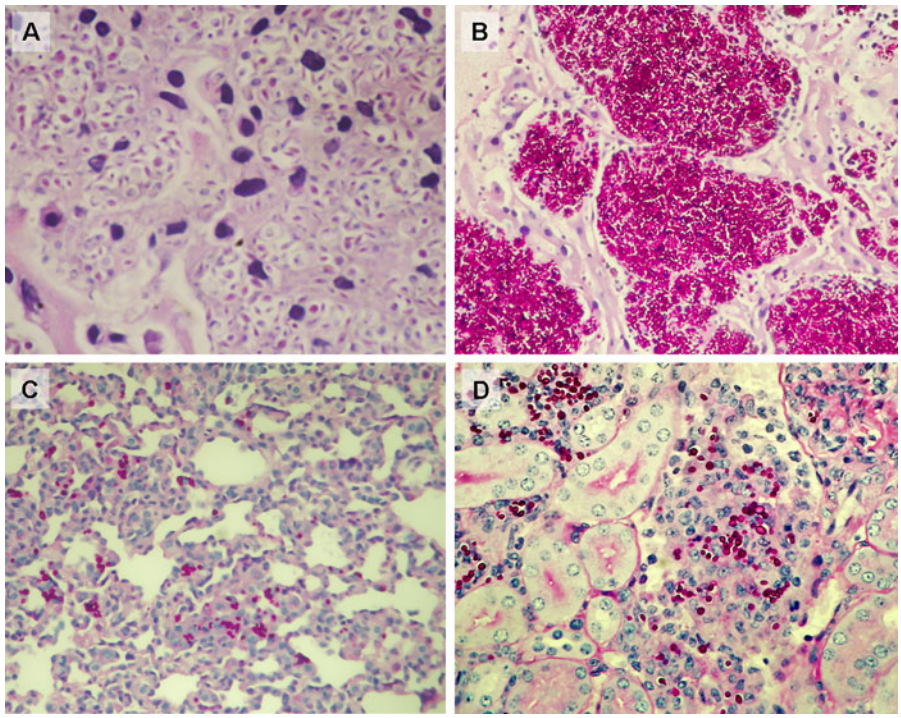

Fig. 3 Histological findings for *S. luriei* in mice infected with 2×10^7 CFU/animals at 15th day. **a** Fungal infiltration by fungal cells in liver but with no inflammatory response. **b** Massive infiltration with yeast-like cells replacing hepatocytes **c** Decrease in the alveolar spaces due to increased interstitial tissue due to the presence of fungal cells. **d** Scattered infiltration of fungal cells in kidney tubules. The stains used are hematoxylin eosin (**a**) and periodic acid–Schiff (**b**–**d**)

showed). At day 15, the number of nodules increased in all organs, especially in liver and spleen where a complete invasion of granulomas was observed. In all studied organs, a massive infiltration of fungal cells was observed particularly in liver where phagocytes and fungal elements invaded the hepatic tissue reducing it to scattered islets of hepatocytes. These fungal elements were mostly located in the Kupffer cells (Fig. 3a, b). In the other organs, there was no alteration in tissue structure but there was a large presence of fungal cells (Fig. 3c, d). The presence of cells with typical "eyeglass" shape, which had been observed in the human cases, was not observed in this experimental infection [10, 11]. It was not possible to carry out the histopathological study 30 days after infection, because all the animals had succumbed to the infection.

Discussion

We have developed a murine model of disseminated infection by *S. luriei* that was used to demonstrate the virulence and the high degree of tissue invasion of that fungus. Similar to a previous study that tested other species of the genus, i.e. *S. brasiliensis*, *S. mexicana* and *S. schenckii* [15], low inocula of *S. luriei* (2×10^5 CFU/animal) was not able to cause mice mortality and the lesions produced disappeared a few days after challenge. In contrast, infection with high doses (2×10^7 CFU/animal) allowed *S. luriei* to invade and proliferate in internal organs causing tissue destruction. Infected mice died between 12 and 16 days after infection, which was a similar, or even shorter, period of time to that obtained in infections carried out with *S. schenckii* or *S. brasiliensis* with the same inoculum size [15]. These histopathological findings were similar to those previously obtained during the experimental infection with *S. brasiliensis* but differed from those seen in the *S. schenckii sensu stricto* infection, which mainly evolved showing granulomas with a necrotic center [15]. Although "eyeglass cells" have been described as the most typical histopathological feature at identifies *S. luriei* in the four clinical cases

reported to date, we did not observe such cells in the tissues of our infected mice.

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4.2. Efficacy of posaconazole in murine experimental sporotrichosis.

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Efficacy of Posaconazole in Murine Experimental Sporotrichosis

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We developed a murine model of systemic sporotrichosis by using three strains of each of the two commonest species causing sporotrichosis, i.e., *Sporothrix schenckii* sensu stricto and *Sporothrix brasiliensis*, in order to evaluate the efficacy of posaconazole (PSC). The drug was administered at a dose of 2.5 or 5 mg/kg of body weight twice a day by gavage, and one group was treated with amphotericin B (AMB) as a control treatment. Posaconazole, especially at 5 mg/kg, showed good efficacy against all the strains tested, regardless of their MICs, as measured by prolonged survival, tissue burden reduction, and histopathology.

Sporotrichosis is the most common and cosmopolitan subcutaneous mycosis (13). The most usual clinical manifestation of the disease is the subacute or chronic lymphocutaneous form, followed by fixed cutaneous infections (5); however, disseminated infections have also been described for those with underlying alcoholism and for immunosuppressed patients, especially those with AIDS or those receiving chemotherapy or corticoids (11, 13, 17). Sporotrichosis is caused by a group of species belonging to the *Sporothrix schenckii* complex which have various individual *in vitro* responses to antifungal agents. The differential antifungal activity among *Sporothrix* species could explain the variability in antifungal susceptibility reported for *S. schenckii* in studies prior to the recent recognition of the *S. schenckii* complex (5, 20, 21).

The recommended procedures for the management of sporotrichosis include local measures such as hyperthermia and systemic measures such as administration of a saturated solution of potassium iodide or administration of azoles (itraconazole and fluconazole), amphotericin B, or terbinafine (15). Itraconazole has become the drug of choice for treatment of the lymphocutaneous and cutaneous forms. In those patients with intolerance to itraconazole, fluconazole is the recommended alternative. Amphotericin B is the first choice for the treatment of disseminated sporotrichosis, and itraconazole is recommended as the step-down therapy after patients respond to the initial treatment with amphotericin B (18).

Posaconazole has shown activity *in vitro* against the species of the *S. schenckii* complex (21, 25). Since treatment options for disseminated sporotrichosis are limited and there is only scarce information on the effectiveness of posaconazole *in vivo*, we evaluated the response to this drug in a murine model of disseminated sporotrichosis.

MATERIALS AND METHODS

Fungi were stored in slant cultures covered with sterile paraffin oil and subcultured on potato dextrose agar (PDA) plates at 30°C for 7 days.

In vitro antifungal susceptibility to posaconazole of 5 isolates of *S. brasiliensis* and 10 of *S. schenckii* sensu stricto was determined using a broth microdilution method according to the CLSI guidelines for filamentous fungi (8).

For the *in vivo* studies, strains of *S. brasiliensis* ($n = 3$) and *S. schenckii* sensu stricto ($n = 5$) showing different MIC values were chosen. The inocula were prepared by flooding the surface of an agar plate with saline solution and scraping the sporulating mycelium. The resulting solutions were transferred to 100 ml of potato dextrose broth (PDB) and incubated in an orbital shaker at 150 rpm at 30°C for 5 days. Cultures were then filtered twice through sterile gauze and centrifuged at 325 × *g*. The pellets were washed once with saline solution, and the conidium concentrations were adjusted to the desired concentrations by hemocytometer counting. To verify the viability and size of inocula, 10-fold dilutions were placed in PDA to determine the CFU.

Four-week-old OF-1 male mice (Charles River, Criffa S.A., Barcelona, Spain) with a mean weight of 30 g were used. Animals were housed in standard boxes with corncob bedding and free access to food and water. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Mice were infected intravenously (i.v.) in the lateral tail vein with 2×10^7 CFU of fungi in 0.2 ml of sterile saline. This inoculum level was chosen based upon previous studies with strain FMR 8314 that indicated that this concentration was the minimum dose that killed all the infected animals within 18 days (data not shown).

Posaconazole, provided as Noxafil (Schering-Plough Ltd., Hertfordshire, United Kingdom), was administered at 2.5 or 5 mg/kg of body weight twice a day (BID) by gavage. These doses were chosen based upon preliminary studies using higher (10 and 20 mg/kg BID) and lower (1 and 2 mg/kg BID) doses of posaconazole (Fig. 1). Controls received no treatment. The efficacy of posaconazole was evaluated as prolonged survival, reduced tissue burden, and differences in histopathology. Treatments began 1 day after infection and lasted for 18 days. For survival studies, groups of 10 mice were randomly established for each strain and each treatment and checked daily for 30 days after challenge. For tissue burden studies, groups of 10 mice were also established, and the animals were sacrificed on day 13 postinfection in order to compare the results with controls. The liver and spleen, which are the most affected organs in experimental systemic sporotrichosis (2), were removed aseptically, and one half of each was homogenized in 1 ml sterile saline. Serial 10-fold dilutions of the homogenates were plated on PDA and incubated for 72 h at 30°C. The numbers of CFU/g of tissue were calculated. For the histopathology study, half of each organ was fixed with 10% buffered formalin. Samples were dehydrated, paraffin embedded, and sliced into 2-μm sections, which were then stained with hematoxylin-eosin (H-E), periodic acid-Schiff (PAS) stain, or Grocott methenamine silver and examined in blinded fashion by light microscopy. Additionally, one group of 10 animals was infected with *S. brasiliensis* FMR 8326 and treated i.v. with amphotericin B (Fungizone; Squibb Industria Farmacéutica S.A., Barcelona, Spain) at 0.8 mg/kg for 18 days.

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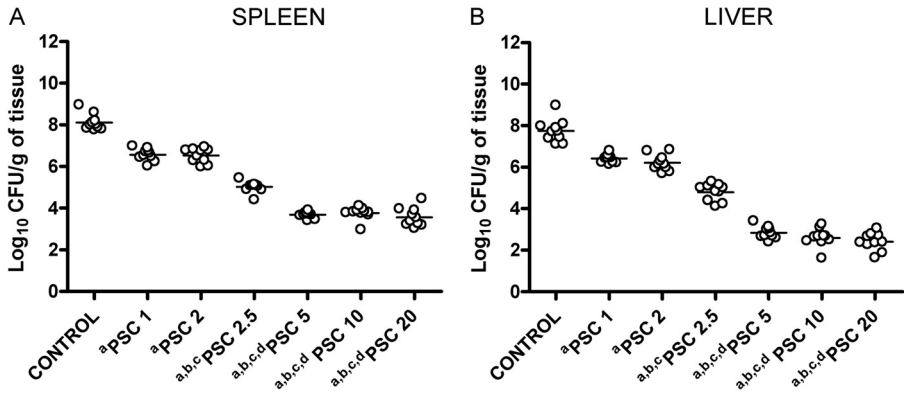

FIG 1 Effects on fungal loads of mice infected with *S. brasiliensis* FMR 8314 (MIC = 1 μg/ml) following antifungal treatment with various doses of posaconazole (PSC). a, $P < 0.005$ versus control; b, $P < 0.05$ versus PSC 1; c, $P < 0.05$ versus PSC 2; d, $P < 0.05$ versus PSC 2.5. Posaconazole was administered BID by gavage at 1 mg/kg, 2 mg/kg, 2.5 mg/kg, 5 mg/kg, 10 mg/kg, or 20 mg/kg. Horizontal lines indicate median values.

Statistical analysis was performed using Graph Pad Prism 5 for Windows. The mean survival time was estimated by the Kaplan-Meier method and compared among groups by using the log rank test. The colony counts from tissue burden studies were analyzed using the Mann-Whitney U test. The observed differences were considered statistically significant for *P* values of <0.05.

RESULTS

Two of 5 *S. brasiliensis* strains tested and 4 of 10 *S. schenckii* sensu stricto strains tested showed posaconazole MICs of 0.5 to 1 μg/ml, which are close to the suggested breakpoint indicative of the susceptibility of filamentous fungi, i.e., ≤1 μg/ml for posaconazole (8). The other strains of *S. brasiliensis* (3) showed a MIC of 2 μg/ml (intermediate susceptibility). Three strains of *S. schenckii* sensu stricto showed intermediate susceptibility, and another 3 showed MICs of >2 μg/ml.

The posaconazole MICs for those strains included in the *in vivo* study were as follows: for *S. brasiliensis*, 0.5 μg/ml (FMR 8319), 1 μg/ml (FMR 8314), and 2 μg/ml (FMR 8326); and for *S. schenckii* sensu stricto, 1 μg/ml (FMR 8606), 2 μg/ml (FMR 8609 and FMR 9018), 4 μg/ml (FMR 9010), and 8 μg/ml (FMR 8604).

Systemic infection caused 100% mortality in untreated control groups within 11 to 18 days after challenge, regardless of the species or strain tested, with the exception of strains of *S. schenckii* sensu stricto with high MICs (FMR 9010 and FMR 8604), which did not cause death; these strains were discarded from the treatment study.

Posaconazole given at 2.5 or 5 mg/kg BID significantly prolonged survival with respect to the controls (Fig. 2), and all treated animals survived to the end of the experiment, regardless of the species and strain tested.

Posaconazole given at 2.5 or 5 mg/kg BID was able to significantly reduce the fungal loads in the liver and spleen in comparison to the control group for animals infected with *S. schenckii* sensu stricto or *S. brasiliensis* (Fig. 3). Moreover, the high dose of posaconazole significantly reduced the fungal loads in both organs in comparison to the low dose ($P < 0.05$). Amphotericin B was effective at reducing the fungal loads in both organs in comparison to controls ($P < 0.0001$), despite the fact that the amphotericin B MIC for the tested strain was 4 μg/ml.

Histopathological studies of control mice showed an abundance of typical cigar-shaped cells in the liver and spleen. In mice infected with *S. schenckii* sensu stricto, these fungal cells were usually surrounded by a granuloma, while in those infected with *S. brasiliensis* a massive infiltration of tissue with fungal cells was observed, with an absence of granulomas (Fig. 4A and B). In general, there was a scarcity of fungal cells in mice treated with posaconazole at 2.5 mg/kg BID (Fig. 4C), with a recovery of tissue structure, and in both those treated with posaconazole at 5 mg/kg BID (Fig. 4D) and those treated with amphotericin B, fungal elements were not observed and tissue structure was normal.

DISCUSSION

Although cases of systemic sporotrichosis are fortunately not frequent, several cases have been described (1, 3, 15, 19, 22, 26). Amphotericin B is the most common drug used in the treatment of disseminated sporotrichosis, but the dosage is limited by its toxicity and by the long-term therapy required.

In this study, we evaluated the activity *in vitro* and efficacy *in vivo* of posaconazole against several strains of *S. schenckii* sensu stricto and *S. brasiliensis*. Only a few previous studies evaluated the *in vitro* activity of posaconazole against *Sporothrix* spp., and they reported very variable results, with MIC values ranging from 0.03 to 16 μg/ml. The MICs obtained in our study are consistent with those obtained by previous authors (12, 14, 21, 25), although they did not establish MICs for each individual species of the complex as was done later by Marimón et al. (21). Although some studies have evaluated the efficacy of some antifungals (16) and/or determined the virulence of strains of various origins (10), all of them were carried out prior to the reclassification of *Sporothrix* organisms as a species complex. Only a comparative study on virulence in mice had been carried out for this complex of species (2). Our study shows that the spleen and liver have high fungal loads, in agreement with previous reports on animal models of sporotrichosis (2, 9, 10). To our knowledge, this is the first study to explore the efficacy of posaconazole in a murine model of disseminated sporotrichosis. Our results demonstrated good efficacy of posaconazole administered at 2.5 or 5 mg/kg BID against disseminated sporotrichosis caused by the two species tested. In addition, lower (1 or 2 mg/kg BID) and higher (10 or 20 mg/kg BID) doses showed *in vivo* efficacy in our model of infection. However, the best statistical significance was observed at 2.5 mg/kg and 5 mg/kg BID. These results should be considered ex-

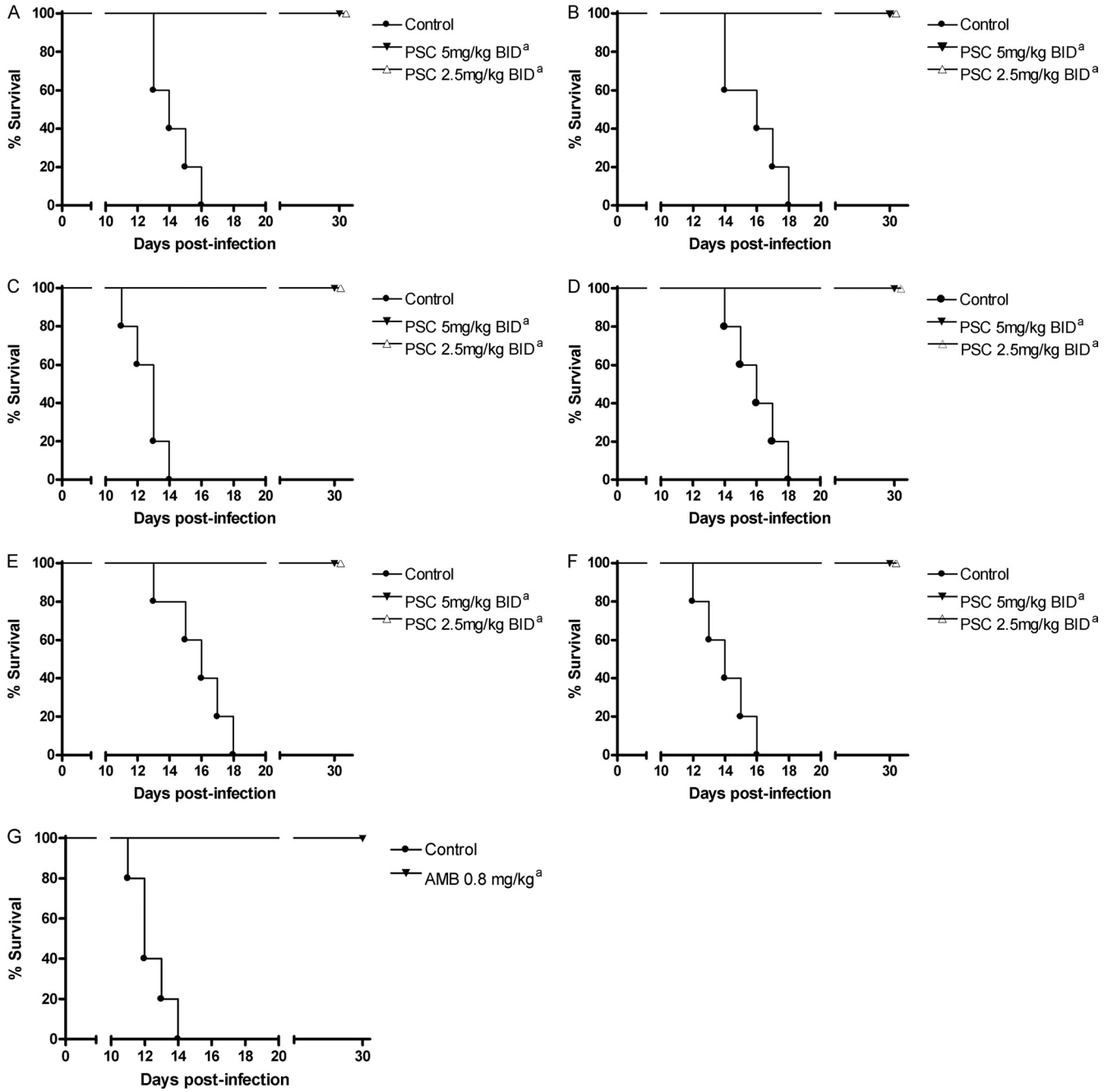

FIG 2 Cumulative mortality of mice infected with the following strains and treated with posaconazole (PSC) (A to F) or amphotericin B (AMB) (G). (A) *S. brasiliensis* FMR 8314 (MIC = 1 μg/ml); (B) *S. brasiliensis* FMR 8319 (MIC = 0.5 μg/ml); (C) *S. brasiliensis* FMR 8326 (MIC = 2 μg/ml); (D) *S. schenckii* sensu stricto FMR 9018 (MIC = 2 μg/ml); (E) *S. schenckii* sensu stricto FMR 8609 (MIC = 2 μg/ml); (F) *S. schenckii* sensu stricto FMR 8606 (MIC = 1 μg/ml); (G) FMR 8326 (MIC = 4 μg/ml). a, $P < 0.005$ versus control. Posaconazole was administered BID by gavage at 2.5 mg/kg or 5 mg/kg. Amphotericin B was administered at 0.8 mg/kg i.v. once a day.

cellent for prolonging survival, since none of the infected mice died during the experiment, even considering that the doses were relatively low in comparison with those tested in other studies of treatment of infections by other fungi under similar conditions (7, 23, 24). For instance, a dose of 20 or 30 mg/kg BID against *Cryptococcus gattii* (7), *Rhizopus oryzae* (23), or the *Aspergillus terreus* complex (24) achieved a survival rate of only 20%, 40%, or 60%, respectively.

Surprisingly, our results indicate that outcome does not seem to correlate with the MIC in the range observed among our strains, since in those strains with a MIC of 0.5 or 2 mg/kg, tissue burden reductions were similar (approximately 4 to 5 log with respect to the control group). Furthermore, we also observed that the efficacies of posaconazole were similar against the two species tested.

As demonstrated experimentally (16) and corroborated in clinical reports (19, 26), amphotericin B is effective for the treat-

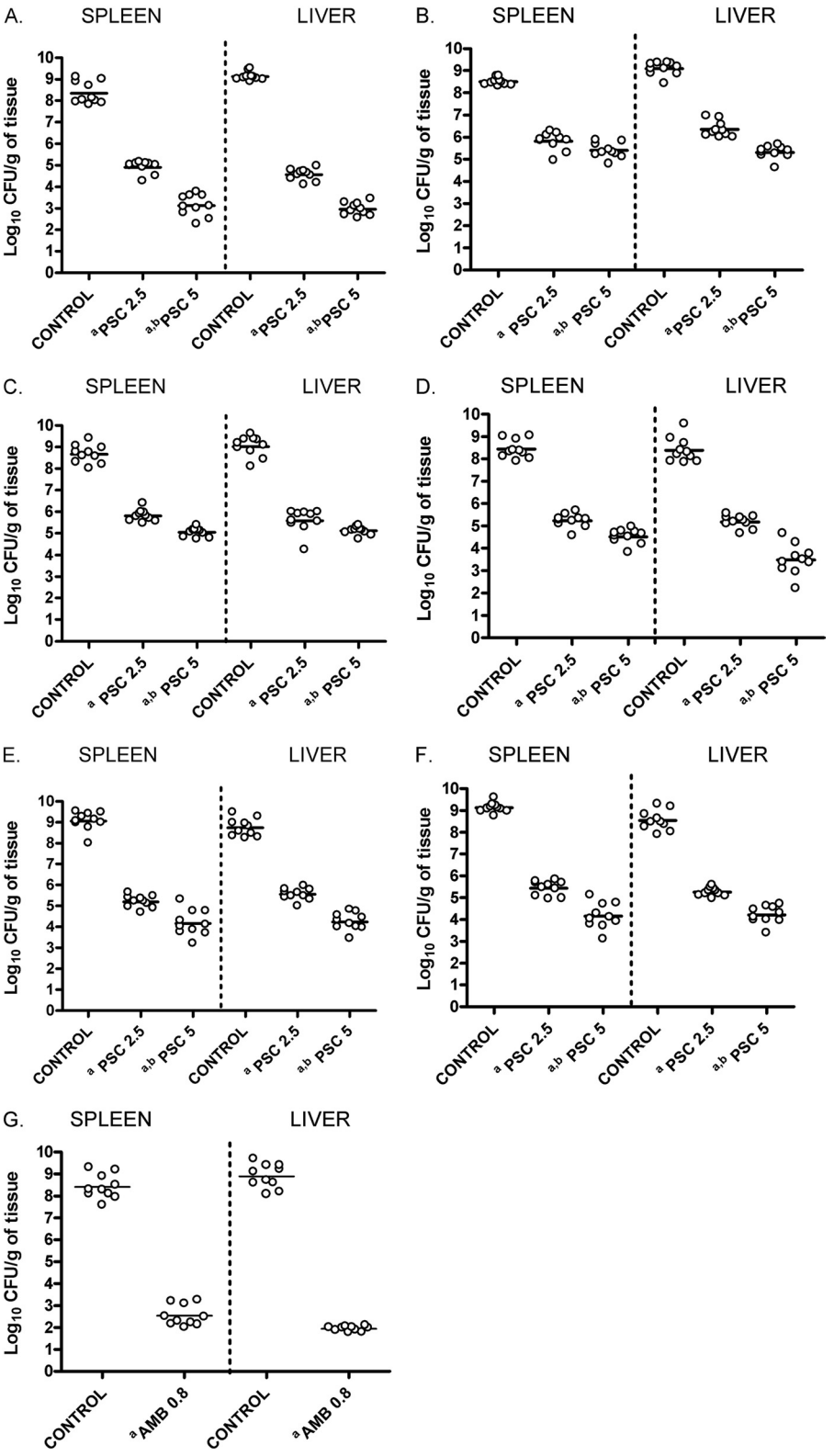

FIG 3 Effects of antifungal treatment with posaconazole (PSC) (A to F) or amphotericin B (AMB) (G) on fungal loads of mice infected with the indicated strains. (A) *S. brasiliensis* FMR 8314 (MIC = 1 μg/ml); (B) *S. brasiliensis* FMR 8319 (MIC = 0.5 μg/ml); (C) *S. brasiliensis* FMR 8326 (MIC = 2 μg/ml); (D) *S. schenckii* sensu stricto FMR 9018 (MIC = 2 μg/ml); (E) *S. schenckii* sensu stricto FMR 8609 (MIC = 2 μg/ml); (F) *S. schenckii* sensu stricto FMR 8606 (MIC = 1 μg/ml); (G) FMR 8326 (MIC = 4 μg/ml). a, $P < 0.005$ versus control; b, $P < 0.05$ versus PSC 2.5. Posaconazole was administered BID by gavage at 2.5 mg/kg or 5 mg/kg. Amphotericin B was administered at 0.8 mg/kg i.v. once a day. Horizontal lines indicate median values.

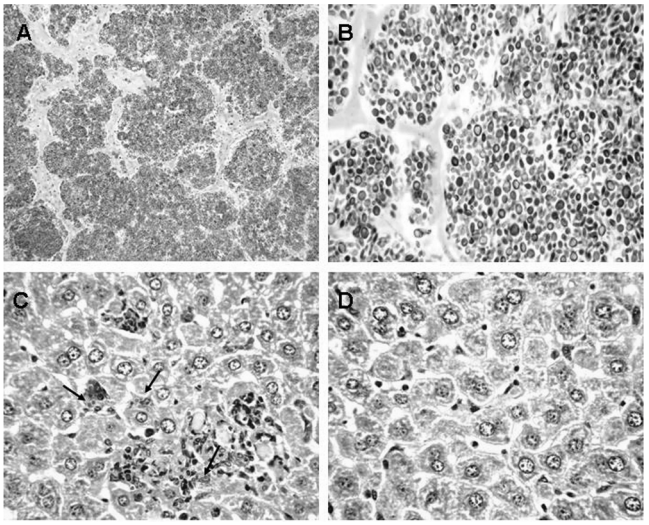

FIG 4 Histopathology of livers of mice infected with *Sporothrix brasiliensis* and treated with posaconazole. (A and B) Liver sections showing abundance of fungal cells with massive infiltration of the hepatic tissue. (A) Grocott stain. Magnification, ×100. (B) Grocott stain. Magnification, ×600. (C) Scarcity of fungal cells in mice treated with posaconazole at 2.5 mg/kg BID (PAS stain). Magnification, ×600. Arrows indicate fungal cells in Kupffer cells. (D) Liver of a mouse treated with 5 mg/kg posaconazole BID showing the absence of fungal elements and the absence of an inflammatory response (PAS stain). Magnification, ×600.

ment of disseminated sporotrichosis. It should be noted that *Sporothrix* infections are frequently chronic and consequently require long-term therapy. Our results suggest that treatment with posaconazole could be an alternative to that with amphotericin B. Despite the reductions in fungal load in posaconazole-treated animals being lower than those achieved by using amphotericin B, the reductions were notably significant with respect to untreated animals, and together with the reduced toxicity reported for posaconazole, this allows us to propose posaconazole as an alternative therapeutic for sporotrichosis. However, further studies including more strains and treatment durations are required in order to establish the usefulness of posaconazole against sporotrichosis.

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4.3. Modest efficacy of voriconazole against murine infections by *Sporothrix schenckii* and lack efficacy against *Sporothrix brasiliensis.*

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Diagnosis, Therapy and Prophylaxis of Fungal Diseases

Original article

Modest efficacy of voriconazole against murine infections by *Sporothrix schenckii* and lack of efficacy against *Sporothrix brasiliensis*

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Summary

The efficacy of voriconazole (VRC) was evaluated against two strains of each of the two most common species causing sporotrichosis, *Sporothrix schenckii sensu stricto* and *Sporothrix brasiliensis*, using a murine model of disseminated infection. Voriconazole was administered at doses of 20 or 40 mg kg^{-1} per day by gavage. The drug showed some efficacy, especially at 40 mg kg^{-1} per day, in prolonging the survival and reducing fungal load in spleen and liver in mice infected with *S. schenckii*, whereas in animals infected with S. *brasiliensis* the drug did not work.

Key words: Voriconazole, sporotrichosis, animal model.

Introduction

Sporotrichosis is a fungal disease caused by several species of *Sporothrix*, a ubiquitous environmental fungus, distributed worldwide, but predominantly in tropical and subtropical areas of Latin America.[1–5] It was demonstrated recently that *Sporothrix schenckii* is a complex of species[6,7] with different *in vitro* antifungal susceptibility [8] and virulence,[9] which have significant clinical and therapeutic implications. Sporotrichosis is characterised by subcutaneous nodular lesions, usually treated successfully with itraconazole.[10] Secondary spread to articular surfaces, bone and muscle is not infrequent and the infection may also occasionally disseminate.[11] Systemic sporotrichosis has increased in recent decades, mainly in immunocompromised patients,[11–16] although also it has been reported in immunocompetent patients.[17] Amphotericin B is the recommended treatment for systemic infections,[10] but its renal toxicity limits its use and treatment outcome remains poor to date. There is no published clinical data on the use of other drugs as first-line therapy. Itraconazole is recommended as step-down therapy after the patient responds to initial treatment with amphotericin B.[10] A previous experimental study showed the efficacy of posaconazole against *S. schenckii* and *S. brasiliensis*,[18] although this drug can only be administered orally and its pharmacokinetics is unpredictable,[19] therefore alternative therapy is required for some patients.

The aim of this study was to evaluate the *in vivo* efficacy of voriconazole (VRC) in a murine model of disseminated sporotrichosis. Although the *in vitro* susceptibility of *Sporothrix* spp. to VRC is low and can vary,[8, 20–24] it remains unknown whether there is any type of correlation between MICs and *in vivo* efficacy.

Materials and methods

Two strains of *S. brasiliensis*, FMR 8326 and FMR 8314, showing VRC MICs of 2 and 8 μg ml^{-1}, respectively, and two of *S. schenckii*, FMR 8609 and FMR 9018, with MICs of 8 and 16 μg ml^{-1}, respectively, were used in this study. The fungi were stored in slant cultures covered with sterile paraffin oil and subcultured on potato dextrose agar (PDA) plates at 30 °C

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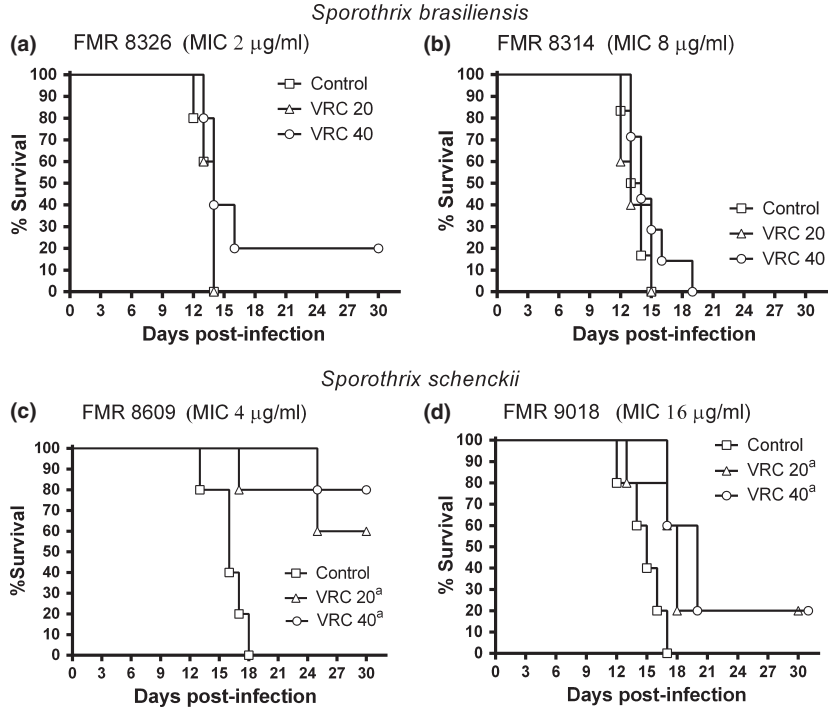

Figure 1 Cumulative mortality of controls or infected mice with *S. brasiliensis* (a, b) or *S. schenckii* (c, d) and treated with voriconazole administered by gavage at 20 mg kg^{-1}or 40 mg kg^{-1}. [a]$P < 0.005$ vs. control.

for 7 days until they sporulated. Male OF1 mice (Charles River, Criffa S.A., Barcelona, Spain) were used. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Mice were challenged intravenously (i.v.) with a conidial suspension of 2×10^7 in 0.2 ml of saline.[9,18]

Voriconazole (Vfend; Pfizer S. A., Madrid, Spain) was administered at 20 or 40 mg kg^{-1} day by gavage.[25] Treatments began 24 h after challenge and lasted for 18 days. From 3 days before infection until the end of the experiment mice were given grapefruit juice instead of water.[26] Groups of 10 animals were established for survival and checked 30 days after challenge. For fungal load study, groups of 10 mice were also established and the animals were sacrificed on day 13 after infection to compare the results with the control group since mice controls start to die on that day. Liver and spleen, two of the most relevant target organs in this infection,[9] were aseptically removed and half of them were homogenised in 1 ml of sterile saline. Serial dilutions were placed on PDA agar and incubated for 72–96 h at 30 °C. The number of CFU g^{-1} of tissue was calculated. The levels of VRC in serum, spleen and liver of mice infected with the strain FMR 8314 and treated with VRC 20 or 40 mg kg^{-1} were determined using a bioassay following previously described methods.[27,28]

For the histopathology study, the half of each organ was fixed with 10% buffered formalin. Samples were dehydrated, paraffin embedded and sliced into 2 μm sections, which were then stained with haematoxylin–eosin (H–E), Periodic acid Schiff (PAS) or Grocott methamine silver and examined in blinded fashion by light microscopy.

The statistical analysis was made using Graph Pad Prism 4 for Windows (Graphpad software, San Diego, CA, USA). The mean survival time was estimated by the Kaplan–Meier method and compared among groups using the log rank test. The CFUs from tissue burden studies and antifungal levels were analysed using the Mann–Whitney *U*-test. The differences were considered statistically significant at $P < 0.05$.

Results and discussion

The results of the survival study are shown in Fig. 1. Systemic infection caused 100% mortality of untreated controls within 18 days after challenge. VRC was able to prolong survival significantly with respect to control group in those animals infected with the two strains of *S. schenckii* ($P < 0.0185$), with no significant differences between doses ($P > 0.2813$). However, there were differences between both strains. For the strain FMR 8609 (MIC = 4 μg ml^{-1}), the survival rates were 60%–80%, whereas that for the strain FMR 9018

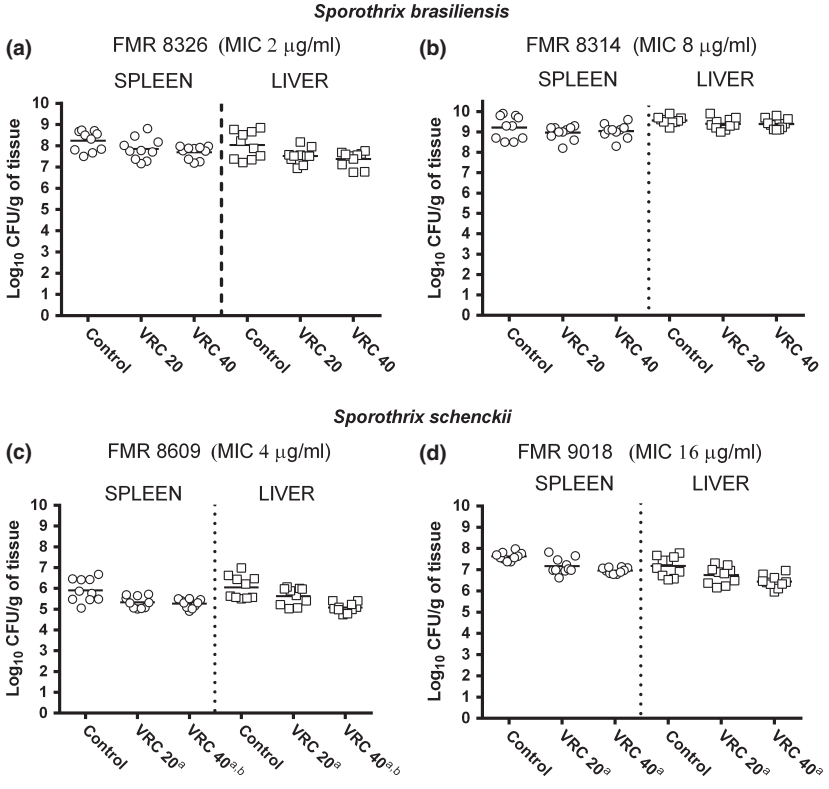

Figure 2 Effects of the antifungal treatment with voriconazole (VRC) on fungal load in spleen (○) or liver (□) of mice infected with *Sporothrix brasiliensis* (a, b) or *S. schenckii* (c, d). [a]$P<0.005$ vs. control, [b]$P<0.05$ vs. VRC 20. VRC was administered by gavage at 20 mg kg^{-1} or 40 mg kg^{-1}. Horizontal lines indicate mean values.

(MIC = 16 μg ml^{-1}) it was of only 20%. Conversely, the drug did not prolong survival of animals infected with any of the two strains of *S. brasiliensis,* all the animals dying within 21 days.

In mice challenged with *S. schenckii,* VRC reduced significantly the tissue burden in both organs evaluated with respect to the control group ($P < 0.0432$), but the efficacy was only modest as the reduction never was higher than 2 $\log_{10}$ CFU g^{-1} of tissue (Fig. 2). By contrast, VRC did not reduce tissue burden of animals infected with any of the two strains of *S. brasiliensis*.

The bioassay results are shown in Table 1. Although the levels of VRC 40 mg kg^{-1} in liver and spleen at day 13 of therapy were higher than serum levels, all of them were above the corresponding MICs.

The histopathology study showed a massive infiltration of liver and spleen tissue of typical cigar-shaped fungal cells in control mice infected with each of both species. However, in mice infected with *S. schenckii*, granulomas were observed with a necrotic centre, with mature round and immature oval and cigar-shaped fungal cells, whereas in those infected with *S. brasiliensis* immune response was not observed. A slight decrease in fungal cell in liver and spleen with respect to control group was observed in mice infected with *S. schenckii* and treated with VRC 40 mg kg^{-1}, whereas in those challenged with *S. brasiliensis* there was no change with respect to control group.

Table 1 Voriconazole levels in serum, liver and spleen from animals infected with strain FMR 8314 of *Sporothrix brasiliensis* on treatment day 13.

Dose (mg kg^{-1})	Serum (μg ml^{-1})[1]	Liver (μg ml^{-1})[1]	Spleen (μg ml^{-1})[1]
20	5.8 ± 0.94	16.1 ± 2.84	12 ± 2.02
40	8.2 ± 2.25	35.0 ± 3.76	29.2 ± 7.01

[1]Results are expressed as the mean ± standard deviation.

To our knowledge, this is the first study that has explored the efficacy of VRC in the treatment of an experimental infection by *Sporothrix* spp. The fungal loads in liver or spleen from mice infected with *S. brasiliensis* were 2 $\log_{10}$ CFU g^{-1} of tissue higher than for those challenged with *S. schenckii* which confirmed the higher virulence of *S. brasiliensis* as reported previously.[9,18] Voriconazole showed a modest efficacy in mice infected with *S. schenckii* while it was ineffective in those infected with *S. brasiliensis,* which demonstrate that outcome depends on the species of *Sporothix* involved.

In antifungal therapy, the correlation of *in vitro–in vivo* is usually difficult to establish; however, in murine

studies on *Aspergillus* infections it has been described that VRC MICs might have a predictive value of *in vivo* results based on currently epidemiological cut-off values.[29,30] Although breakpoints have not been defined for *Sporothrix* spp, interpretative guidelines based on the M38-A2 for other filamentous fungi suggest that VRC MICs ≤ 1 μg ml^{-1} are indicative of susceptibility. Based on this, our MIC values were considerably high and consequently we might expect a lack of efficacy *in vivo*. Our results do show, however, that efficacy, regardless of the MIC, is species dependent. Against *S. brasiliensis* even with the highest VRC levels in serum and organs, the highest VRC dose was ineffective. Our results highlight the importance of the proper identification of *Sporothrix* at species level for an appropriate treatment regimen to be designed.

In conclusion, although further studies are needed to confirm the data shown here, our results suggest that VRC might have potential in the treatment of disseminated infection by *S. schenckii* but not by *S. brasiliensis.*

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4.4. Experimental murine acremoniosis: an emerging opportunistic human infection.

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Experimental murine acremoniosis: an emerging opportunistic human infection

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Acremonium is an emerging fungal pathogen causing severe infections. We evaluated the virulence of three clinically relevant species within the genus, i.e., *Acremonium kiliense* (currently *Sarocladium kiliense*), *Acremonium sclerotigenum-A. egyptiacum* complex and *Acremonium implicatum* in a murine model of disseminated infection. Both immunocompetent and immunosuppresssed mice were infected with two inocula concentrations (2×10^6 and 2×10^8 conidia/animal) of two strains of each species. Tissue burden, mortality rate, histopathology and levels of (1→3)-β-D-glucan were used as virulence markers. None of the species of *Acremonium* tested was able to cause infection in immunocompetent mice. Conversely, severe infections were produced in immunocompromised mice, the spleen being the most affected organ. In general, the virulence of the *Acremonium* species tested was low, *S. kiliense* being the most virulent species.

Keywords virulence, immunosuppressed mice, mycosis, fungal infection, *Acremonium*

Introduction

Opportunistic fungal infections have increased dramatically in recent years and are a leading cause of death in immunocompromised patients [1]. Although *Acremonium* is less common than other opportunistic molds, such as *Aspergillus*, *Fusarium* or *Scedosporium*, some species can cause invasive infections in humans [2,3. *Acremonium* is a cosmopolitan environmental fungus commonly found in soil, plant debris and rotting mushrooms, as well as some species having been reported to be pathogens of plants, insects and mammalians [4,5]. In humans, mycetoma, keratitis and onychomycosis are the most common clinical presentations of *Acremonium* infections [3,6,7], although disseminated infections have also been reported in immunosuppressed patients [8–11] and rarely in immunocompetent patients [12]. *Acremonium* is a complex and polyphyletic genus comprising approximately 150 species. Summerbell *et al.* [15] recently carried out a comprehensive review of the genus based on the ribosomal DNA sequences of a large number of species which resulted in *A. kiliense* being relocated to the genus *Sarocladium*. *S. kiliense* (*Acremonium kiliense*) is the most commonly recovered from clinical samples followed by *A. sclerotigenum-A. egyptiacum* complex and *A. implicatum* [13,14]. Due to the difficulty in identifying *Acremonium* isolates morphologically and to the general absence of reliable sequences in public databases, the etiological agent was not identified or at least wrongly identified in most clinical cases [16,17]. Therefore, the real incidence of the involvement of *Acremonium* spp. in the clinical setting is unknown.

The use of molecular tools in recent years has enlarged the range of fungal species that can be identified in a clinical situation. Concerning *Acremonium*, a recent molecular study investigated its prevalence in a large set of clinical isolates from the USA, which resulted in the finding that the most common were *S. kiliense*, *A. sclerotigenum-A. egyptiacum* complex, *A. implicatum*, *A. persicinum* and *A. atrogriseum* [14]. Since there are no recommended therapies for *Acremonium* infections and their resistance to the available drugs [3], it is crucial to develop appropriate models to evaluate possible therapies. In this study, we have investigated the virulence of the most clinically

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relevant species of *Acremonium* in mice by developing experimental models of acremoniosis.

Materials and methods

Six clinical isolates of the most common *Acremonium/Sarocladium* species were included in the investigation, i.e., *S. kiliense*, *A. implicatum* and *A. sclerotigenum-A. egyptiacum* complex (Table 1). They were grown on potato dextrose agar (PDA) at 25°C for 7 days, with the plates then flooded with sterile saline solution, the surface scraped with a culture loop and the resulting suspension collected with a sterile Pasteur pipette to prepare the inocula suspensions. The latter were transferred to 100 ml of potato dextrose broth, incubated at 25°C for 7 days, filtered twice through several layers of sterile gauze to remove large clumps of hyphal fragments and centrifuged at 3000 rpm for 20 min. The resulting pellets were suspended in sterile saline and the conidial inocula concentrations adjusted through counts made with a haemocytometer. The viability of the components of the inocula was confirmed by growing 10-fold dilutions on PDA.

Both immunocompetent and immunosuppressed four-week-old OF-1 male mice (Charles River, Criffa SA, Barcelona, Spain), weighing 28–30 g, were used. The animals were housed in standard boxes with corncob bedding and free access to food and water. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Animals were immunosuppressed by the administration of a single intraperitoneal injection of cyclophosphamide (200 mg/kg) plus i.v. injection of 5-fluorouracil (150 mg/kg) one day before infection [18,19].

In a preliminary study on immunosuppressed animals, three different inocula were assayed (2×10^4, 2×10^6 and 2×10^8 conidia/animal) of *S. kiliense* UTHSC 03-3197 to establish the inoculum that could cause acute infection, with animals succumbing within 10 days (data not shown). All infections were established by administering 200 µl of the inoculum via the lateral tail vein of groups of 10 animals. For the survival study, animals were checked daily for 30 days, with survivors and animals meeting the criteria for discomfort euthanized by CO_2 inhalation.

Fungal load and (1→3)-β-D-glucan serum levels were determined only in the group of immunosuppressed animals infected with each of the two inocula. Additionally a control group of uninfected mice was included. Mice were anesthetized by sevoflurane inhalation (Sevorane; Abbott, Madrid, Spain), blood collected by cardiac puncture, centrifuged at 3500 rpm and the serum obtained stored at − 80°C until its use for determining (1→3)-β-D-glucan levels. Next, animals were euthanized and brain, kidneys, liver, lung and spleen were aseptically removed. Approximately half of each organ was weighed, mechanically homogenized in 1 ml of sterile saline and serially 10-fold diluted. Dilutions were inoculated onto PDA plates and incubated at 25°C for 7 days in order to determine the colony forming units (CFU) per gram. Serum levels of (1→3)-β-D-glucan were determined using the Fungitell kit (Associates of Cape Cod, East Falmouth, MA, USA) following the manufacturer's instructions. The other half of each organ was fixed with 10% buffered formalin, dehydrated, paraffin-embedded, sliced into 2 µm sections, stained with haematoxylin and eosin (H-E), periodic acid-Schiff and Grocott methamine silver and examined in blinded fashion by light microscopy.

Mean survival time (MST) was estimated using the Kaplan-Meier method and compared among groups with the log rank test. Differences in levels of (1→3)-β-D-glucan between species and inocula were determined by the ANCOVA test using the SPSS program. When multiple comparisons were carried out, the Bonferroni correction was used to avoid an increase in type I error. Organ burden data was analyzed using the Mann-Whitney U-test using GraphPad Prism 5 for Windows. *P*-values ≤ 0.05 were considered statistically significant.

Table 1 Clinical isolates of *Acremonium* spp./*Sarocladium* included in this study and their origins.

Species	Strains	Origin
Sarocladium kiliense	UTHSC 03-3197	Vitreous humor
	UTHSC 07-550	Blood
Acremonium	UTHSC 01-194	Blood
sclerotigenum-A. egyptiacum complex	UTHSC 05-2270	Blood
Acremonium implicatum	UTHSC 07-3260	Bone
	UTHSC 07-3667	Bronch wash

Results

The inocula escalation study carried out on *S. kiliense* strain UTHSC 03-3197 showed that 2×10^6 and 2×10^8 conidia/animal were both able to cause fatal acute infections in mice (data not shown). We therefore used these inocula levels for challenging both immunocompetent and immunosuppressed mice with the six fungal strains included in the studies. None of the infected immunocompetent animals succumbed to the infection or showed clear signs of disease.

Figure 1 shows that at low inocula there were no observable differences in survival rates between strains of the same species ($P > 0.05$), although there were significant differences in MST. The infection with *A. implicatum* strains killed 30% of the animals with an MST of 22.5–23.7 days, demonstrating less virulence than infections

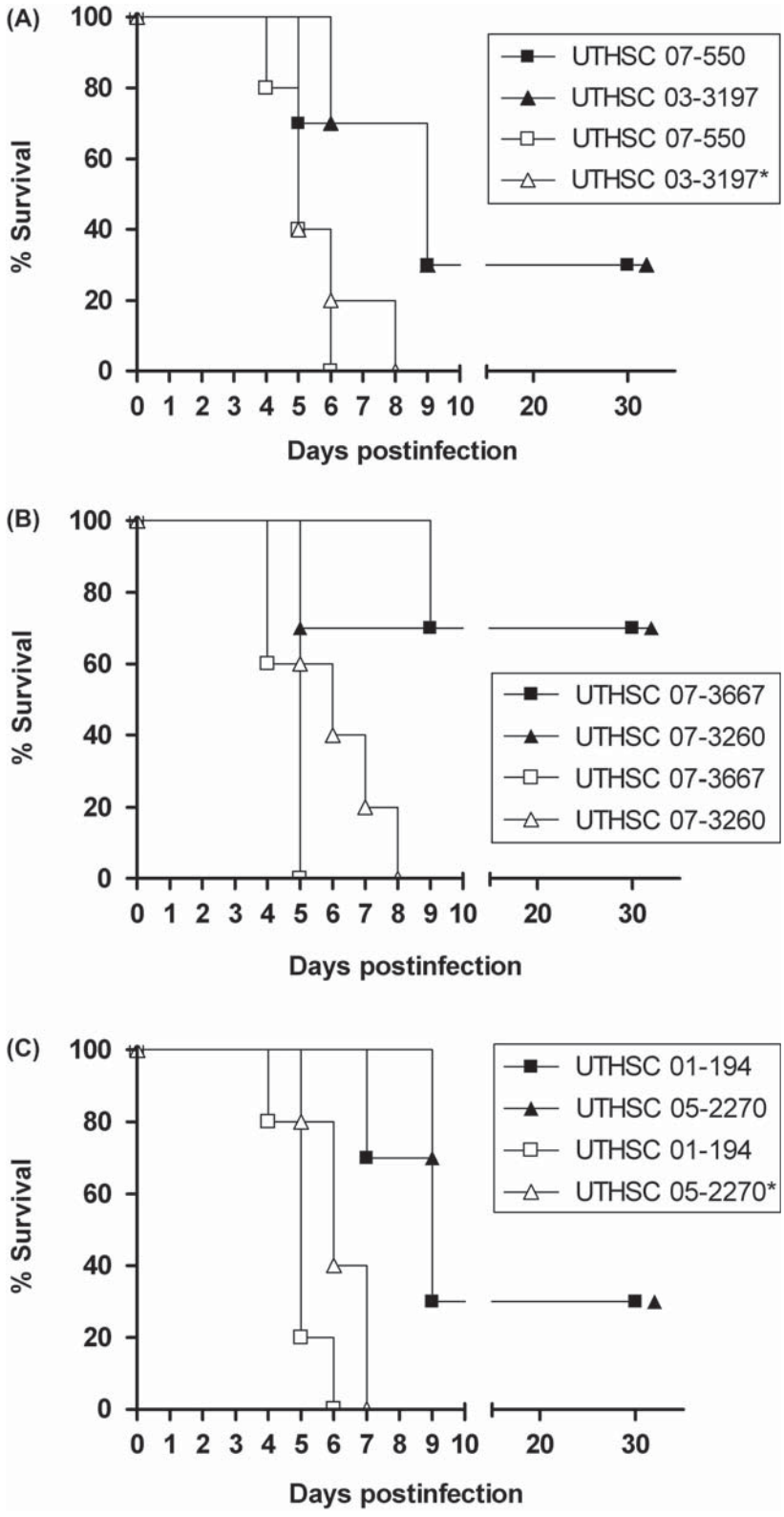

Fig. 1 Survival of immunosuppressed mice infected with low (2×10^6 conidia/animal) (filled symbols) and high (2×10^8 conidia/animal) (clear symbols) inoculum of (A) *Sarocladium kiliense* (*S. k*), (B) *Acremonium sclerotigenum-A. egyptyacum* complex (*A. s-e*) and (C) *A. implicatum* (*A. i*). *significant differences between strains of the same species at same inoculum size. $P \leq 0.05$.

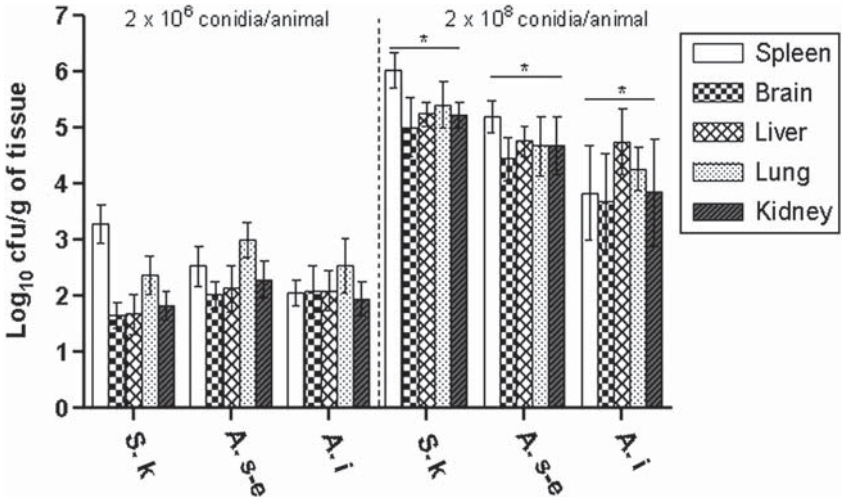

Fig. 2 Fungal load of different organs recovered from immunosuppressed mice infected with low (2×10^6 conidia/animal) and high (2×10^8 conidia/animal) inoculum of *Sarocladium kiliense* (*S. k*), *Acremonium sclerotigenum-A. egyptyacum* complex (*A. s-e*) and *A. implicatum* (*A. i*). ∗significant differences between low and high inoculum. $P \leq 0.05$.

caused by *S. kiliense* or the *A. sclerotigenum-A. egyptiacum* complex, which killed 70% of the animals with MST ranges of 14.1–14.4 and 14.7–16.1 days, respectively. Infections at a high inocula resulted in the death of all animals regardless of species, with a MST range of 4.6–6.2 days (Table 1).

As there were no signs of disease or death in immunocompetent mice, we determined the fungal load and levels of (1→3)-β-D-glucan in immunosuppressed animals infected with both previously tested inocula. Viable fungal cells were recovered from all the studied organs and the number of CFUs recovered from depended on inocula concentrations with significantly higher counts found in animals receiving high doses than in those challenged with low inocula levels (Fig. 2). In mice challenged with low inoculums numbers there were no significant differences in fungal load between the two strains of the same species ($P > 0.05$) (Fig. 3), i.e., the lungs were the most affected organs in those animals infected with *A. implicatum* or *A. sclerotigenum-A. egyptiacum* complex and the spleen in those infected with *S. kiliense* (Fig. 2). When high inoculum levels were used, the liver was the most affected organ in animals infected with *A. implicatum* and the spleen in those challenged with *S. kiliense* or the *A. sclerotigenum-A. egyptiacum* complex. Regardless of inoculum size and strain, the brain was the least affected organ (Fig. 2). With the high inoculum, significant differences between the strains of *A. implicatum* were found in brain ($P = 0.0001$), liver ($P = 0.0005$), lung ($P = 0.0010$) and kidney ($P = 0.005$), while for *S. kiliense*, differences were in brain ($P = 0.0001$) and lung ($P = 0.0464$) and for the *A. sclerotigenum-A. egyptiacum* complex, differences were only found in kidney ($P = 0.0031$) (Fig. 3).

The concentration of (1→3)-β-D-glucan in sera of uninfected mice were 54.04 ± 0.38 pg/ml in contrast to those obtained from mice infected with *Acremonium* spp. which were ≥80 pg/ml. These results were dependent on the species ($P = 0.023$) and the inocula concentrations ($P < 0.0001$), being significantly higher for high inoculum ($P \leq 0.001$), with no interaction between them ($P = 0.618$) (Fig. 4). When the means for each species were evaluated there were significant differences between *S. kiliense* and *A. sclerotigenum-A. egyptiacum* ($P = 0.020$) but none between *A. implicatum* and *S. kiliense* or the *A. sclerotigenum-A. egyptiacum* ($P > 0.05$).

Organs from mice infected with any of the three species assayed were similar histopathologically, regardless of the inoculum size tested. In general, the infection did not produce a detectable inflammatory response, necrosis, edema or angioinvasion. However, in kidneys glomerular invasion by hyphae with no parenchyma destruction was observed (Fig. 5A) and focal and low presence of round shaped fungal cells was found in brain, lung, spleen and liver (Fig. 5B).

Discussion

We have developed a murine model of disseminated acremoniosis for evaluating and comparing virulence in the most clinically relevant species. In recent years, numerous animal models have been developed for common pathogenic yeasts and molds, which have been useful for evaluating antifungal therapies [20]. However, despite an increase in invasive infections by *Acremonium* [2], no animal models are available for this fungus, probably because of the complex taxonomy of *Acremonium* [3,14,15], which

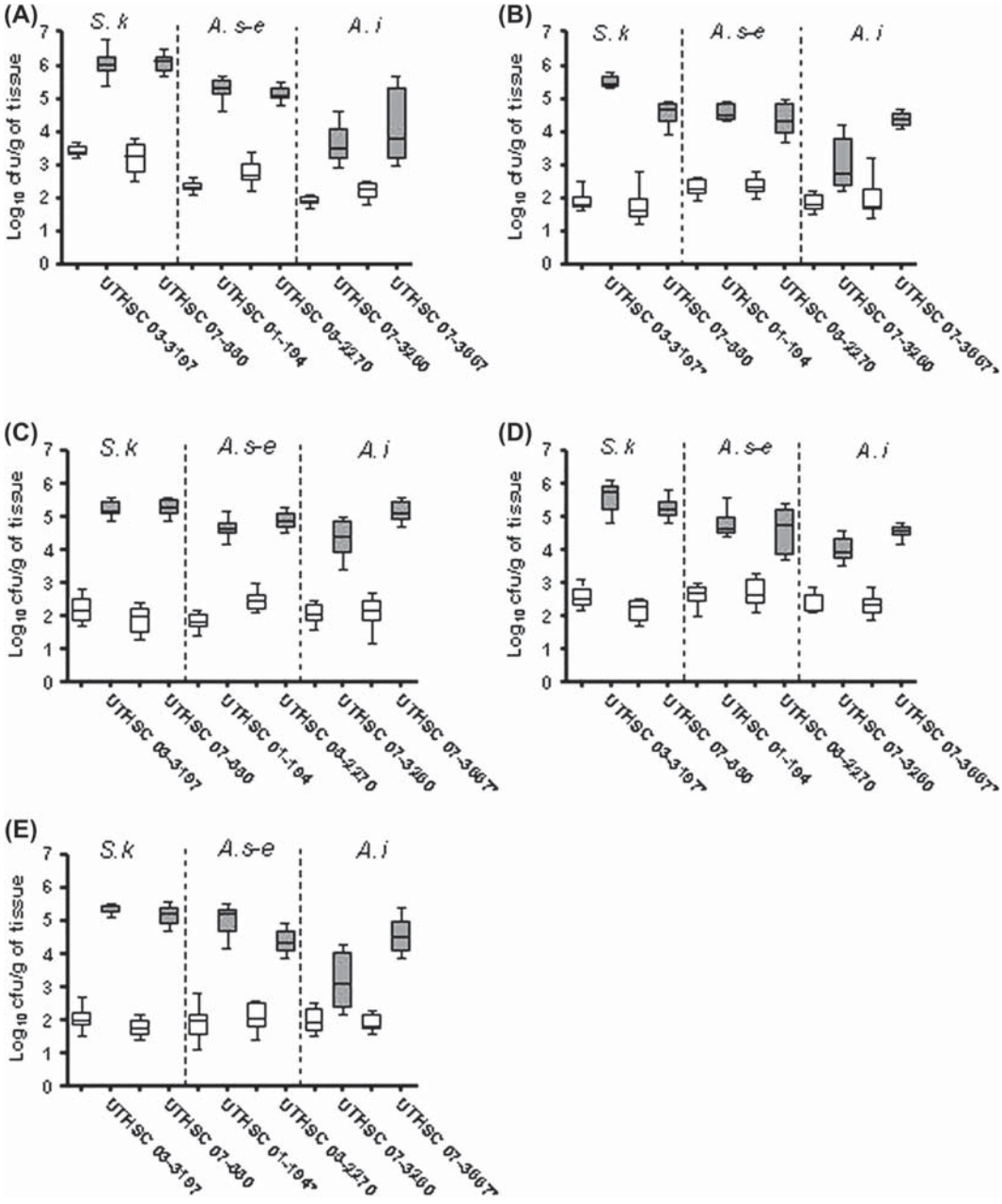

Fig. 3 Quantitative fungal recovery from spleen (A), brain (B), liver (C), lung (D) and kidney (E) from immunosuppressed mice infected with low (2×10^6 conidia/animal) (white box) and high (2×10^8 conidia/animal) (gray box) inocula of *Sarocladium kiliense* (*S. k*), *Acremonium sclerotigenum-A. egyptiacum* complex (*A. s-e*) and *Acremonium implicatum* (*A. i*). *significant differences between strains of the same species at high inoculum ($P \leq 0.05$). Horizontal lines indicate the mean.

makes it very difficult to determine representative isolates of clinical relevant species for studying in animal models.

In general, our results indicate a low virulence of the three species tested, *S. kiliense* being slightly more virulent than the others, at least at low inocula levels. All the immunocompetent mice survived the infection despite the high inocula concentrations employed, which mirrors the natural course of the infection if patients are not immunocompromised [2]. Disseminated infection by *Acremonium* has been reported to involve multiple organs, including liver, spleen, kidney and brain [8,9,11,21]. In our study, we recovered fungi from all organs studied, including brain at both inoculum in all the species tested.

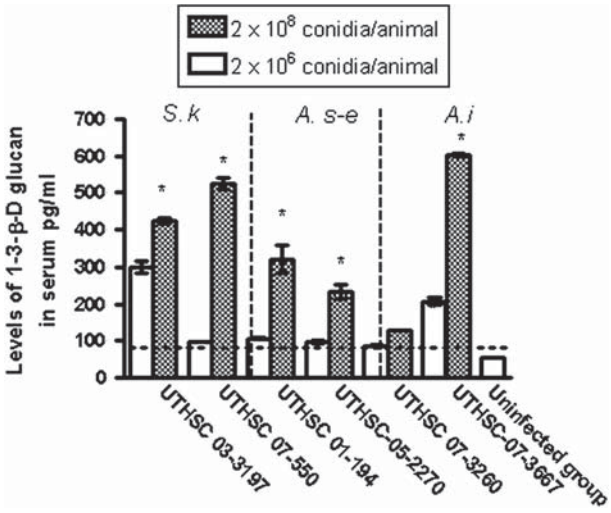

Fig. 4 Serum levels of (1→3)-β-D-glucan in mice infected with *S. kiliense* (*S. k*), *A. sclerotigenum-A. egyptiacum* complex (*A. s-e*), *Acremonium implicatum* (*A. i*) and uninfected mice as control group on day 5 post-infection. The horizontal line indicates the cut-off for positivity (≥ 80 pg/ml). *significant differences ($P \leq 0.05$) between low (2×10^6 conidia/animal) and high (2×10^8 conidia/animal) inoculum.

To some extent, the clinical behavior and even the morphology of *Acremonium* are similar to that of *Fusarium*, both fungi producing a disseminated infection almost exclusively in neutropenic patients and being highly refractory to antifungal drugs [22]. One of the typical characteristics of *Acremonium* that it shares with *Fusarium*, *Scedosporium* and other genera, is its ability to sporulate in tissue, called 'adventitious sporulation', which explains the relative high frequency of recovering these fungi from the blood of infected patients [23]. *In vivo* sporulation has been described in a case of disseminated *Acremonium* infection at day 79 [8], but in our study we did not observe such characteristics, probably because the organs were removed too soon after infection. Moreover, since we did not attempt to culture blood we could not evaluate the presence of fungemia.

Current serological methods used in fungal diagnosis are generally based on the detection of cell-wall components [24]. We have evaluated the usefulness of (1→3)-β-D-glucan as a marker of infection and whether there are any significant differences among the species of *Acremonium* tested. Our results reveal high serum levels, even when low inocula were used. Significant differences were observed among the species when the mean value was calculated, with *S. kiliense* showing the highest values. Other authors obtained similar high values in experimental fungal infections, from mice and guinea pigs in invasive pulmonary aspergillosis in serum and BAL fluid, respectively [25,26]. Although those animal models were different from our study, they highlight the usefulness of (1→3)-β-D-glucan for evaluating the progression of infection and its response to antifungal therapies. Although we cannot confirm fungemia because blood cultures were not carried out, the high levels of this antigen in serum obtained in our study might be related to the angioinvasive ability reported for *Acremonium*, which would facilitate its release directly into the bloodstream [23]. Therefore, although it must be taken into account that such antigen is a panfungal marker [27], detection of (1→3)-β-D-glucan could be considered a useful tool for the prompt detection of disseminated infections by uncommon fungi like *Acremonium* once aspergillosis has been discarded.

Murine models have been used extensively to test experimental antifungal therapy against clinical important fungi, and past experience demonstrated that efficacy in animal models is usually predictive of clinical efficacy [28]. To our knowledge, this is the first animal study that has

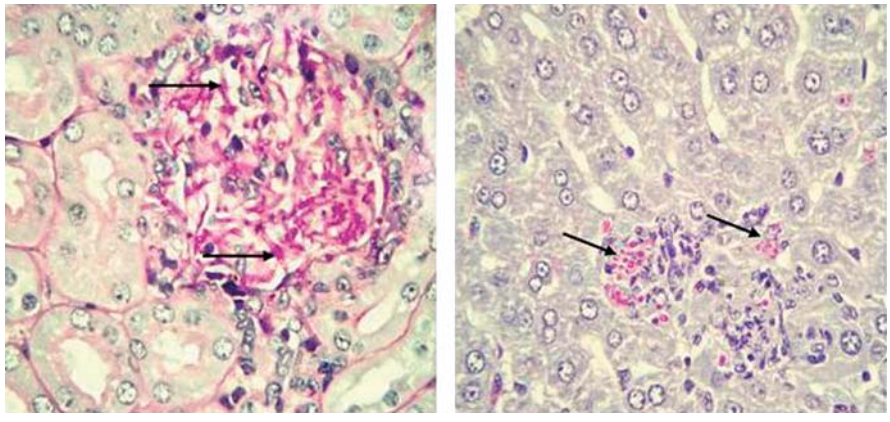

Fig. 5 Histological section at 5th day post-infection of kidney and liver of mice immunosuppressed infected with 2×10^8 conidia/animals of the strain UTHS 03-3197 of *S. kiliense*. (A) Kidney section showing glomerular invasion by hyphae cells (arrow) with no alteration of parenchyma or necrosis. (B) Liver section showing scarcity of round-shaped fungal cells (arrow). This Figure is reproduced in color in the online version of *Medical Mycology*.

explored the virulence of *Acremonium*, which could be useful for future studies directed to establish a suitable antifungal therapy for the management of these refractory infections.

In conclusion, our results demonstrate a low virulence of *Acremonium/Sarocladium*, even in immunosuppressed animals. *S. kiliense*, the most common species in the clinical setting was the most virulent here.

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

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4.5. Antifungal therapies in a murine model of disseminated infection by the emerging opportunistic fungus S*arocladium (Acremonium) kiliense*.

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Antifungal therapies in a murine model of disseminated infection by the emerging opportunistic fungus *Sarocladium (Acremonium) kiliense*

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Key words: *Acremonium,* murine model, antifungal therapy

Running title: Antifungal therapies for *Sarocladium kiliense*

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Abstract

We evaluated and compared the efficacy of different antifungal drugs against *Sarocladium kiliense* (formerly *Acremonium kiliense*), *a* clinically relevant opportunistic fungus in a murine model of systemic infection. Three clinical strains of this fungus were tested and the therapies administered were as follows: posaconazole at 20 mg/kg (twice daily), voriconazole at 40 mg/kg, anidulafungin at 10 mg/kg, or amphotericin B at 0.8 mg/kg. The efficacy was evaluated by prolonged animal survival, tissue burden reduction and by (1→3)-β-D-glucan serum levels. In general, the four antifungal drugs showed high MICs and poor *in vitro* activity. The efficacy of the different treatments was only modest, since survival rates never were higher than 40% and no drug was able to reduce fungal load in all the organs for the three strains tested. Posaconazole, in spite of its high MICs (≥16 μg/ml), showed the highest efficacy. The (1→3)-β-D-glucan serum levels were equally reduced by all drugs evaluated.

Introduction

Acremonium kiliense is a saprobic fungus with a worldwide distribution (1-4), which has been reported in the last years as an emerging opportunistic pathogen able to cause a wide range of human infections (5, 6). Localized infections, such as mycetoma, keratitis or onychomycosis are acquired mainly by immunocompetent patients through trauma (7-13). Invasive infections generally affect immunosuppressed hosts such as those undergoing transplantation or those with AIDS, resulting in a high degree of fatality (14-18). More rarely, though, invasive infections have also been reported in immunocompetent individuals (19, 20). Recently, based on molecular studies, this fungus has been transferred to the genus *Sarocladium* as *S. kiliense* (1). This species is the most clinically relevant of the genus and apparently also the most virulent (4, 21, 22). Considering that the morphological identification of these fungi is difficult it is likely that some clinical isolates of this species have been misidentified as *A. strictum*, another clinically important species of *Acremonium* (21). Infections by *Acremonium* spp. are difficult to treat due to the intrinsic resistance to the current antifungal agents and an effective treatment has not yet been determined (2, 4, 21). Amphotericin B (AMB) is the most commonly used drug to treat severe fungal infections caused by opportunistic moulds (4). Despite its poor *in vitro* activity and variable clinical results, this drugs still remains the first therapeutic choice against invasive *Acremonium* infections (2, 4, 21). Clinical experience with other drugs, such as posaconazole (PSC), voriconazole (VRC) or the echinocandins, is very poor.

To evaluate possible therapeutic strategies, we tested the efficacy of PSC, VRC, anidulafungin (AFG) compared to AMB against *S. kiliense* in a recently developed murine model of disseminated infection (22).

Material and Methods

Three clinical strains of *Sarocladium kiliense,* UTHSC 01-2238, UTHSC 03-3197 and UTHSC 07-550, were tested. The strains were stored in slant cultures covered with sterile paraffin oil and subcultured onto potato dextrose agar (PDA) plates at 25ºC for 7-10 days. *In vitro* susceptibilities to AMB, PSC, VRC and AFG were tested by using a broth microdilution reference method (23).

For the *in vivo* study, the inocula were prepared flooding the surface of the agar plate with sterile saline solution, scraping the sporulating mycelium with a culture loop, and drawing up the resultant suspension with a sterile Pasteur pipette. The conidial suspensions were transferred to 100 ml of potato dextrose broth (PDB) and incubated at 25ºC for 7 days. Suspensions were filtered twice through several layers of sterile gauze to remove large clumps and hyphal fragments and then centrifuged at 3000 rpm for 20 minutes. The resulting pellets were suspended in sterile saline and the inocula adjusted to the desired concentration by haemocytometer counts and by serial plating onto PDA to confirm viability. Four-weeks-old OF-1 male mice (Charles River, Criffa SA, Barcelona, Spain) weighing 28-30 g were used. Animals were housed in standard boxes with corncob bedding and free access to food and water. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Mice were immunosuppressed 1 day prior to the infection by intraperitoneal (i.p.)

administration of a single dose of 200 mg/kg of cyclophosphamide plus a single dose of 150 mg/kg of 5-fluorouracil given intravenously (i.v.) (24).

Groups of 8 animals were established for the different studies. For the survival study, animals were challenged with 2×10^8 conidia injected into the lateral tail vein and checked daily for 30 days. Survivors and those meeting the criteria for discomfort were euthanized by CO_2 inhalation. For tissue burden and (1→3)-β-D-glucan serum level determinations mice were infected with $2x10^6$ conidia in 0.2 ml of sterile saline. This inoculum was chosen in order to avoid the rapid killing obtained with the highest inoculum and to allow the fungal load of treated groups be compared with controls at same day. Mice received AMB at 0.8 mg/kg/day i.v. (25) once daily, PSC at 20 mg/kg orally (p.o.) twice a day (BID) by gavage (26), VRC at 40 mg/kg p.o. once daily (27) or AFG at 10 mg/kg i.p. once daily (28). Mice that received VRC were given grapefruit juice instead of water 3 days before being infected (29). All treatments were started 24 h after the challenge and lasted for 7 days. Animals were checked for survival twice daily for 30 days.

Fungal load and (1→3)-β-D-glucan serum levels were determined after 5 days of treatment in order to compare the results with the control group, since control mice start to die on that day. Additionally, a group of 3 uninfected mice was included as negative controls for the (1→3)-β-D-glucan determination. Mice were anesthetized by sevoflurane inhalation (Sevorane; Abbott, Madrid, Spain). Blood was collected by cardiac puncture and centrifuged at 3500 rpm and the serum obtained stored at -80ºC until its use for determining (1→3)-β-D-glucan levels. Then, animals were euthanized and kidneys, liver, lung and spleen were aseptically removed (22). Approximately half of each organ was weighed,

mechanically homogenized in 1 ml of sterile saline and serially 10- fold diluted. Dilutions were plated on PDA plates and incubated at 25ºC for 7 days in order to determine the number of colony forming units (CFU) per gram. Serum levels of (1→3)-β-D-glucan were determined using the Fungitell kit (Associates of Cape Cod, East Falmouth, MA) following the manufacturer´s instructions. The other half of each organ was fixed with 10% buffered formalin, dehydrated, paraffin-embedded, sliced into 2 μm sections, stained with haematoxylin and eosin (H-E), periodic acid-Schiff and Grocott methamine silver and examined in a blinded fashion by light microscopy.

Mean survival time (MST) was estimated using the Kaplan-Meier method and compared among groups with the Log Rank test. Differences in levels of (1→3)-β-D-glucan between treatments and strains were determined by Mann-Whitney U-test pairwise comparisons and the Dunnett's method, considering control group as reference, was used to adjust their p-values. Organ burden data differences between treatments were detected by Mann-Whitney U-test pairwise comparisons and the Bonferroni method was used to correct p-values. Data analysis was performed with GraphPad Prism 4 for Windows. P-values ≤ 0.05 were considered statistically significant.

Results

For the UTHSC 01-2238 strain the MICs of AMB, PSC, VRC and minimal effective concentration (MEC) of AFG were 8, 16, 2 and 8 μg/ml, respectively , for the UTHSC 03-3197 strain they were 4, 16, 0.5 and 4 μg/ml, respectively, and for UTHSC 07-550 strain were 2, >16, 2 and >16 μg/ml, respectively.

The results of the survival studies are shown in figure 1. All the controls died within 7 days after fungal inoculation. PSC and AFG were the only drugs able to significantly prolong survival for the three strains assayed (P<0.0012), although at the end of the experiment the survival never was over 40%. VRC increased the survival with respect to the control group only for one strain (UTHSC 03-3197) (P=0.0015). AMB did not show efficacy against any strain (P>0.150).

The spleen was the most affected organ and its fungal load was at least two log units higher than liver, lung and kidneys (Fig 2). In general the efficacy of the different therapies depended on the strain and on the organ tested, and even in the cases that tissue burden was reduced, it was only modest. PSC, with a few exceptions, was able to reduce fungal load in all organs and strains, and in some cases was more effective than the other drugs. PSC reduced tissue burden in 9 of the 12 groups evaluated (4 different organs x 3 strains), VRC in 7 of them, AMB in 7 also, and AFG only in 4.

The concentration of (1→3) β-D glucan in the serum of non-infected mice was 39.28 ± 0.42 pg/ml in contrast to that obtained from infected control mice which ranged from 210.2 to 499.8 pg/ml. All treatments were able to significantly reduce the (1→3)-β-D-glucan serum concentrations in comparison with that for the control group. However, differences between drugs were not observed (Fig 3).

The histopathological studies of untreated controls showed a low and focal presence of fungal cells in all organs studied with exception of kidney, which showed glomerular invasion by hyphae with no signs of inflammatory response,

necrosis, edema or angioinvasion. A decrease of fungal cells in kidney section was observed only in those mice treated with PSC.

Discussion

Until now only a few studies on the antifungal susceptibility of *Acremonium* have been done, all of them being *in vitro* (3, 4, 21). Our azoles MICs are different from those reported by Khan *et al*., using E-test (4) which were significantly lower, which could be explained by the different methods used in both works. To our knowledge, this is the first study that has explored the efficacy of antifungal drugs in the treatment of an experimental infection by *S. kiliense* and unfortunately the results can not be compared with other studies. In our study PSC displayed the best results, prolonging the survival of the mice infected by each of the three strains tested and reduced fungal load of most of the organs tested. Until now the clinical experience on the use of PSC in disseminated infections by *Acremonium* is scarce. However, that drug resolved a pulmonary infection by *A. strictum* in a leukemic patient after failure of AMB (30).

The variable outcomes of the reported *Acremonium* infections, have not allowed a conclusion about the most suitable treatment. Perhaps this variability may be explained by the different virulence of the strains. However, in our study although all strains tested show similar virulence, some variability on the effectiveness of treatments was also observed.

AMB has been the drug most commonly used to treat *Acremonium* infection with very variable results (14, 19, 31-36). While a disseminated infection by *Acremonium* sp. in a patient with Addison´s disease was successfully treated with that drug (36), failure has been reported mainly in cases of fungemia or

disseminated infections(19, 32, 33). Although in our study this drug was able to reduce fungal load in some organs it was ineffective in prolonging survival for any of the strains tested, which suggest that the use of this drug in human infection should be taken with caution. Clinical experience with VRC is more limited; however, some cases of successful treatment with that drug have been reported (37, 38). Although unfortunately the *Acremonium* isolates were not identified at species level (38). Similarly to AMB, VRC showed discouraging efficacy in mice survival prolonging in our study.

Although only few isolates were tested, there was a clear lack of correlation between the *in vitro* and *in vivo* results observed in our study. AMB and VRC showed the lowest MICs, however their efficacy was poor, and PSC which showed MICs≥16μg/ml, indicative of resistance, demonstrated the highest efficacy.

The relatively poor drug efficacy shown here is, to some extent, similar to that shown with other morphologically similar fungi causing hyalohyphomycosis such as *Fusarium*, *Paecilomyces* or *Scedosporium*, which are refractory to most of antifungal agents (39). All these fungi share the common characteristic of sporulating in the infected tissue, which is a rare feature in pathogenic moulds (40). Perhaps, this ability to sporulate facilitates a quick dissemination to very distant organs limiting the effectiveness of the antifungal drugs. Nevertheless, we did not observe this feature in our histologic sections, most likely be because the organs were removed too soon after infection.

Different biomarkers have been tested to help in the diagnosis of fungal infections (41). Detection of (1→3)-β-D-glucan has been useful for the diagnosis and therapeutic monitoring in experimental and clinical studies on invasive

fungal infections (42- 46). In our studies, the high serum levels of that marker in control mice were similar to those observed previously with *Acremonium* (4, 22), but also in experimental invasive fungal infections by *Fusarium* and *Aspergillus* (44-46). Although the animal models used in those studies were different than our models, since the infection was by conidial inhalation their results also showed a good correlation between the (1→3)-β-D-glucan levels, the progression of infection and the response to antifungal therapies (46). Our (1→3)-β-D-glucan levels from mice treated with different drugs showed that the treatments were able to significantly reduce these levels with respect to the control group; however no differences were noted between the different antifungal agents. Although it must be taken into account that (1→3)-β-D-glucan is a panfungal marker (41) and the cutoff value is not determined for animal models, our results suggest the potential use of this marker for the prompt detection of infection and for monitoring treatment efficacy.

In conclusion, the results of this work suggest that further studies are needed to explore the potential efficacy of PSC in the treatment of refractory human infections incited by *S. kiliense*.

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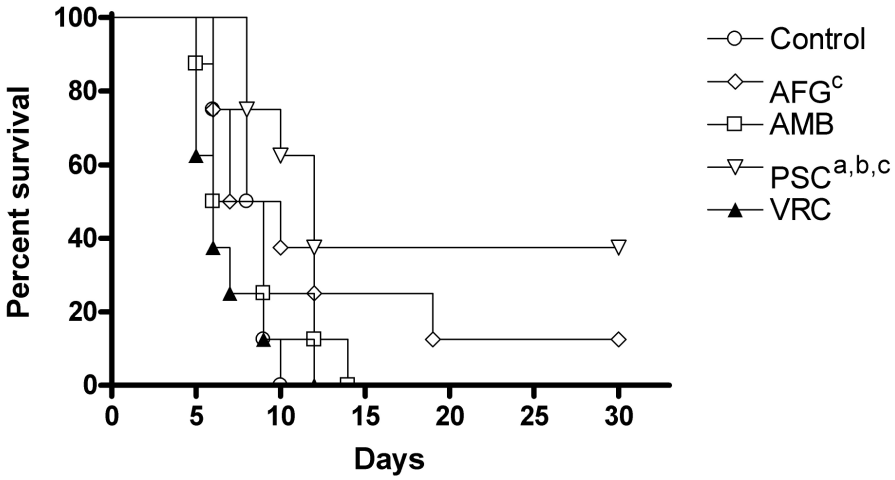
Fig 1. Cumulative mortality of mice infected with $2x10^8$ conidia/animal of three strains of *Sarocladium (Acremonium) kiliense,* UTHSC 01-2238 (A), UTHSC 03-3197 (B) or UTHSC 07-550(C). $^{a}P< 0.05$ versus control; $^{b}P< 0.05$ versus amphotericin B (AMB); $^{c}P< 0.05$ versus voriconazole (VRC).

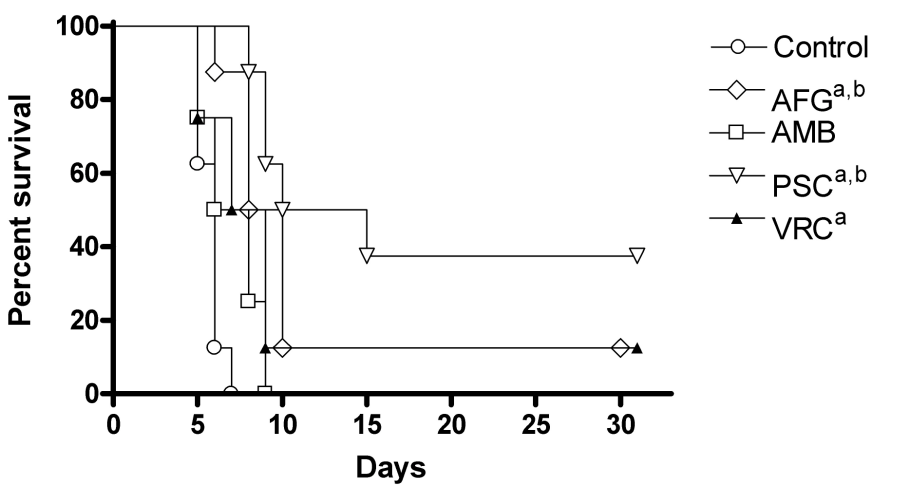
Fig 2. Effects of the antifungal treatments on colony counts in mice infected with $2x10^6$ conidia/animal of three strains of *Sarocladium kiliense,* UTHSC 01-2238, UTHSC 03-3197 or UTHSC 07-550, in spleen, liver, lung and kidneys of mice. Amphotericin B (AMB) at 0.8 mg/kg; posaconazole (PSC) at 20 mg/kg twice a day; voriconazole (VRC) at 40 mg/kg; anidulafungin (AFG) at 10 mg/kg. ^{a}P value< 0.05 versus the control; ^{b}P value <0.05 versus AFG; ^{c}P value <0.05 versus AMB; ^{d}P value <0.05 versus VRC. Horizontal lines indicate mean values.

Fig 3. (1→3)-β-D-glucan serum levels of mice infected with $2x10^6$ conidia/animal of the strains of *Sarocladium kiliense,* UTHSC 01-2238, UTHSC 03-3197 or UTHSC 07-550, on day 5 after challenged. Amphotericin B (AMB) at 0.8 mg/kg; posaconazole (PSC) at 20 mg/kg twice a day; voriconazole (VRC) at 40 mg/kg; anidulafungin (AFG) at 10 mg/kg. ^{a}P value< 0.05 versus the control. Discontinuous lines indicates values cutoff ≥ 80pg/ml.

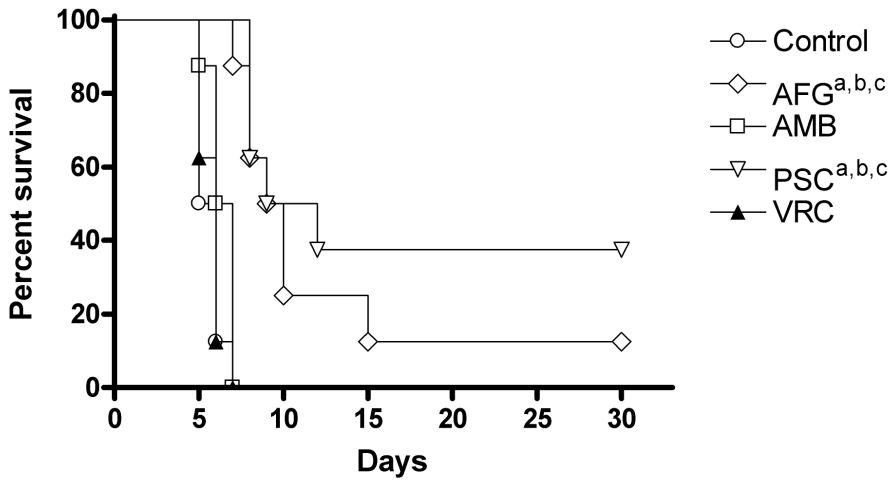
A.

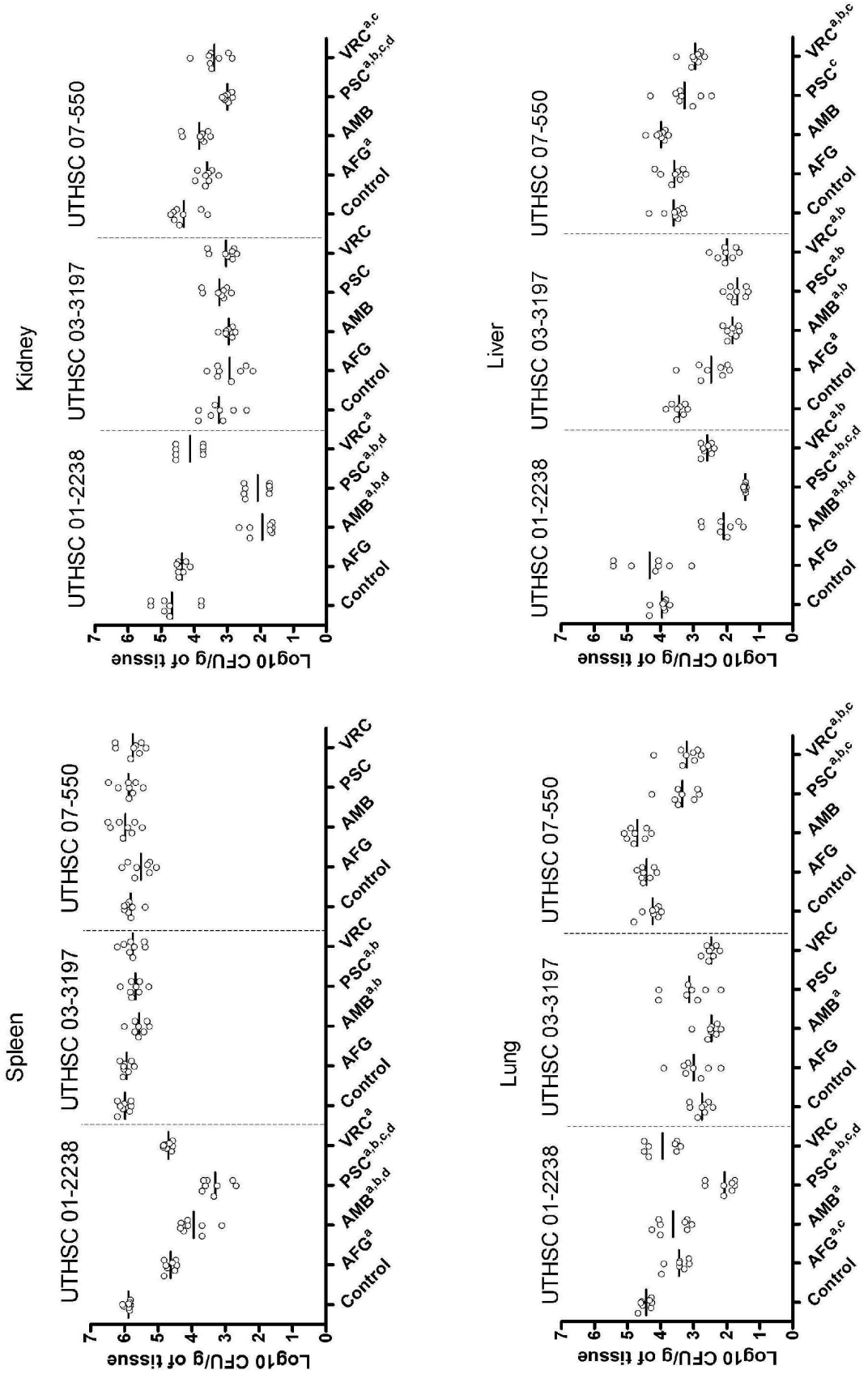

Percent survival
100
80
60
40
20
0
0
5
10
15
20
25
30
Days
Control
AFG[c]
AMB
PSC[a,b,c]
VRC

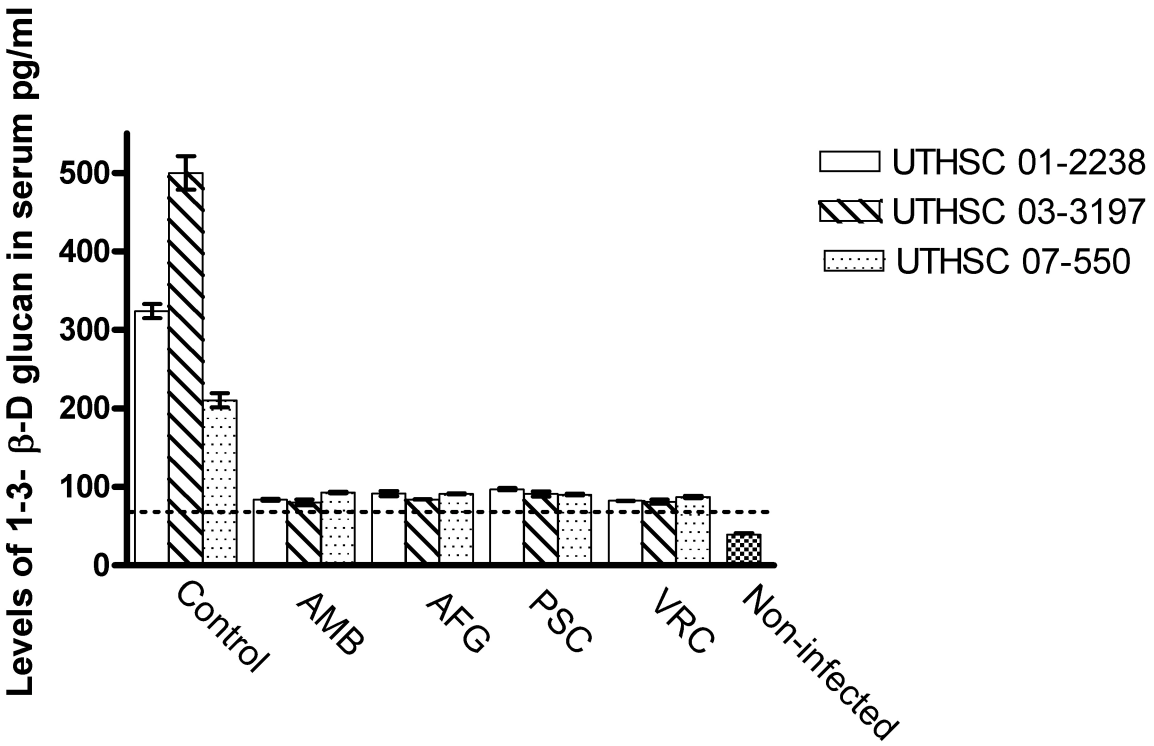
B.


Percent survival
100
80
60
40
20
0
0
5
10
15
20
25
30
Days
Control
AFG[a,b]
AMB
PSC[a,b]
VRC[a]

C.


Percent survival
100
80
60
40
20
0
0
5
10
15
20
25
30
Days
Control
AFG[a,b,c]
AMB
PSC[a,b,c]
VRC




Levels of 1-3- β-D glucan in serum pg/ml
500
400
300
200
100
0
Control
AMB
AFG
PSC
VRC
Non-infected
UTHSC 01-2238
UTHSC 03-3197
UTHSC 07-550

4.6. *In vitro* evaluation of combinations of antifungal drugs against *Sarocladium* (*Acremonium*) *kiliense*, an opportunistic emergent fungus resistant to antifungal therapies.

Fernández-Silva F., Capilla J., Mayayo E., Sutton D., Guarro J. (In preparation).

In vitro evaluation of combinations of antifungal drugs against *Sarocladium* (*Acremonium*) *kiliense,* an opportunistic emergent fungus resistant to antifungal therapies.

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Sir,

Sarocladium kiliense, formerly known as *Acremonium kiliense* (1), is a ubiquitous soil saprophyte commonly found in the environment and occasionally infecting humans (2). Its pathogenicity in immunocompetent patiens is low and usually is related to inoculation of the fungus via a penetrating injury that often leads to a granuloma formation. However, the presence of underlying immunological disorders can predispose to development of systemic infection usually fatal (3). The optimal treatment for these infections is unknown; however, amphotericin B seems to be the most efficacious drug, although therapeutic failure has been also reported (3, 4). In addition this drug shows important side effect that commonly are incompatible with the poor healthy conditions of the patients. Therapeutic data available are based on a few clinical cases where the etiologic agent was only identified at genus level or misidentified (5). Antifungal *in vitr*o studies have shown that *S. kiliense* is resistant to almost all antifungal drugs (2, 6). In addition recent murine studies have demonstrated that all the therapies tested against this fungus, i.e voriconazole, posaconazole, amphotericin B and anidulafungin exerted very poor efficacy (7). Regarding that, it is crucial to explore new therapeutic strategies for the treatment of the severe invasive infection caused by *S. kiliense*. Therefore, the aim of this study was to evaluate the *in vitro* activity of drug combinations against a set of S*. kiliense* strains from clinical source, previously identify by sequencing of the internal transcribed spacer region (ITS) of the rRNA gene (2). We tested combinations of anidulafungin (AFG), amphotericin B (AMB), voriconazole (VRC), posaconazole (PSC) and terbinafine (TRB) (Table 1). To determine the *in vitro* activity we used a

checkerboard microdilution method based on the CLSI methodology for filamentous fungi (8). The minimal inhibitory concentration (MIC) for azoles, AMB and TRB and minimal effective concentrations (MEC) for AFG were determined according to CLSI broth microdilution methods. For combinations between AFG and azoles, the endpoint was calculated by taking into account both the MEC of the echinocandin and the MIC-2 (~50% reduction in turbidity compared to the growth control) for azoles, which has shown good correlation with the MEC (9). Studies were performed in duplicate and the final results were expressed as the means of these replicates. The fractionary inhibitory concentration index (FICI) was used to classify drug interaction, which were defined as synergistic when FICI was ≤0.5 synergy, as antagonistic when FICI was >4.0 and no interaction, when FICI was >0.5 but <4 (10).

In general, most of the combinations showed an indifferent effect. Synergism was observed in the combinations of VRC plus AFG for three strains (25%) and PSC plus TRB for one strain (8.3%). Antagonism was detected in 16.6% of the strains for AMB plus PSC and PSC plus AFG.

Although, some of our results are promising i.e. combination between anidulafungin and voriconazole, further experiments that evaluate the *in vivo* efficacy of this antifungal combination are warranted in order to provide new therapeutic alternatives for the treatment of this resistant pathogen.

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Table 1. Interaction showed by antifungal drug combinations against 12 clinical isolates of *Sarocladium* (*Acremonium*) *kiliense*

Antifungal combination[a]	**FICI**[b,c]	**Nº (%) strains showing**		
		Synergism	**Indifference**	**Antagonism**
AMB + AFG	1.43	0 (0)	12 (100)	0 (0)
AMB + PSC	2.08	0 (0)	10 (83,3)	2 (16,6)
AMB + VRC	2.00	0 (0)	12 (100)	0 (0)
VRC + AFG	0.68	3 (25)	9 (75)	0 (0)
VRC + TRB	1.43	0 (0)	12 (100)	0 (0)
PSC + AFG	3.04	0 (0)	10 (83,3)	2 (16,6)
PSC + TRB	2.29	1 (8.3)	11 (91,6)	0 (0)

[a,]MECs were calculated for AFG and MIC-2 (~50% reduction in turbidity compared to the growth control) were calculated for azoles. MICs were calculated for AMB and TRB.

[b,] FICI, fractionary inhibitory concentration index, ≤0.5,synergistic; >0.5 to ≤4, indifferent;>4, antagonism.

[c], Mean of FICI determined for 12 *S. kiliense* isolates.

4.7. Combination therapy in the treatment of experimental invasive fungal infection by *Sarocladium (Acremonium) kiliense.*

Fernández-Silva F, Capilla J, Mayayo E, Sutton D, Guarro J.

(In preparation).

Combination therapy in the treatment of experimental invasive fungal infection by *Sarocladium* (*Acremonium*) *kiliense*.

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Abstract

Human infections by *Sarocladium* (*Acremonium*) *kiliense* are usually refractory to traditional amphotericin B treatment. The *in vivo* efficacy of anidulafungin plus posaconazole or voriconazole and amphotericin B plus voriconazole was evaluated in a murine model of disseminated infection by that fungus. Anidulafungin combined with posaconazole was the only treatment able to prolong survival and reduce fungal load in spleen and kidney.

Introduction

Invasive infections by *Sarocladium kiliense*, formerly known as *Acremonium kiliense* (Summerbell et al 2011) are difficult to treat and often have a fatal outcome (Guarro et al 1997; Khan et al 2011). Up to now, the only drugs that have shown some beneficial effect at least in animal infections by *S. kiliense* have been posaconazole (PSC) and to lesser extent anidulafungin (AFG) (Fernández-Silva et al 2013a). However, in the clinical setting, the usefulness of these drugs has not been evaluated. Amphotericin B (AMB) has been the drug most commonly used to treat *Acremonium* infections and in some cases voriconazole (VRC) (Mattei et al 2003), but therapeutic failure has been reported mainly in cases of fungemia or disseminated (Beaudreuil et al 2003; Guarro et al 2009; Hitoto et al 2010; Khan et al 2011). Regarding the resistance of this fungus and the cases of therapeutic failure reported, other approaches should be considered. Combined therapy could be a new alternative to explore, particularly for immunosuppressed patients with disseminated infections. Therefore the aim of this study was evaluate the *in vivo* efficacy of the combination of the most active drugs in experimental study (PSC and AFG) and the most used drugs in the clinical setting (AMB and VRC).

Material and Methods

Three clinical strains of *Sarocladium kiliense*, UTHSC 01-2238, UTHSC 03-3197 and UTHSC 07-550, were tested. The inocula were prepared as previously described (Fernández-Silva et al 2013a). The *in vitro* susceptibilities of the isolates to AFG, AMB, VRC and PSC were determined by a microdilution reference method (CLSI 2008). Drug interactions were assessed using a checkerboard method and

the fractionary inhibitory concentration index (FICI) was used to classify drug interaction (Dannaoui et al 2004; Johonson et al 2004).

Four-weeks-old OF-1 male mice (Charles River, Criffa SA, Barcelona, Spain) weighing 28-30 g were used. Mice were housed in standard boxes with corncob bedding and free access to food and water. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Mice were immunosuppressed one day before to the infection by intraperitoneal (i.p.) administration of a single dose of 200 mg/kg of cyclophosphamide plus a single dose of 150 mg/kg of 5-fluorouracil given intravenously (i.v.) (Ortoneda et al 2004).

The efficacies of the different treatments were evaluated through prolongation of survival and fungal tissue burden reduction in spleen and kidney (Fernández Silva et al 2013b). Groups of 8 mice were established for the different studies. For the survival study, animals were challenged with 2×10^8 conidia in 0.2 ml injected into the lateral tail vein and checked daily for 30 days (Fernández Silva et al 2013b). Survivors and those mice meeting the criteria for discomfort were euthanized by CO_2 inhalation. For tissue burden determination mice were infected with $2x10^6$ conidia in 0.2 ml. This inoculum was chosen in order to avoid the fast killing obtained with the highest inoculum and to allow the fungal load of treated groups be compared with controls at same day (Fernández-Silva et al 2013b). The different groups were treated as follow: AMB at 0.8 mg/kg/day i.v. once daily (Graybill, 2000); PSC at 20 mg/kg orally (p.o.) twice a day (BID) by gavage (Rodriguez et al 2009), VRC at 40 mg/kg p.o. once daily (Rodriguez et al 2010) or

AFG at 10 mg/kg i.p. once daily (Calvo et al 2011). AFG plus PSC; AFG plus VRC and VRC plus AMB. The doses and the routes of administration used in the combined therapies were the same as in the monotherapies. From 3 days before the infection, the mice that received VRC were given grapefruit juice instead of water (Graybill et al 2003). All treatments were started 24 h after the challenge and lasted for 7 days. Animals were checked for survival twice daily for 30 days.

Fungal load was determined after 5 days of treatment in order to compare the results with the control group since mice controls start to die on that day. Then, animals were euthanized and kidneys and spleen were aseptically removed. Approximately half of each organ was weighed, mechanically homogenized in 1 ml of sterile saline and serially 10- fold diluted. Dilutions were plated on PDA and incubated at 25ºC for 7 days in order to determine the number of colony forming units (CFU) per gram. Mean survival time (MST) was estimated using the Kaplan-Meier method and compared among groups with the log rank test. Organ burden data was analysed using the Mann-Whitney U-test using GraphPad Prism 4 for Windows. P-values ≤0.05 were considered statistically significant.

Results

Table 1 shows the *in vitro* activity of the different drugs combinations assayed and their interactions against the three strains of *S. kiliense*. In general the FICI showed an indifferent effect for all the combinations and strains evaluated, with the exception of the UTHSC 03-3197 and UTHSC 01-2338 strains against which the combinations of AFG/PSC and AFG/VRC showed antagonism and synergy, respectively.

The results of the survival study are shown in Figure 1. In general almost all the treatments prolonged the survival with respect to control group, with the exception of VRC and AMB given alone. The combinations AFG/PSC and AFG/VRC improved the survival in comparison with their respective monotherapies for all the strains tested, with exception of strain UTHSC 03-3197, for which AFG/PSC combination works equal than its respective monotherapies.

The results of the tissue burden study are shown in Figure 2. The combined therapy of AFG/PSC reduced for two strains (UTHSC 03-3197 and UTHSC 07-550) the fungal load in kidney and spleen with respect to the control and the respective monotherapies and the other combinations, while that for the strain UTHSC 01-2238 the combination did not improve the results obtained with the monotherapies. The efficacy of combined therapy of AFG/VRC and AMB/VRC was modest in comparison to AFG/PSC combination; they were able to reduce significantly with respect to the control group and their respective monotherapies the fungal load only in spleen of those mice infected with strains UTHSC03-3197 and UTHSC 07-550.

Discussion

Nowadays the antifungal therapy for *Acremonium* infections has not been established yet. Lack of reliable sequences for identification of the species involved (Perdomo et al 2010) the scarce clinical reports (Khan et al 2011; Das et al 2010) as well as the few antifungal susceptibility studies (Das et al 2010; Perdomo et al 2011) are the main causes of that situation. Recently, *S. kiliense* has been reported as the most common species of *Sarocladium* (*Acremonium*) in clinical

samples (Perdomo et al 2011). Also recently experimental models determined the virulence (Fernández- Silva et al 2013b) and the efficacy of different antifungal therapies against different *Acremonium* or *Sarocladium* species (Fernández- Silva et al 2013a). Such studies demonstrated that *S. kiliense* is the most virulent species and PSC the most effective antifungal drug, better than VRC or AFG and AMB, which is currently the first therapeutic option in cases of disseminated infection by *S. kiliense* (Khan et al 2011). To our knowledge, this is the first study that has explored the *in vitro* activity and the *in vivo* efficacy of different antifungal combinations for the treatment of an experimental infection by *S. kiliense.* Here the interactions showed mostly an indifferent effect. However, against one strain (UTHSC 01-2238) the combination of AFG/VRC showed *in vitro* synergism. Synergisitic effect for this combination has been reported previously *in vitro* (Perkhofer et al 2008) as well as *in vivo* (Calvo et al 2011) for *Aspergillus* infections. However, our *in vivo* results showed that there is not correlation between the synergistic effects *in vitro* with the efficacy *in vivo*, since this combination did not improve the result from the monotherapies improving the survival time or reducing the fungal load. Similar results were obtained with the combination AMB/VRC. Although only a reduced number of strains were tested the study shows the poor predictive value of the in vitro drug combinations. In our study the combination AFG/PSC showed the higher FICI which were indicative of indifference or antagonism displayed the best results, it works better than the respective monotherapies; prolonging the survival of the mice infected by each of the three strains evaluated and reduced the fungal load in the organs tested. The

promising results obtained from AFG plus PSC combination provide relevant data for the treatment of this refractory infection.

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Figure 1.Cumulative mortality of mice infected with 2x108 conidia/animal of three strains of Sarocladium (Acremonium) kiliense, UTHSC 01-2238 (A), UTHSC 03-3197 (B) or UTHSC 07-550(C). [a]P< 0.05 versus control; [b]P< 0.05 versus amphotericin B (AMB); [c]P< 0.05 versus voriconazole (VRC); [d]P< 0.05 versus posaconazole(PSC).

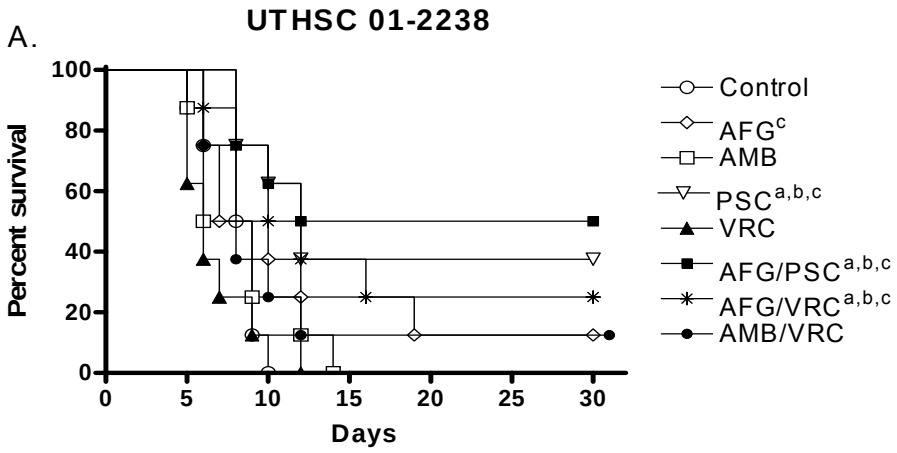
Figure 2. Effects of the antifungal treatments on colony counts in mice infected with $2x10^6$ conidia/animal of three strains of *Sarocladium kiliense,* UTHSC 01-2238, UTHSC 03-3197 or UTHSC 07-550, in spleen and kidneys of mice. Amphotericin B (AMB) at 0.8 mg/kg; posaconazole (PSC) at 20 mg/kg twice a day; voriconazole (VRC) at 40 mg/kg; anidulafungin (AFG) at 10 mg/kg. [a]P value< 0.05 versus the control; [b]P value <0.05 versus AFG; [c]P value <0.05 versus AMB; [d]P value <0.05 versus PSC; [e]P value <0.05 versus VRC; [f]P value <0.05 versus AFG/VRC; [g]P value <0.05 versus AMB/VRC. Horizontal lines indicate mean values.

Table 1. In vitro antifungal activity of antifungal drugs and their interactions against three clinical isolates of *Sarocladium* (*Acremonium*) *kiliense.*

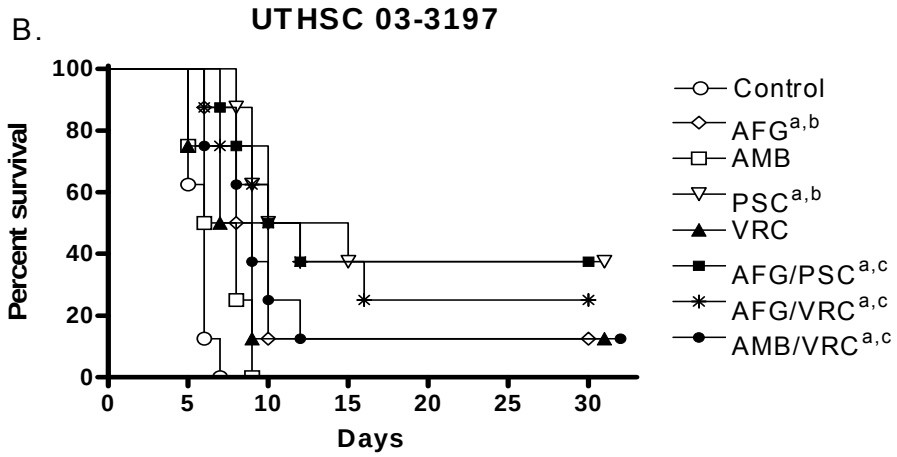
Antifungal drug	**MIC/ MEC(µg/ml)[FICI][a]**		
	UTHSC 01-2238	**UTHSC 03-3197**	**UTHSC 07-550**
AMB	8	4	4
AFG	8	4	16
PSC	16	1	1
VRC	2	4	16
AFG/PSC	8/8[2]	8/8[5]	8/8[2]
AFG/VRC	1/0.5[0.37]	2/0.25[1.25]	4/0.25[0.75]
AMB/VRC	8/8[2]	4/4[2]	4/4[2]

[a]FICI, fractional inhibitory concentration index ≤0.5, synergistic; >0.5to <4 , indifferent; ≥4, anatagonistic

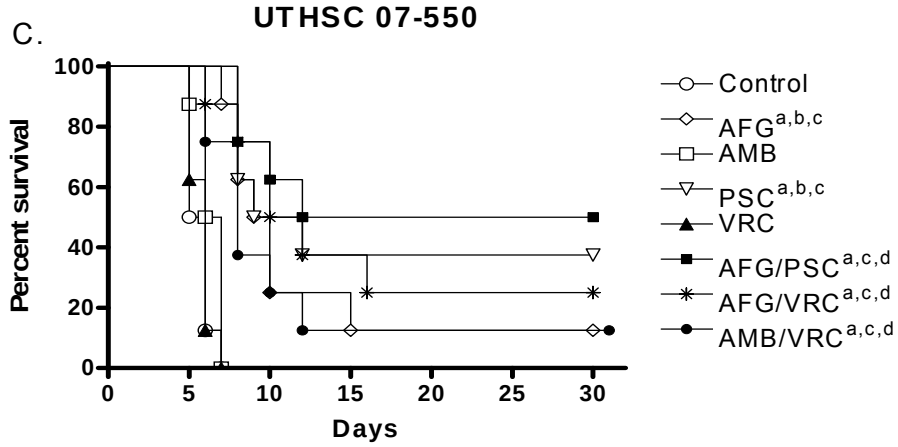
A.

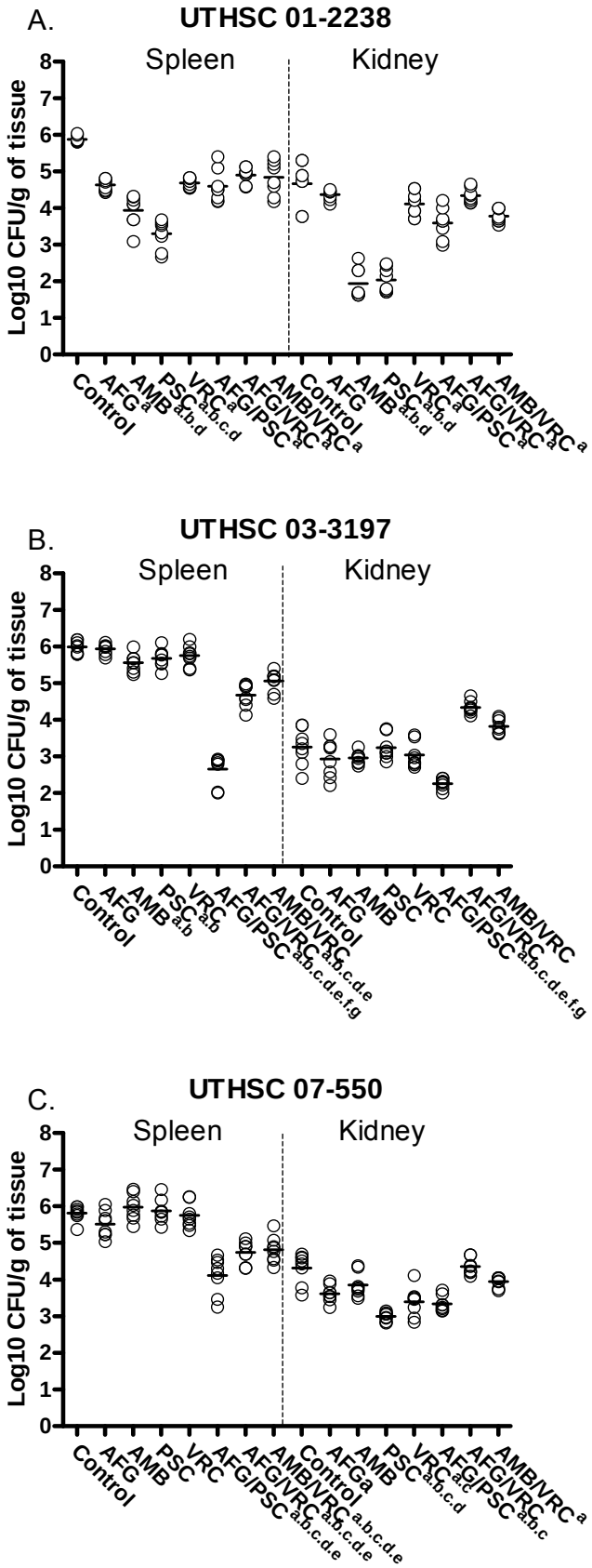


B.



C.





4.8. Azole–echinocandin combination as a potential therapeutic option against scedosporiosis: an *in vitro* and murine-model study.

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(In preparation)

Azole-echinocandin combination as a potential therapeutic option against scedosporiosis*:* an *in vitro* and murine-model study.

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Keywords: *Scedosporium*, antifungal combination therapy, fungal infection,

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Abstract

The *in vitro* activity and the *in vivo* efficacy of the combination of micafungin (MFG) with posaconazole (PSC) or voriconazole (VRC) were evaluated against strains of *P. boydii* and *P. apiosperma*, two of the most common species belonging to former *P. boydii* complex. For the *in vitro* study two methods were evaluated E-test® and checkerboard method based on the document from the CLSI M38-A28. The *in vivo* efficacy was evaluated in a murine model of disseminated infection. Mostly of the *in vitro* interactions resulted in an indifferent effect; however, some strains showed antagonism and synergism. Both combinations were able to prolong the survival for species evaluated, being better than their respective monotherapies. The combination of VRC plus MFG showed the best results for *P. boydii*, being able to reduce the fungal load in brain and kidney better than the respective monotherapies. In contrast, for *P.apiosperma* none of both combinations were able to improve the results obtained with the monotherapies.

Introduction

Pseudallescheria boydii sensu lato is a complex of species, beside *S. dehoogii* that have been reported to cause invasive infections in human, particularly in immunocompromised patients, which have increased in the last few decades (Cortez et al., 2008, Guarro et al., 2006). Currently, there have been numerous taxonomical changes within these groups, several new species being reported (Gilgado et al 2008, Harun et al 2009). The most clinical relevant species of the complex are *P. boydii, P. apiospermum* and *Scedosporium aurantiacum* (Gilgado et al 2009, Harun et al 2009), but the real incidence and the clinical significance of these organisms in human infections is still under investigation (Cortez et al., 08, Guarro et al.,06). Members of the *P. boydii* sensu lato complex were described as intrinsic resistant towards most available systemic antifungal compounds, with the exception of voriconazole (VRC) and in lesser extent to posaconazole (PSC) and micafungin (MFG), which have show *in vitro* activity at least against part of the *Pseudallescheria*/*Scedosporium* population (Lackner et al., 2012). The increasing frequency and high mortality rates in invasive infection caused by these fungi required a new therapeutic approach and combined therapy could be helpful, in particular for patients with: impaired immunity, therapeutic failures, or relapses. Therefore, the aim of this study was to evaluate the *in vitro* activity and *in vivo* efficacy of VRC in combination with MFG and PSC in combination with MFG.

Material and Methods

Strain set. The tested strains set of this study included 43 strains associated to the *P. boydii sensu lato* complex*;* out of those 17 were identified as *P. boydii* and 26 as *P. apiosperma.* Strains were identified to species level in a previous study (Lackner et al., 2012) using amplified fragment length polymorphism (AFLP).

In vitro. For susceptibility testing, strains were cultured on potato dextrose agar (PDA) for 10 days at 30ºC. To evaluate the *in vitro* activity of azole-echinocandin combinations, a two-dimensional checkerboard method based on the document from the CLSI M38-A28 (CLSI 2008) and modified by Dannaoui et al. (Dannaoui et al., 2004) and E-test®, was applied. The minimal inhibitory concentration (MIC) for azoles and minimal effective concentrations (MEC) for MFG were determined according to CLSI broth microdilution methods (CLSI 2008). For combinations the endpoint was calculated by taking into account both the MEC of the echinocandin and the MIC-2 (definition: ~50% reduction in turbidity compared to the growth control) for azoles, which was found to correlate with the MEC (Calvo et al., 2012). In the case of E-test® (company, Country), the MIC was determined following the manufacture´s instructions. To both methods the fractionary inhibitory concentration index (FICi) was calculated as followed: $\sum$ FICI=(A/MIC_A+ B/MIC_B),where A and B are the concentrations of the drugs A and B in combination and MIC_A and MIC_B are the MICs of the drugs A and B alone. Drugs interactions were classified based on the FICI as synergistic when FICI was ≤0.5, as antagonistic when FICI was >4.0 and no interaction, when FICI was >0.5 but <4 (Dannaoui et al 2004, Johnson et al 2004).

To determined the method that are most sufficient to evaluated the in *vitro* interaction of azoles and echinocandins, both methods were applied to a randomly chosen pilot strain set (n=9). Both assays were evaluated in respect to influence of operator (objectivity) and number of unreadable or unclear results.

In vivo. To evaluate the efficacy of the echinocandin-azole combination *in vivo* a total of four representative strains (two per species) were selected based on their *in vitro* FICI index. The inocula were prepared flooding the surface of the agar plate with sterile saline solution, scraping the sporulating mycelium with a culture loop, and drawing up the resultant suspension with a sterile Pasteur pipette. The conidial suspensions were filtered twice through several layers of sterile gauze to remove large clumps and hyphal fragments and the resulting pellets were suspended in sterile saline and the inocula were adjusted to the desired concentration by haemocytometer counts and by serial plating on PDA in aim to confirm viability.

Four-weeks-old OF-1 male mice (Charles River, Criffa SA, Barcelona, Spain) with a bodyweight between 28.0 g – 30.0 g were used as model organisms. Animals were housed in standard boxes with corncob bedding and free access to food and water. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

The in vivo set up was as follows: 3 days (day -3) prior to infection mice within the VRC treatment groups received grapefruit juice instead of water (Graybill et al., 2001) for drinking, all mice were immunosuppressed one day before to the infection (day-1) via intraperitoneally (i.p.) administration of a single dose of 200 mg/kg of cyclophosphamide plus a single dose of 150 mg/kg of 5-fluorouracil given intravenously (i.v.)(Ortoneda et al., 2006), animals were infected at day 0 with 5.0×10^3 conidia/mouse via lateral tail vein injection, all treatments started at day +1 lasted for until day +11. The mice were chosen randomly for the different treatment groups. The group (8 mice per group) set up was as follows: MFG (Mycamine) at 10 mg/kg i.p. once daily, PSC (as Noxafil) at 20 mg/kg orally (p.o) by gavage twice a day; VRC (Vfend) at 40 mg/kg p.o by gavage once daily (Rodriguez et al., 2010); MFG plus PSC and MFG plus VRC. The doses and the routes of administration used in the combined therapies were the same as in the mono-therapies. For the survival study the mice were checked

daily for a total of 30 days. Survivors and animals meeting the criteria for discomfort were euthanized by CO_2 inhalation Fungal load was determined at day +6 in order to compare the results with the control group; as control mice start to die on day +6. Kidneys and brain were aseptically removed. Approximately half of each organ was weighed, mechanically homogenized in 1 mL of sterile saline and serially 10- fold diluted. Dilutions were plated on PDA and incubated at 25ºC for 7 days in order to determine the number of colony forming units (CFU) per gram of organ.

The efficacies of the different treatments were evaluated through prolongation of survival and fungal tissue burden reduction in brain and kidney of infected mice (Rodriguez et al., 2010).

Statistical analyses. Mean survival time (MST) was estimated using the Kaplan-Meier method and compared among groups with the log rank test. Organ burden data was analysed using the Mann-Whitney U-test using GraphPad Prism 4 for Windows.

Results

Pilot experiment. For reasons of practicability and objectivity checkboard broth microdilution was used, as E-test® determination of MEC for MFG was highly operator dependent and often bot clearly readable (data not show).

In vitro. Most strains (88 and 90%) had indifferent effect (FICI between 0.54-2.0 and 0.51-2.24) for both combinations (VRC/MFG and PSC/MFG, respectively). No significant difference was observed between the species *P. apiosperma* and *P. boydii*, for details see Table 1. However, for some strains the combination of VRC/MFG and/or PSC/MFG the combination had a synergistic effect and for individual strains antagonism was detected.

In vivo. To determine the efficacy of the combinations, and the correlation between the *in vitro* and *in vivo* results two strain of each species with different FICI were evaluated. For *P. apiosperma* strain FMR 13015 and CBS 117419 with FICI values to VRC/MFG

and PSC/MFG of 0.5/0.019 and 3/2.24, respectively, were evaluated and for *P. boydii* strain FMR 12741 and FMR 13004 with FICI values to VRC/MFG and PSC/MFG of 0.48/0.37 and 8.24/0.74, respectively.

Significant ($P<0.0322$) differences between the survival groups in respect to the control group were found for both combination therapies (PSC/MFG, VRC/MFG) and the azole monotherapies, but not for MFG, independent from the FICI of the challenging strain. The combination treatment VRC/MFG significantly ($P<0.0276$) prolong the survival in respect to MFG monotherapy for *P. boydii* y *P. apiosperma* strains independent from the FICI of the strain. Moreover VRC as monotherapy and its combination are better prolonging the survival than PSC or MFG monotherapy, independent from the strain and the species. Detailed data on survival results are given in Figure 1.

The second *in vivo* design that was chosen to evaluate the efficacy of the azole-echinocandin combinations *in vivo* was the fungal load in brain and kidneys (Figure 2).

The results from the survival study were found in both concordance with those found for fungal burden. For *P. boydii* strains the combination of VRC/MFG works significantly ($P<0.0281$) better than the monotherapies independent from the FICI of challenging strains for reducing the fungal load in kidney and brain. MFG was not able to reduce the fungal load neither the kidneys nor in the brain did not show a significant reduction in tissue burden with respect to control group. However, in combination they show a synergistic effect reducing the tissue burden significantly, in particular for strain FMR 12741. For *P. apiosperma* all treatments with exception of MFG were able to reduce the fungal load in both kidney and brain in comparison to the control group; however the combinations were not superior in respect to the monotherapies.

Even though *in vitro* results showed an antagonistic effect for *P. boydii* strain FMR 13004, there was no correlation *in vitro-in vivo*. No antagonistic effect was observed neither in survival time nor in fungal load reduction.

Discussion

We have evaluated the *in vitro* activity and the *in vivo* efficacy of the combination of VRC or PSC with MFG in a murine model of disseminated infection by *P. boydii* and *P. apiosperma,* the most prevalent species of *P. boydii sensu lato* complex. Both species were found to have similar susceptibility profile with the lowest MICs/MECs for VRC and MFG (Lackner et al. 2012). For PSC, part of the *P. apiosperma* population was found to have a reduced susceptibility and is therefore less susceptible than *P. boydii.*

Several studies *in vitro* have been carried out in order to determine the best antifungal drugs. VRC has showed until now the best activity *in vitro* with favourable outcome in mostly of the cases reported (Gilgado et al., 2006, Alastruey-Izquierdo et al., 2007). In addition combination studies have been performed (Cuenca-Estrella 2008). However, in cases of severe infections with CNS involvement, failure therapeutic has been reported. In addition scarce clinical data exist on the efficacy of other antifungal drugs. To our knowledge this is the first study that evaluated the *in vivo* efficacy of azole-MFG combination for *Ps. apiosperma* and *Ps.boydii*. Previously that combination was evaluated for *Scedosporium prolificans* the most resistant fungus related to this complex (Rodriguez et al., 2009). The combination MFG plus VRC worked better than the respective monotherapies. Similar results were obtained in this study for *Ps. boydii* strains, particularly for strain FMR 13004, against which the interaction *in vitro* showed an antagonistic effect. However, for *Ps apiosperma* none of the combinations improved the result of the monotherapies. One of the most important finding in this study was we did not observe antagonism in vivo, in spite that the in vitro activity showed a strong antagonistic effect of the VRC/MFG combination for strain FMR 13004. Our results are promising, however more studies are required .

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Table 1. Percent of *Pseudallescheria boydii* and *Pseudallescheria apiosperma* strains showing different effect against the interaction between voriconazole plus micafungin (VRC/MFG)and posaconazole plus micafungin (PSC/MFG).

Species(Nº strain)	VRC/MFG			PSC/MFG		
	Synergism	Indifference	Antagonism	Synergism	Indifference	Antagonism
P. boydii (17)	5.88	88.24	5.88	11.76	82.35	5.88
P. apiosperma (26)	11.54	84.62	3.85	7.69	92.31	0

Table 2. Strains of *Pseudallescheria boydii* and *Pseudallescheria apiosperma* selected for the in vivo study of efficacy of antifungal drugs alone and in combination and their Fractionary inhibitory concentration index (FICI).

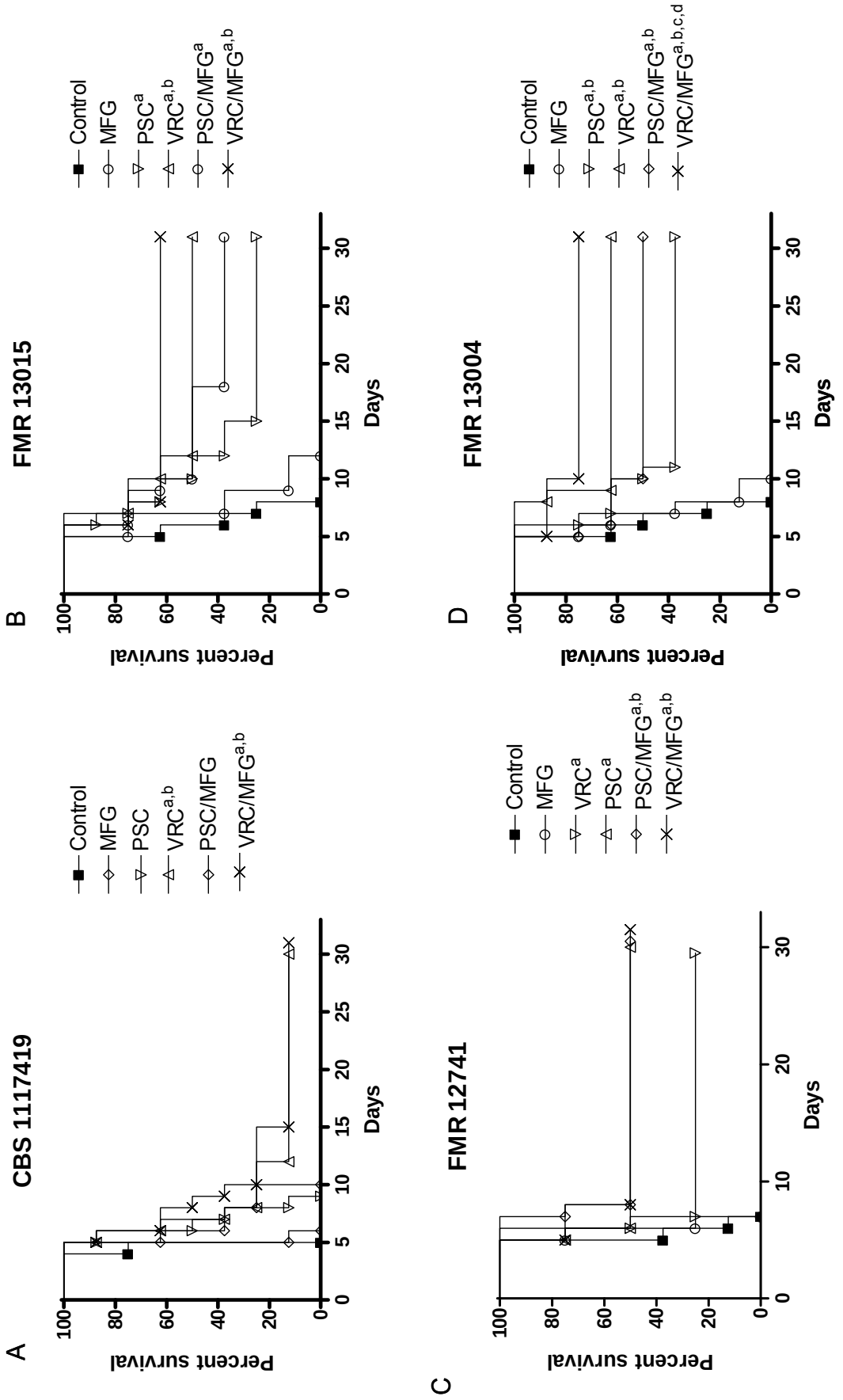
Species	**Strains**	**FICI[a]**	
		PSC/MFG[b]	**VRC/MFG[b]**
P. boydii	FMR 12741	0.37	0.48
	FMR 13004	0.74	8.24
P. apiosperma	CBS117419	0.019	0.5
	FMR 13015	2.24	3

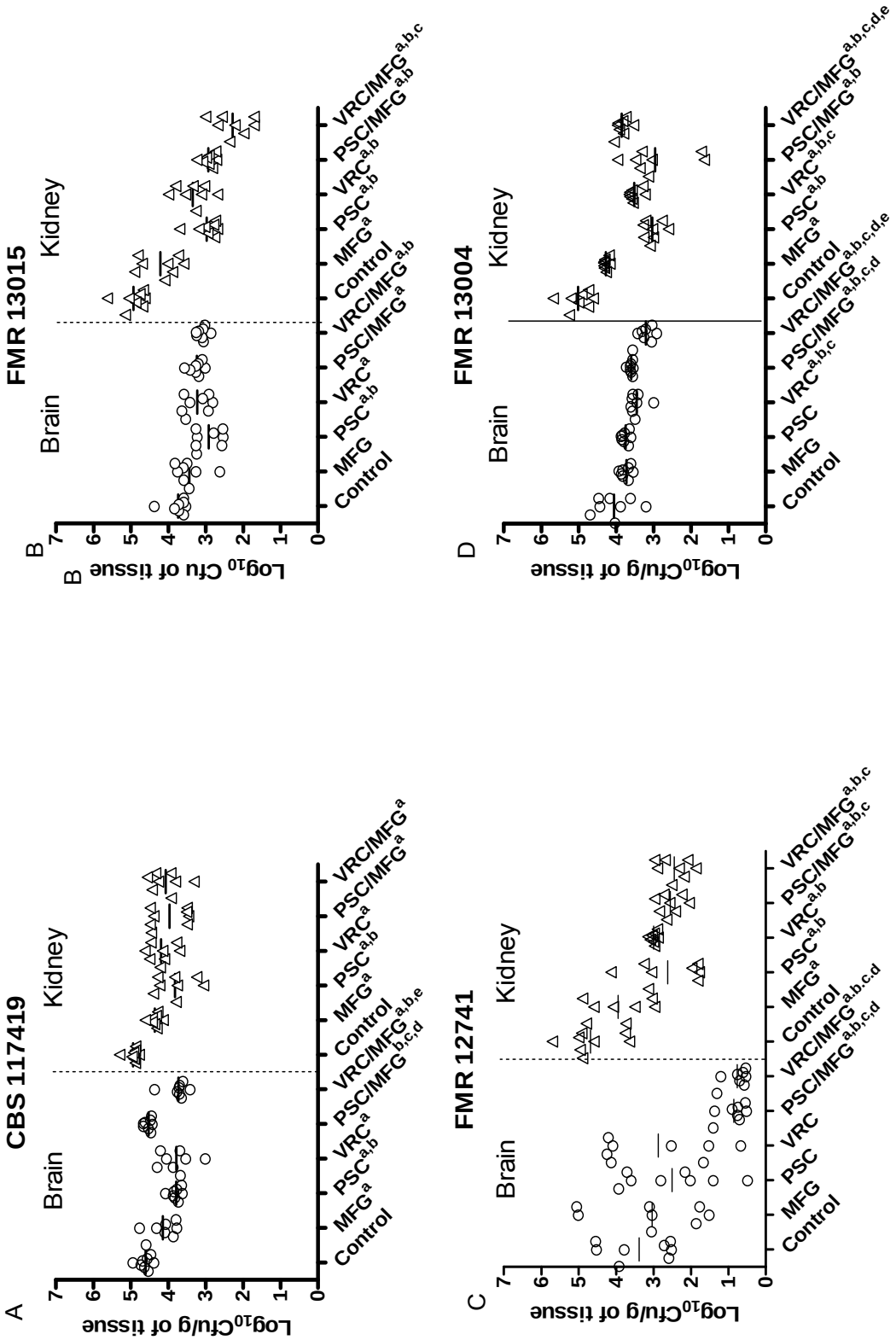
[a,] FICI, fractionary inhibitory concentration index, ≤0.5,synergistic; >0.5 to ≤4, indifferent;>4, antagonism.

[b,] MEC was calculated for MFG and MIC-2 (~50% reduction in turbidity compared to the growth control) were calculated for azoles.

Figure 1. Cumulative mortality of mice infected with $5x10^3$ conidia/animal of *Pseudallescheria apiosperma* (A-B) or *Pseudallescheria boydii* (C-D). $^{a}P< 0.05$ versus control; $^{b}P< 005$ versus micafungin (MFG); $^{c}P< 0.05$ versus posaconazole(PSC), $^{d}P< 0.05$ versus voriconazole (VRC), $^{e}P< 0.05$ versus posaconazole plus micafungin combinations (PSC/MFG).

Figure 2. Effects of the antifungal treatments on colony counts brain and kidneys of mice infected with $5x10^3$ conidia/animal of *Pseudallescheria apiosperma* (A-B) *or Pseudallescheria boydii* (C-D). Micafungin (MFG) 10 mg/kg; posaconazole (PSC) at 20 mg/kg twice a day; voriconazole (VRC) at 40 mg/kg; voriconazole plus micafungin (VRC/MFG) at 40mg/kg and 10mg/kg, respectively and posaconazole plus micafungin (PSC/MFG) at 20 mg/kg twice a day and 10mg/kg, respectively. $^{a}P< 0.05$ versus control; $^{b}P< 0.05$ versus MFG; $^{c}P< 0.05$ versus PSC, $^{d}P< 0.05$ versus VRC, $^{e}P< 0.05$ versus PSC/MFG.





4.9. Evaluation of correlation of *in vitro* antifungal susceptibility and presence of FKS mutations with *in vivo* response to caspofungin in a murine infection by *Candida glabrata*.

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(In preparation)

Evaluation of correlation of *in vitro* antifungal susceptibility and presence of *FKS* mutations with *in vivo* response to caspofungin in a murine infection by *Candida glabrata.*

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Key word: Candida glabrata, caspofungin, animal model, epidemiological cut-off value

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UNIVERSITAT ROVIRA I VIRGILI
EVALUATION OF VIRULENCE AND NEW EXPERIMENTAL THERAPEUTIC STRATEGIES FOR EMERGING AND UNCOMMON MEDICALLY IMPORTANT FUNGAL PATHOGENS
Fabiola Vanessa Fernández Silva
Dipòsit Legal: T.1329-2013

Abstract

We evaluated the *in vivo* efficacy of caspofungin against a large panel of C*andida glabrata* strains with MICs ranges between 0.03 µg/mL to >16 µg/mL. In addition the presence or abscence of *FKS* mutations were determined by sequencing the hot spot 1(HS) of *fks*1 and *fks*2 gene.

Three new mutations were detected. Two mutations were placed in the *fks*1 (n= 2; L737S, S624F) and one was located in the *fks*2 gene (n =1; S707L). Caspofungin at a dose of 1.0 mg/kg was active in all animals that were infected with strains that carried a MIC ≤ 0.5µ g/ml and in some animals that were infected with strains with a MIC of 1µ g/ml. In contrast, animal infected with strains with MICs > 1.0µ g/ml did not respond to caspofungin treatment.

Our results demonstrate *FKS* mutations are present in strain that have lower MICs for caspofungin than the epidemiological cutoff value (ECV), but these mutations have no influence on the in vivo efficacy of the drug. In vitro MIC values correlate better with the in vivo outcome than the presence or absence of *FKS* mutations.

1. Introduction

Candida glabrata is an important agent of invasive candidiasis (IC) and in frequency the second most prevalent after *C. albicans* (Pfaller 2007, Arendrup 2013). Voriconazole and the lipid amphotericin B can be used for the treatment of IC, but for *Candida glabrata* with decreased azoles-susceptibility echinocandins (caspofungin, micafungin, and anidulafungin) are the preferred front line therapy (Pappas 2009, Aguado 2009, Ullman 2013).

Caspofungin (CSP) has been used successfully in the treatment of oesophageal candidiasis and IC (including candidemia), even in patients with previous antifungal drug exposure (Pappas2009, Aguado 2010, Denning 2003). Although *in vitro* caspofungin-resistance among *C. glabrata* strains is rare, infections with poor or no response to treatment have been reported (Zaas 2006, Katiyar 2006, Cleary 2008, Thompson 2008, Lortholary 2011, Pfaller 2011).Strains causing therapeutic failures were associated with the presence of mutations in two hot spot (HS) regions of the *FKS* genes. These genes encode the major subunit of the 1,3-β-D-glucan synthase complex which produces a major cell wall component (Denning 2003, Perlin 2007).

For avoiding clinical failure, the following variables: epidemiological cut-off values (ECVs) and clinical interpretative breakpoints (CBP) were established based on clinical, molecular, and microbiological data (Pfaller 2011). The proposed ECV of CSP for *C. glabrata* is 0.12 µg/ml, while the CBP is set at ≤ 0.12 µg/ml for susceptible isolates and at ≥ 0.5 µg/ml for resistant isolates (Pfaller 2011).

The aims of this study were to evaluate the proposed CSP using an invasive murine model and whether MICs of caspofungin and presence or absence of *FKS* mutations in clinical isolates of *C. glabrata* are predictive of *in vivo* outcome.

2. Material and Methods

Strains. Clinical *C. glabrata* strains (N = 17) representing a wide MICs range (0.06 µg/ml to >16 µg/ml) of CSP were included in the study (Table 1). MIC values were determined using a microdilution approach according to the CLSI standards (CLSI 2008).

DNA sequence analysis of *FKS* genes

C. glabrata strains were grown at 37°C overnight on Sabouraud dextrose agar (SDA). DNA was extracted and purified as previously described (Lackner 2012). The hot spot 1 (HS-1) of the fks1 and fks2 genes were amplified and sequenced using previously described primers to detect possible mutations (Castanheira 2010). The sequence quality was checked, the alignment were made and mutations detected using the BioNumerics Software V 6.6. Translation of nucleic acid sequence into amino acid sequence was performed using EBI Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) and amino acid alignments were made using ClustalW2 too (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Animals. Male OF1 mice (Charles River, Criffa S.A., Barcelona, Spain) weighing with a weight of 30 g were used. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Mice were housed under standard conditions and immunosuppressed one day before the infection by a single 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) (Ortoneda 2006).

Infection. All isolates were grown on SDA for 48 hours. Then cultures were suspended in sterile saline and adjusted to the desired concentration by haemocytometer counts and serial plating on SDA to confirm viability. Mice were

infected with 2 x 10^8 colony forming units (CFU) of each strain suspended in in 0.2 ml of sterile saline and injected via the lateral tail vein.

Treatment. CSP (Cancidas, Merck and Co., Inc., Whitehouse Station, N.J, USA) was administered at 1 mg/kg/d i.p., based on previous pharmacokinetic studies (Arendrup 2012, Howard 2012). The treatment was started 24 h after infection and lasted for seven days. All animals received 5 mg/kg/d of ceftazidime subcutaneously to prevent bacterial infection. The therapy efficacy was evaluated through prolonging survival time and fungal tissue burden reduction. For the survival studies, groups of six mice were randomly established for each strain and checked daily for 30 days after infection. For the tissue burden studies, groups of six mice were used and the animals were sacrificed five days post infection; in order to compare the results with the control group**.** Kidneys were aseptically removed, weighed and mechanically homogenized in 1.0 mL of sterile saline**.** Serial 10-fold dilutions of the homogenates were placed on SDA and incubated for 48 h at 35 ºC to determine CFUs per gram of tissue.

Statistics. Mean survival time was estimated by the Kaplan-Meier method and compared among groups using the Log Rank test. Colony counts in kidneys were analyzed using the Mann-Whitney U test. A P value ≤ 0.05 was considered statistically significant.

Results and Discussion

The MICs of tested strains and *FKS* mutations detected are shown in Table 1. Only three of the tested strains (FMR 11381, UTHSC 11-68, and UTHSC 10461) were wild type (i.e. absence of SNPs in *FKS* gene). However, two strains (UTHSC 11-68 and UTHSC 10461) showed MIC >ECV. Two mutations in the *fks*1 (L737S and S624F) and one in the *fks*2 (S707L) genes, which had not been previously reported were

detected. In addition, a nucleotide insertion in *fks*1 in the strain FMR 8498 was also shown. Surprinsingly, these new mutations were exclusively found in strains with MIC ≤ 0.5 µg/ml (Table 1).

All strains were found virulent as the caused acute infection with mortality rates of 60% to100% (data no shown). CSP could not prolong the survival time of animals challenged strains that expressed in vitro a MIC ≥ 1 µg/ml (n =9); but prolonged the survival of mice infected strains infected with MIC ≤ 0.5 µg/ml in 50 % of all strains (Table 1).

Fungal load data are shown in Table 1. The strains were distributed in three groups, i.e. eight strains (MIC< 1 µg/ml), which responded to CSP treatment in vivo as tissue burden in infected mice was significantly reduct; four strains (MIC> 1 µg/ml) were refractory to caspofungin as tissue burden in infected mice was not reduced and five strains (MIC=1 µg/ml) showed variable responce towards CSP treatment as fungal burden was only reduced in animals infected with the strains JMI 297 and JMI 2092, but not for mice challenged with JMI 206, JMI 211 and JMI 760.

In both survival and tissue burden studies the presence of newly described *FKS* mutations did not influence the response to the treatment with CSP.

Studies attempting to correlate CSP susceptibility, *FKS* mutations, and CBP with the clinical outcomes of IC by *C. glabrata* are scarce and have yielded conflicting results (Shields 2012, Lepak 2012). Shields et al (2012) demonstrated that the presence of *FKS* mutations has a higher predictive value for echinocandin treatment failure than the *in vitro* determined MICs. In contrast, Lepak *et al*., demonstrated using a murine model of invasive *Candida glabrata* infection that the CSP efficacy was closely linked to *in vitro* MIC rather than to the presence of *FKS* mutations (Lepak et al., 2012). Our results agree with the latter since the presence of *FKS* mutation did not exclusively influence the response to CSP treatment. In strains with MICs< 1µg/ml, CSP reduced

tissues burden regardless of the presence or absence of SNPs in the *FKS* gene. But, for strains with MICs ≥ 1 µg/mL no relationship between CSP therapeutic failure and the presence or absence of SNPs in *FKS* were found.

Based on survival time and tissue burden of infected and CSP treated mice, MICs ≤ 0.5 µg/mL were predictive for the in vivo therapeutic response. In contrast all animals infected with strains with a MICs ≥2 µg/ml did not response to the CSP treatment. For mice infected with strains with MICs of 1 µg/ml the *in vivo* response was variable. These findings agree with those reported by Lepak *et al.* (Lepak 2012). Although studies with more strains are needed, our data suggest that the current CBP for CSP and *C. glabrata* (≤ 0.12µg/ml) is set too low to discriminate susceptible from resistant isolates. Three new mutations in the *FKS* were detected, located in *fks*1 (L737S and S624F) and *fks2* (S707L) that have no influence on the CSP resistance. Whether or not these mutations result in anidulafungin or micafungin resistance remain to be evaluated.

The authors are aware that, apart from microbiologic resistance, many other factors such as host immune status, underlying diseases, intravenous catheter removal, adjunctive surgical interventions, and pharmacokinetic parameters also play significant roles in the response to antifungal therapy among patients with IC (Rex 2002) and therefore murine models cannot one to one to predict the patients *in vivo* response

One limitation of our study is that CSP susceptibility testing shows significant interlaboratory variations. An extraordinary amount of wide MIC ranges, truncated and bimodal MIC distributions prevented EUCAST to set up interpretative cutoffs for this agent (Arendrup 2012a, Arendrup 2012b). For *C. glabrata* wilde type MICs ranged from 0.016 µg/ml – 2.0 µg/ml. Many factors such as CSP powder source, stock solutions solvent, powder storage time, length and temperature, and MIC

determination testing parameters, may be the cause of such variability. For this reason EUCAST has only established breakpoints for the other two candins anidulafungin and micafungin, but not for CSP. It is expected that CLSI will reach similar conclusions (Espingel-Ingroff et al. 2013).

In conclusion, our results demonstrate that CSPs MIC correlate better with therapeutic outcome than with the presence of *FKS* mutations and suggest that CBP and ECV of CSP against *C. glabrata* are set too low.

Table 1 Isolates of *Candida glabrata*, in vitro activity of Caspofungin, mutations on *FKS* gene(new mutations are given in bold letters) and mean survival time (MST) and fungal load on kidney.

Strains	CSP MIC (µg /ml)	Mutation		Category[a]	Reducing fungal load[b]	MST[d] (95%CI)		Mean (±standard deviation) Log 10 CFU/gr of tissue	
		FKS1	FKS2			Controls	Treated group	Control	Treated group
FMR 11381	0,06			S	+	18.1 (4.56-31.78)	22.17 (9.43-34.91)	6.367(±0.333)	5.397±0.227[c]
UTHSC 08-134		**L737S**		S	+	10.5 (0.42-20.58)	18.67 (5.63-31.70)	4.762±0.226	1.623±0.110[c]
FMR 8489	0.12	**S624F**		S	+	18.1 (4.56-31.78)	30.00 (30.00-30.00)[c]	8.318±0.393	6.005±0.262[c]
FMR 8498		**NA-INSERT**		S	+	18.5 (5.26-31.70)	19.00 (6.34-31.66)	6.968±0.567	4.03±0.549[c]
UTHSC 11-149	0.25		**S707L**	I	+	13.8 (0.67-27.00)	30.00 (30.00-30.00)[c]	6.827±0.371	5.685±0.101[c]
UTHSC 11-68				I	+	10.6 (0.67-20.66)	25.17 (17.30-33.03)[c]	7.018±0.383	5.712±0.156[c]
UTHSC 073662	0.5		**S707L**	R	+	14.0 (0.97-27.02)	30.00 (30.00-30.00)[c]	7.427±0.548	4.732±0.304[c]
UTHSC 10461				R	+	17.6 (3.48-31.85)	18.5 (5.263-31.74)	6.377±0.368	5.152±0.076[c]
JMI-206	1		F659S	R	-	16.3 (4.87-27.80)	23.00 (11.62-37.38)	7.174±0.094	7.044±0.416
JMI-211			S663P	R	-	7.1 (5.02-9.30)	13.83 (0.66-27.00)	6.711±0.587	6.391±0.179
JMI-297		F629P		R	+	15.0 (2.71-27.29)	21.83 (8.55-35.11	5.436±0.269	3.558±0.061[c]
JMI-760			F663P	R	-	8.0 (6.67-9.33)	16.00 (4.14-27.85)	6.706±0.539	6.782±0.364
JMI-2092		L630I		R	+	15.6 (3.85-27.48)	22.17 (9.43-34.91)	4.955±0.656	3.665±0.136[c]
JMI-10956	2		F659V	R	-	18.3 (4.85-31.82)	19.67 (7.78-31.55)	5.34±0.155	4.882±0.340
JMI-14378	4	S629P		R	-	7.5 (5.53-9.46)	12.00 (9.79-14.20)	7.669±0.428	7.046±0.546
JMI-127	16	S629P		R	-	14.8 (2.32-27.35)	7.16 (5.62-8.71)	5.00±0.528	5.587±0.387
JMI-729	>16		F663P	R	-	6.66 (4.95-8.38)	9.33 (8.47-10.19)	5.599±0.170	5.381±0.171

[a], Susceptibility categorización according to CBP(Pfaller, 2011)
[b], Efficacy on reducing the fungal load with respect to control group.
[c], *P* value < 0.05.
[d], MST, mean survival time in days

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V. Discussion

5.1 *Sporothrix.*

Until 2006, *S. schenckii* was considered a well-defined species causing most of the subcutaneous chronic disease, but based on molecular studies Marimón *et al*. concluded that *S. schenckii* is a complex of species which includes *S. brasiliensis*, *S. mexicana*, *S. globosa* and *S. schenckii* sensu stricto (Marimón *et al*., 2007). In this sense, molecular studies performed by Marimón *et al* (Marimón *et al*., 2008a) showed that although, until 2008, *S. luriei* used to be considered a variety of *S. schenckii,* in reality this species did not belong into the *S. schenckii* complex. In fact, *S. luriei* has rarely been reported in human infections, although it is likely that these species were involved in more cases than those reported, but identification to species level have been underestimated the presence of this species due to the morphological similarity between species. We evaluated the virulence of *S. luriei* in order to assess its potential pathogenicity. Our study revealed that *S. luriei* is able to produce systemic spread in immunosuppressed mice causing a high mortality. The high fungal load reported, particularly in liver and spleen, where a massive infiltration of fungal cells without inflammatory response was found, demonstrated high *in vivo* proliferation of *S. luriei* and tissue tropism, both similar to *S. brasiliensis* - the most virulent species of the complex. These results highlighted the importance of correct identification since we have determined that *S. luriei* is a species with a high virulence and could be the causative agent of disseminated infections.

The identification of *Sporothrix* at species level has also an interest for the management of the disease. The new designed species not only differs in terms of virulence, but they also show different sensibility to antifungal agents

(Marimón *et al*., 2008b). This has helped to explain the variability in the susceptibility reported prior to the reclassification of *Sporothrix schenckii* as a complex. Currently, there is a guideline for the management of the sporotrichosis which includes AMB and itraconazole in those cases of disseminated and systemic sporotrichosis (Kaufman *et al*., 2007). While cases of systemic sporotrichosis are fortunately not frequent, its treatment requires long-term therapy increasing the risk for the development of toxicity associated to the antifungal therapy.

Although VRC has shown efficacy in the treatment of invasive infections by other opportunistic fungi such as *Aspergillus*, little or no clinical experience has been reported about its efficacy against sporothrichosis. Unfortunately, the vast majority of *in vitro* studies and case reports were made prior to the reclassification of *Sporothrix* as a species complex being difficult to know the activity of the antifungal agents by species. However, at genus levels, the *in vitro* assays have demonstrated poor activity of VRC (Espinel-Ingroff *et al*., 2001; Marimón *et al*., 2008). To our knowledge this is the first assay of the efficacy of VRC in an animal model of disseminated sporothrichosis. Although the strains tested in this study showed MIC> to 2 µg/ml, being considered resistant, we could observe a dose-response effect dependant of species. VRC was modestly active against *S. schenckii* increasing the survival and reducing the fungal loads of mice especially when administered at high doses such as 40mg/kg. Surprisingly, the drug showed no efficacy against the *S. brasiliensis* infection and not advantage over the untreated animals was found. Furthermore, by bioassay studies we determined that the VRC level reached were above MIC values of the tested strains in both serum and organs.

Although it has been recently described that the MICs for VRC could be predictive for effective treatment, our results indicate that the efficacy of VRC may be determined by the species involved in the infection rather than by the MIC. Nevertheless, further studies must be done, including strains showing wider MIC range.

Although the clinical experience with PSC, as treatment for sporotrichosis is scarce, has been reported as favourable (Brunce *et al*., 2012). In addition, in vitro data has also showed a good activity of this drug (Marimón *et al*., 2008b, Silveira *et al*., 2010). Results about the *in vitro* activity of PSC against 34 strains of *S. schenckii* and 23 of *S. brasiliensis* showed MIC90 of 8 and 1 µg/ml, respectively (Marimón *et al*., 2008b). Despite the good *in vitro* activity of PSC against the two most frequent *Sporothrix* species no data about its clinical efficacy has been reported. We tested the efficacy of PSC in an animal model of systemic sporotrichosis by both species demonstrating its efficacy. PSC administered at 5 or 10 mg/kg/day showed an excellent efficacy in prolonging the animals' survival since 100% of animals survived the challenge which caused the death of the animals in 14 to 18 days. The high effect in prolonging the survival contrasts with experimental infections by *Cryptococcus gattii*, *Rhizopus oryzae*, or *Aspergillus terreus* where survival rates were not superior to 60% (Calvo *et al*., 2009; Rodriguez *et al*., 2010; Salas *et al*., 2011) even at lower doses than those reported for other fungi like *Aspergillus* (Salas *et al*., 2011). In addition, high reduction on fungal load was observed in those animals receiving PSC, being this reduction dose-dependent but not superior to those observed with AMB. Although PSC showed no benefits over AMB it can be a therapeutically option in those cases where AMB toxicity can aggravate the

patients´s conditions. Susceptibility break point of PSC has not been established for *Sporothrix*, however in the case of *Aspergillus fumigatus* values have been proposed being ≥ 1 µg/ml considered as resistant. Our results showed that up to MIC = 2 µg/ml PSC have efficacy against *S. brasiliensis* and *S. schenckii*; however, studies including a wide range of strains showing higher MICs needs to be done in order to correlate the efficacy observed in our study with strains with high MICs.

5.2 *Acremonium.*

Acremonium is an opportunistic pathogen that has increasingly been described in recent years (Walsh *et al*., 2004; Das *et al*., 2010, and Khan *et al*., 2011). It is difficult to discriminate *Acremonium* species because the morphological similarity between them complicates the correct identification, which is why in most of the cases the identification is only at genus level (Perdomo *et al*., 2011). *Acremonium* is able to cause a wide spectrum of clinical manifestations as well as response to the therapies used (Khan *et al*., 2011). Recently, molecular studies clarified the spectrum of species involved in the clinic setting, being *A. kiliense*, *A. sclerotigenum-A. egyptiacum* complex and *A. implicatum* those with high incidence and also allowed the re-identification of isolates that were misidentified (Novick *et al*., 2003). It was unknown whether these species had a differential pathogenicity as was described for *Sporothrix*. *Acremonium* is commonly associated to mycetoma (Das *et al*., 2010); however, in recent years, it has been the casual agent of invasive infections mainly affecting immunocompromised patients undergoing hemodialysis, diabetes or neutropenia. The study also showed that the species with the highest virulence was *A. kiliense,* which was consistent with the results of the molecular study

(Perdomo *et al*., 2011), which showed to *S. kiliense* as the most prevalent *Acremonium* species isolated from systemic infections. Moreover although the other species evaluated showed a lower virulence than *A. kiliense* it were also able to cause infection in animals but not as severe as that caused by *A. kiliense.* These differences were noted in the study of the 1,3 β-D glucan levels which showed that although the three species raised the levels, *A. kiliense,* showed the highest values. This would be related to the angioinvasive ability reported for these fungi (Schell, 1995). However, in our study we did not perform blood culture to detect it. On the other hand one of the most important findings was that one of the most affected organs by *A. kiliense* was the kidney, which was demonstrated by s fungal load and histopathology studies. These results are consistent with those reported in clinic, where it has been isolated from patients with renal disease (Das *et al*., 2010). These results allowed to determine the virulence of the species associated with clinical samples as well as to develop a model of disseminated infection in the neutropenic mouse which we used for antifungal therapy evaluation.

Acremonium, particularly *Acremonium kiliense* (reclassified as *Sarocladium kiliense*), is related to *in vitro* resistance to almost all antifungal agents and in consequence there is a lack of optimal treatment for the disease (Khan *et al*., 2011). Knowledge of the species most frequently isolated in human infections and their pathogenicity has allowed the study of antifungal therapies in order to determine the best experimental treatment of disseminated infection by *S. kiliense*, the species with the highest incidence in clinical setting (Perdomo *et al*., 2011). The susceptibility of *S. kiliense* isolates studied in the animal models showed that the most active drugs were AMB and VRC while

PSC and AFG were the less active. The results of the *in vivo* experiments showed that, in general, the efficiency of the four drugs tested was modest, being PSC the drug with better efficacy; however its effectiveness was not able to improve the survival rates over 40%. An important finding of our study was that AMB, which is currently used as the therapy of choice, was ineffective for improving the survival of the animal and the reduction of fungal load was strain dependent. Our results are consistent with clinical data of disseminated infection by this fungus in which a high rate of therapeutic failure was found. Additionally, we also assessed the efficacy of VRC, which has been used several times as replacement therapy in cases of AMB treatment failure or adverse effects. Similar to that observed with AMB, our results indicate that the efficacy of VRC is strain dependent, and that its *in vitro* activity, although elevated, does not correlate with its *in vivo* efficacy. Up to now there is no clinical evidence of the use of echinocandins for infections by this fungus, may be due to its high MEC values (Khan *et al*., 2011; Perdomo *et al.,* 2011). Our results showed that despite its high MIC the drug is able to act *in vivo*, even though their efficacy is modest, was better than that observed for AMB and VRC. These results indicate once more that the results of the *in vitro* activity are not predictive of the *in vivo* efficacy of drugs

Regarding the low activity observed for the monotherapies in the treatment of experimental disseminated infections by *S. kiliense*, it is crucial to explore new therapeutic strategies for the treatment of the severe invasive infection caused by this fungus. Therefore we have evaluated the effectiveness of antifungal drugs showing different mechanism of action in dual combinations *in vitro* and *in vivo* using the previously established model of disseminated infection by this

pathogen in mice. The *in vitro* combination of VRC, PSC, AFG, AMB and terbinafine (TER) showed mostly an indifferent effect; however synergy in 8.3% and 25% for the combination PSC plus TER and VRC plus AFG, were detected respectively. In addition an antagonistic effect was detected in a 16.6 % of the strains for AMB plus PSC and PSC plus AFG combinations

Considering the results of the *in vitro* interactions and *in vivo* results of themonotherapies we decided to evaluate three different combinations: PSC plus AFG, AFG plus VRC and AMB plus VRC in animal model of disseminated infection by *S. kiliense*. Our results showed that the combination of the two most effective monotherapies previously evaluated AFG and PSC had a synergistic effect improving the survival rates and reducing fungal burden compared to monotherapies for the three strains tested. Furthermore, our study showed that the combination of drugs frequently used in human infections, AMB plus VRC, does not improve the efficacy of monotherapies and in some cases has an antagonistic effect, which has been previously described in the literature (Johnson *et al*., 2004). This antagonistic effect is due to the fact that both drugs share the same fungal target, the ergosterol, although with different mechanism of action. So when one of them acts on the ergosterol either directly (AMB) or indirectly (VRC) decreases the target of action for the other drug. Some clinical cases have reported that the change of AMB to VRC improved the outcome of the infection. However, this is not considered as a combination because both drugs have not been administered together but sequentially. In addition, the combination of VRC and AFG even though it has been reported synergistic for *Aspergillus*, our study showed only a modest efficacy, improving survival compared to monotherapies, but not decreasing the fungal load in the organs

tested. Nevertheless, the efficacy of the combinations studied for the treatment of disseminated infection by *S. kiliense* in mice was evaluated at high doses, similar to that used in monotherapy, our results indicate that the combination of PSC and AFG could be an alternative therapy for these infections.

5.3 *Scedosporium.*

Invasive infections caused by fungi of the *Scedosporium* are difficult to treat and are responsible for a high mortality rate, especially in immunocompromised patients (Guarro *et al*., 2006), requiring a search for new therapeutic alternatives. *Pseudallescheria boydii* and *Pseudallescheria apiosperma* are the species with a higher incidence in clinical setting (Rainer *et al*., 2000). These fungi are highly resistant to conventional antifungal AMB. The new azoles, VRC, PSC have shown promising results *in vitro* (Cuenca-Estrella *et al* 1999; Gilgado *et al*., 2006; Guarro *et al*., 2006; Alastruey-Izquierdo *et al*., 2007; Cuenca-Estrella *et al*., 2008; Lackner *et al*., 2012a) in some clinical reports (Mellinghoff *et al*., 2002; Apostolova *et al*., 2005; Porte *et al*., 2006) and *in vivo* model (Rodriguez *et al*., 2010). To establish a species-specific susceptibility pattern has not been possible for *Scedosporium* spp. Recently Lackner *et al*., (Lackner *et al.,* 2012) in an *in vitro* study demonstrated that for all almost antifungal there is a not normal distribution of the MIC showing mostly two subpopulations of strains, one of them very susceptible and the other highly resistant to almost all antifungal drugs, with the exception of VRC and lesser extent PSC and MFG. the clinical experience with VRC has shown a good efficacy and tolerance of that drug in the management of *Scedosporium* infections (Troke *et al.,* 2008), however, some reports of therapeutic failure has been reported. Currently, there is only a one report of successful treatment of *S.*

apiospermum with VRC in combination with an echinocandin, caspofungin (Troke *et al*., 2008), but no experimental data using azole–MFG combination for *Scedosporium* infection are available. Therefore we evaluated the *in vitro* activity and the interaction between MFG and PSC or VRC and their efficacy in a murine model of disseminated infection by these fungi. The *in vitro* test is the first step to assess potential antifungal combinations. Currently there are three methods commonly used for this purpose; checkerboard, E-test and time kill curves. In our study we evaluated the utility of two methods checkerboard and E-test for both combinations (MFG plus VRC and PSC plus MFG) in order to determine which of these methods provided a better reproducibility of the data. In a pilot study we evaluated only 9 isolates from different species *Scedosporium*, the results indicated that the best method was the checkerboard, since to determine by E-test® the MECs for MFG against these fungi was difficult due to the fungistatic effect, since to determine morphological alterations on the fungi´s growth on plate showed variable results. By the chekerboard method we assayed 43 isolates of *Scedosporium,* the results showed mostly an indifferent effect for the interaction. However some stains showed a synergist effect for PSC plus MFG and VRC plus MFG combinations. Based on those results we selected some strains in order to evaluate the *in vivo* efficacy of such combinations and for trying to correlate the *in vitro* results with *in vivo* efficacy. The results of both *in vivo* combinations showed to be species-dependent and in all cases were better than the MFG as monotherapy. However, only the combination of VRC plus MFG turned out to be better than both monotherapies and more effective for *P. boydii*. By the contrast for *P. apisopermum* combinations efficiency was not better than monotherapy. The

results from *in vivo* study are promising and could provide a new therapeutic alternative for those serious cases of scedosporiosis.

5.4 *Candida glabrata.*

Candida glabrata has become an important agent of IC. Currently, echinocandins, particularly caspofungin, is the first choice therapy for the treatment of IC by this species due to its intrinsic resistance to azoles, especially to fluconazole (Pappas *et al*., 2009). Traditionally, echinocandins have a high *in vitro* activity against *C. glabrata*; however, since their introduction in the clinical practice, there has been an increase in the resistance to this drug (Pfaller *et al*., 2010). Resistance to echinocandins has been associated with the presence of mutations in two hot spot (HS) regions of the *FKS* genes, which encode the major subunit of the 1,3 β-D-glucan synthase complex in the cell wall, which is the target for echinocandins (Denning, 2003) This has led to the current clinical breakpoints and epidemiological cutoff to be evaluated and adjusted in order to make them more predictive of the therapy outcome (Pfaller *et al*., 2010; Pfaller *et al*., 2011). Many *in vitro* and *in vivo* studies have evaluated the influence of these mutations on the susceptibility of the strains A total of 17 *C. glabrata* strains were evaluated, some of them were wild type, i.e. lacked mutations and other non-wild type, with mutations in the *FKS* and covering a wide range of MIC. Consistent with previous studies it was shown that caspofungin is effective in reducing fungal burden and prolonging survival in animals infected with *Candida glabrata* strains with MIC > ECV (0.12μg/ml) and that currently are classified as resistant by the CBP(> 0.5μg/ml). Our results also indicate that the most predictive parameter would be the MIC more than the presence of mutations, since, as previously noted, the treatment was

effective in non-wild type strains. These results are consistent with with those published by Lepak *et al*, (Lepak *et al*., 2012), who evaluated the efficacy of CSP against a *C. glabrata* showing a wide range of MICs similar to our study, but they also determined the pharmadinamic target for each echinocandin against strain displaying or not *FKS* mutations. Those results suggest that the current CBP are too low and that could rise since they are not predictive for the therapeutic success or failure

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VI. Conclusions

6.1 We evaluated the virulence of *Sporothrix luriei* in an animal model of disseminated infection.

- *S. luriei* has showed to have a high virulence, being able to cause a high mortality, tissue burden and tissue damage, especially in liver and spleen.

6.2 We evaluated the *in vitro* activity of posaconazole and voriconazole against clinical isolates of *Sporothrix schenckii* sensu stricto and *S. brasiliensis*, as well as the effectiveness of these drugs in a model of disseminated sporotrichosis.

- Posaconazole showed to have a better *in vitro* activity against the two *Sporothrix* species evaluated than voriconazole. In the experimental treatment of disseminated sporotrichosis, posaconazole showed high efficacy, prolonging survival and reducing fungal burden in the organs studied without differences between the species tested.
- Voriconazole showed a reduced and species-dependent efficacy in the treatment of disseminated sporotrichosis. This drug was only effective for *S. schenckii* at high doses

6.3 We evaluated and compared the virulence of clinical isolates of *Sarocladium* (*Acremonium*) *kiliense*, *Acremonium implicatum* and *Acremonium sclerogtigenum-egyptiacum* complex in models of disseminated infection in immunocompetent or neutropenic mouse.

- The virulence of the *Acremonium* species tested was low. The infection was only established in the neutropenic mouse model. *Sarocladium kiliense* was the most virulent species causing a higher mortality compared to the other species, as well as high fungal load and tissue damage in spleen and kidney in comparison to the others species evaluated.

6.4 We evaluated the *in vitro* activity of anidulafungin, voriconazole, terbinafine, posaconazole and amphotericin B and their interactions with 12 clinical *Sarocladium kiliense* isolates.

- Terbinafine, voriconazole and amphotericin B showed the highest *in vitro* activity. Conversely, posaconazole and anidulafungin were inactive. Their interactions were mostly indifferent. However, the combinations of posaconazole plus terbinafine and voriconazole plus anidulafungin showed synergistic effects.

6.5 We evaluated the efficacy of posaconazole, voriconazole, anidulafungin and amphotericin alone and in combination against *Sarocladium kiliense* in a model of disseminated infection in neutropenic mice.

- Posaconazole was the most effective drug to treat disseminated infection by *S. kiliense* reducing fungal load, the levels of 1,3β-D glucan and prolonging survivance, in comparison with the other monotherapies. The combination of posaconazole and anidulafungin showed the highest efficacy in prolonging survival and reducing the fungal load.

6.6 We evaluated the *in vitro* activity of the combination of micafungin and voriconazole or posaconazole against *Pseudallescheria boydii* or *Pseudallescheria apiosperma.*

- The *in vitro* interactions have generally showed indifference; however, synergism and antagonism was detected for both combinations as well as for both species.

6.7 The efficacy of voriconazole, posaconazole and micafungin alone and in combination were assayed in a murine model of disseminated infection by *Pseudallescheria boydii* or *Pseudallescheria apiosperma.*

- Voriconazole showed the highest efficacy against the two species tested. It was able to prolong the survival and to reduce the fungal burden in kidney and brain.
- The combinations *in vivo* did not show antagonistic interactions conversely, the combinations improved the results of monotherapy, especially for micafungin and, in some cases, for azoles.
- The combinations showed an efficacy species-dependent. Micafungin plus voriconazole showed higher efficacy than the corresponding monotherapies and than the combination of posaconazole and micafungin. The effectiveness of that combination (voriconazole plus micafungin) was better for *Ps. boydii* than for *Ps. apiosperma*. These results suggest that the use of voriconazole plus micafungin therapy might be an alternative therapy in the *Pseudallescheria*/*Scedosporium* infection.

6.8 We evaluated the *in vitro* activity of caspofungin and its *in vivo* efficacy against clinical *Candida glabrata* isolates. In addition the presence of mutations in the hot spot 1 (HS1) of *fks*1 and *fks*2 gen was determined. The efficacy of caspofungin was evaluated in a murine model of invasive candidiasis. Thus, it was intended to determine whether the MIC and the presence of *FKS* mutations are predictive of therapeutic success or failure. Three new mutations were detected: two in the *fks*1 gene of strains with MIC lower than the current epidemiological cutoff value (ECV) (0.12 µg/ml) and one in the *fks*2 gene in strains with MIC over the ECV.

VI. CONCLUSIONS

- The *in vivo* results demonstrated that the presence of *FKS* mutations in strains with caspofungin MICs lower than the ECV do not affect the efficacy of the drug.
- Caspofungin MIC correlated better with therapeutic outcomes than with *FKS* mutations.
- These data suggest that current ECV are really low, and that the level should increase.

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VII.REFERENCES

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VIII. Annex

Asunto: AAC01484-13: Manuscript Received

De: asm@msubmit.net (asm@msubmit.net)

Para: fafernad@yahoo.es;

Fecha: Miércoles 10 de julio de 2013 19:35

Dear Dr. Fernández-Silva,

On July 10, 2013, we received the manuscript "Antifungal therapies in a murine model of disseminated infection by the emerging opportunistic fungus Sarocladium (Acremonium) kiliense" by Fabiola Fernández-Silva, Javier Capilla, Emilio Mayayo, Deanna Sutton, Pilar Hernández, and Josep Guarro. The submission form indicates that this paper should be processed as a(n) Full-Length Text intended for publication in the section Experimental Therapeutics.

The manuscript has been assigned the control number AAC01484-13. Take note of this number, and refer to it in any correspondence with the Journals Department or with the editor.

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