# In vitro activity and post-drug-exposure effect of antifungal agents and other drugs in *Exophiala spinifera* and filamentous fungi

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# In vitro activity and post-drug-exposure effects of antifungal agents and other drugs in *Exophiala spinifera* and filamentous fungi

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*To my parents , Graciela and Pedro*
*my brother, Daniel*
*my parents in law, Elsa and Salvador*
*and*
*my husband Javier*

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Chapter

1

# *General Introduction*

1) Introduction

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## 1.1 General Introduction

Fungi are eukaryotic organisms that belong to the Kingdom Eumycota. Fungal diseases have gained much importance during the last decade due to the growing population of immunocompromised individuals with hematological malignances, solid organ transplants, hematopoietic stem cell transplants (HSCT), those receiving immunosuppressive therapy or broad-spectrum antibiotics or patients diagnosed with AIDS. Of the 50,000-250,000 species of fungi, only about 200 are associated with human disease, which is related to their ability to grow at body temperature. In general invasive infection is acquired from the environment by inhalation, ingestion or via traumatic inoculation.

Prediction of *in vivo* outcome is difficult particularly with less common agents of disease. Only scattered information on antifungal susceptibility is available when rare fungal pathogens are involved. More importantly, taxonomic borderlines of such species may have been investigated insufficiently and may lack statistical support. Strains held under a particular name in a culture collection do not necessarily represent the core of that species as it exists in nature. Studies of its clinical behavior should therefore be preceded by a molecular verification of taxonomy and phylogeny of the entity to be studied. In many cases this will lead to significant taxonomic rearrangements, resulting in entities that consist of strains with the desired mutual resemblance in physiology, ecology and clinical potential.

The most common invasive fungal infections are caused by yeasts such as *Candida* species or filamentous fungi such as *Aspergillus* species, others are uncommon such as zygomycetes and *Scedosporium* species or rare such as *Exophiala* and *Cladophialophora* species.

Aspergillosis can be defined as damage to living tissue caused by species of *Aspergillus*. The damage may be caused by invasion of tissues, allergic mechanisms or colonization of cavities resulting in several diseases of which the severity depends on the state of the host defenses. *Aspergillus* is worldwide distributed and is abundant in the environment. The

conidia are frequently inhaled and therefore, the lung is the most frequently affected site. Individuals with normal defense mechanisms rarely develop invasive disease.

Neutropenia and the administration of high doses of corticosteroids are two major risk factors for the development of invasive aspergillosis (31). Invasive aspergillosis in transplant patients has a mortality of up to 90% depending on the patient group infected (37). The incidence of invasive aspergillosis is 20% in HSCT (38); 14 to18% in lung and heart-lung transplantation (23); 1.5 to14.7% in liver transplantation but can be as high as 67% (7). The case-fatality rate was described as being 86.7% in HSCT (11), 67.6% in liver and 62.5% in kidney transplant patients (20, 47).

Members of the class zygomycetes also cause human disease. The main risk factors for developing invasive zygomycosis include diabetes with ketoacidosis, neutropenia, receipt of a transplant or administration of corticosteroids in patients with leukemia. The mortality in patients suffering from disseminated zygomycosis is 95% (2). Therapy failure may occur due to the divergent degrees of susceptibility of the fungus, therefore, accurate and rapid diagnosis are essential. Current treatment of invasive zygomycosis consists of high doses of amphotericin B and surgery. Other drugs such as the triazoles or the echinocandins appear not to be very active (21, 39).

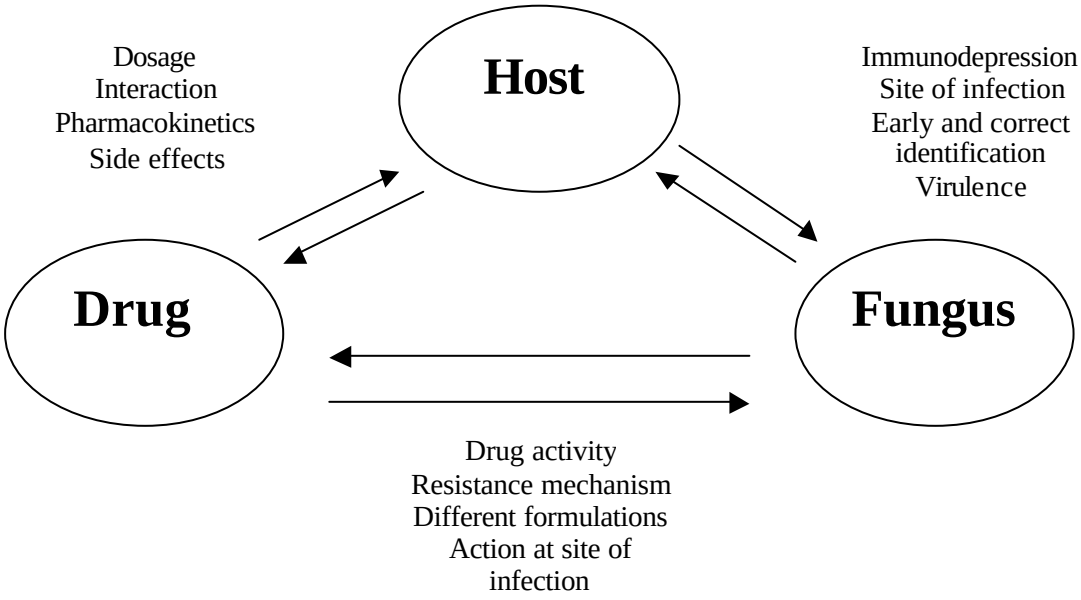
Dematiaceous fungi are characterized by the presence of melanin in the wall of hyphae and/or conidia. There are three orders of melanized fungi. The Pleosporales (e.g. *Alternaria*, *Curvularia*) are widely distributed in soil and on plant material. The Dothiales (e.g. *Aureobasidium*) are particularly found as colonizers of surfaces, such as stone, glass or plant leaves. Members of both orders are occasionally found as opportunistic on humans. The third order, the Chaetothryales, contain the pathogens black yeasts and their relatives, e.g. *Exophiala*, *Cladophialophora* and *Fonsecaea*. They are frequently involved in human mycoses, but their natural niche is hitherto insufficiently understood. These fungi may cause phaeohyphomycosis, chromoblastomycosis or black grain mycetoma (16, 30). They are widely distributed, particularly in wood, soil and other plant matter (36, 41). In normal hosts, localized lesions of skin or subcutaneous tissues are involved. Sinusitis and pneumonia are being increasingly recognized and disseminated infections arise, particularly in immunocompromised patients including organ transplant recipients (43). Although rare, *Exophiala spinifera* is recognized as one of the most aggressive in

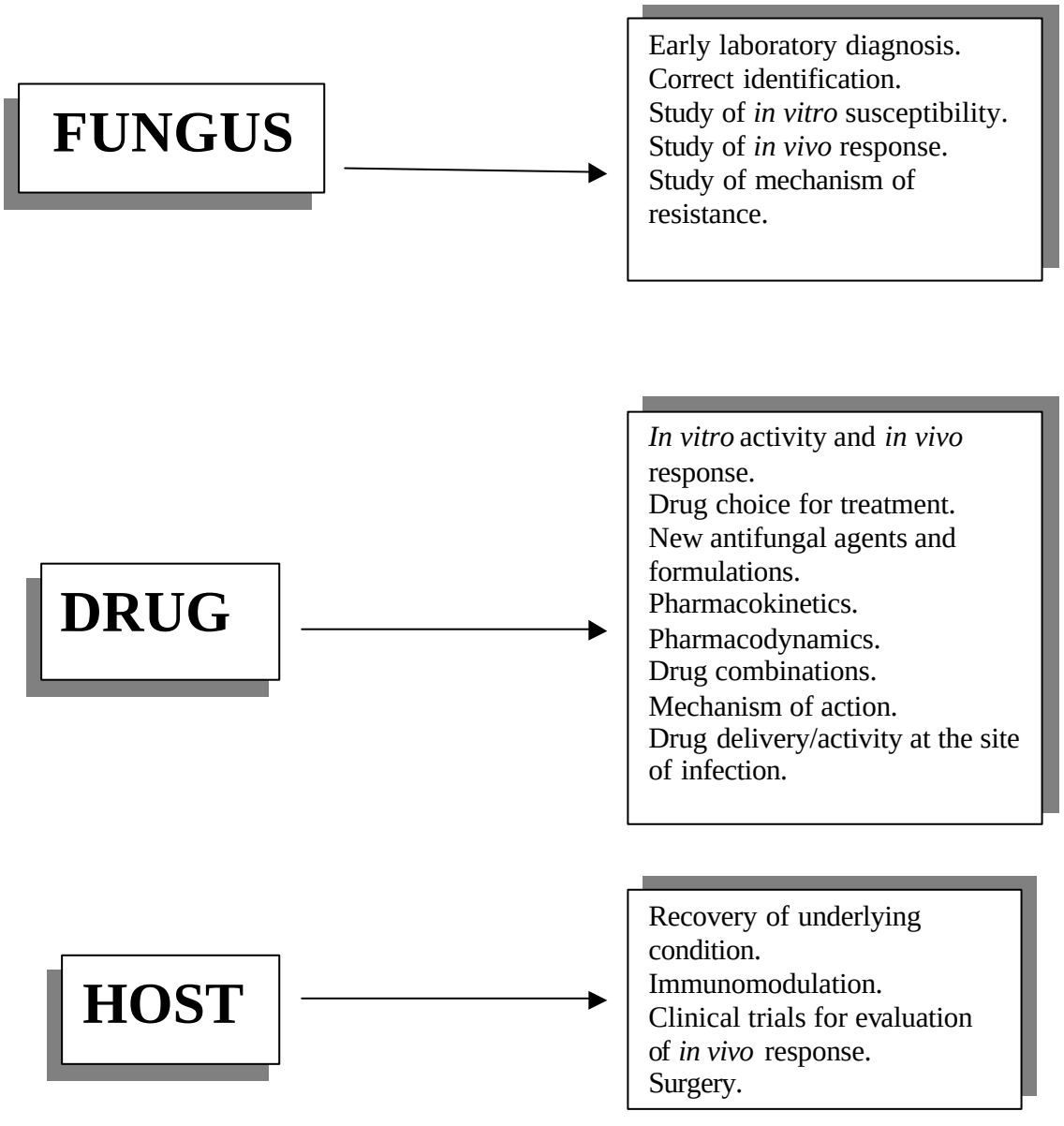
the group of black yeasts, with localized lesions in adults but disseminated infections in children or adolescents, always with a fatal outcome (10).

Therapeutic measures used in cases of *E. spinifera* infection have been highly diverse and the optimal treatment is still in dispute (43). The efficacy of the currently used antifungal therapy regimens is variable since relapses have frequently been observed. (3, 18, 26, 32, 35, 36).

*Scedosporium* species are filamentous fungi with worldwide distribution and which may cause hyalohyphomycoses. They have been reported from white grain mycetoma, disseminated infections, CSF infection and subcutaneous infections (5, 9). With the increment of immunocompromised patients, the species can disseminate in patients such as those with hematological malignances (29) or organ transplant recipients (5). Particularly, *S. apiospermum* has a predilection for the central nervous system (9).

Given the poor responses to the above mentioned fungi novel approaches to improve outcome are clearly needed. Given the interaction between fungus, host and drug in relation to outcome (Figure), each can be used as target for novel approaches to management.




FUNGUS
Early laboratory diagnosis.
Correct identification.
Study of *in vitro* susceptibility.
Study of *in vivo* response.
Study of mechanism of resistance.
DRUG
*In vitro* activity and *in vivo* response.
Drug choice for treatment.
New antifungal agents and formulations.
Pharmacokinetics.
Pharmacodynamics.
Drug combinations.
Mechanism of action.
Drug delivery/activity at the site of infection.
HOST
Recovery of underlying condition.
Immunomodulation.
Clinical trials for evaluation of *in vivo* response.
Surgery.

## Antimicrobial agents

The arsenal of agents used for antifungal therapy is limited compared with antibacterial drugs. The available drugs include polyenes, azoles and allylamines although new compounds are under investigation such as echinocandins, with exception of caspofungin that is already approved.

**Polyenes**. The mechanism of action is the interaction of the antifungal with the membrane sterol, ergosterol, resulting in pores that form a channel, leading to altered permeability, leakage of vital cytoplasmic components and death of the microorganism. Amphotericin B represents the standard therapy for several systemic fungal infections. This drug is active against most *Candida* species, *Cryptococcus neoformans*, *Aspergillus* species, zygomycetes, dimorphic fungi and some dematiaceous fungi (28).

**Azoles**. The target for azoles is the heme protein which catalyzes cytochrome P-450 dependent, 14α-demethylation of lanosterol. Inhibition of this enzyme leads to depletion of ergosterol and accumulation of precursors, resulting in the formation of a plasma membrane with altered structure and function (17).

Itraconazole is a triazole used in humans with a broad spectrum of activity including *Candida* species, *Aspergillus* species, dimorphic and dematiaceous fungi. It is commonly used as prophylactic drug in patients at high risk for invasive aspergillosis. Since an intravenous formulation has become available, it is also used for primary treatment of invasive aspergillosis (6, 44, 45).

Fluconazole is well absorbed and has a favorable toxicity profile but has a narrow spectrum of activity with reduced action against some non-albicans *Candida* species and moulds (19).

Voriconazole a new triazole has a similar spectrum of activity as itraconazole. Other new azoles under investigation are ravuconazole and posaconazole that have been shown to be active against a broad range of yeasts and filamentous fungi (28).

## Other antimicrobial drugs than antifungal agents

Besides the currently used antifungal drugs, other agents have been shown to exhibit antifungal activity either alone or in combination with other drugs.

**Quinolones**. These drugs have a broad spectrum of activity as inhibitors of a DNA gyrase, type II topoisomerase, that is present in prokaryotes and eukaryotes. The presence of high levels of topo-isomerase I and II was reported in pathogenic fungi (42). Although quinolones exhibit no antifungal activity, they have been shown to augment the activity of amphotericin B and azoles (33, 43).

**Phenotiazines**. These compounds are antipsychotic agents that inhibit calmodulin leading to multiple cellular effects like modification of membranes, alteration of cyclic nucleotide metabolism and intercalation into DNA (24). Genetic studies suggest that calmodulin, is essential for growth of fungi (22). Phenotiazines have been reported to exhibit antibacterial and antifungal activity (1, 12, 44, 46). Of special interest is the observation that the *cis*-geometric stereoisomerism has antimicrobial and neuroleptic activity, whereas the *trans*-geometric stereoisomerism has only an antimicrobial effect (25) being an option for further investigation.

## Laboratory diagnosis

The laboratory diagnosis of fungal infections includes standard techniques that include direct microscopic examination, culture, identification including macroscopic and microscopic examination, physiological characteristics, and molecular biological techniques such as genomic sequencing. Preferably microbiological identification of the fungal pathogen should be combined with histological examination of tissue in order to prove the fungal infection.

Correct and fast identification is very important in order to initiate treatment with the optimal drug promptly. Nevertheless, in neutropenic and transplant patients diagnosis of infections due to (uncommon) fungi may be difficult. In such cases therapy is given empirically or pre-emptively, awaiting the final diagnosis.

For in vitro susceptibility testing the National Committee for Clinical Laboratory Standards (NCCLS) provides a guideline for testing the activity of polyenes, flucytosine and azole antifungal agents against a number of conidium-forming filamentous fungi (34). The minimal fungicidal concentration (MFC) is in evaluation in order to find its role as a good parameter to predict the clinical outcome together with the MIC (15).

In addition to testing the activity of single drugs, the study of drug interactions both in vitro and in vivo has gained interest given the increased arsenal of antifungal agents and the overall poor outcome of monotherapy. In vitro several tools are available including time kill methods, checquerboard techniques or E-tests (4, 27).

The clinical usefulness of *in vitro* susceptibility testing results in treating fungal infections remains controversial, due to lack of correlation with clinical response for some drugs. Lack of correlation between *in vitro* and *in-vivo* results could be due to many factors. For instance, MIC is not a physical or chemical measurement, all *in vitro* tests are performed with conidial suspensions while hyphae are the form that is present *in vivo*, variation of pH or temperature, medium and incubation period might also have influence. Microorganisms are exposed to a constant level of the drug in *in vitro* systems while *in vivo* they are exposed to fluctuating levels of the antimicrobial agents, resulting in other possible factors for the observed lack of correlation. In addition, host factors are very important in determining clinical outcome.

Therefore, *in vitro* tests do not always predict successful therapy in susceptible microorganisms. In bacteriology, the 90-60 rule has been put forward, indicating that 90% of patients infected with a susceptible isolate will respond to appropriate therapy, whereas as much as 60% will respond when infected with an isolate resistant to the drug administered. It has been shown that this ratio is also applicable for yeasts (40).

The post-drug-exposure effects are another point to take into consideration. This phenomenon was shown to be the cause for treatment success with certain drugs that were administered intermittently, even when the concentration of the drug fell below the MIC of the pathogen. The explanation was suggested to be the persistent suppression of

microbial growth after clearance of the drug. This effect was first described for bacteria and called post antibiotic effect (PAE), but was later also described for *Candida* species and *C. neoformans* where it was named as post-antifungal effect (PAFE) (8, 13, 14). The study of PAE provides additional information on the action of antimicrobial agents that can not be obtained from standard methods of susceptibility testing.

The clinical significance of this effect has not been established, but could have an impact on antimicrobial dosing regimens as was described for some antibiotics (8).

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## 1.2 Outline of the thesis

In order to gain more insight in the potential of alternative approaches to the management of invasive mycoses, we investigated the in vitro activity of several drugs with antifungal activity against a selection of common and uncommon fungal pathogens.

This study was carried out in terms of evaluation of the minimal inhibitory concentration (MIC), minimal fungicidal concentration (MFC) and drug interactions. Furthermore an *in vitro* system was developed to study the post-drug-exposure effect of antifungal and non-antifungal drugs against filamentous fungi and black yeasts.

Since identification is very important as was highlighted above, we performed classic and molecular identification methods, using *E. spinifera* as a model, because the group of black yeasts are one of the most difficult to treat and to identify.

The classical strategy against severe or resistant bacterial or viral infections has been combining different classes of antimicrobial agents. By analogy, the combination of antifungal drugs with other antifungal or antimicrobial agents could represent a promising approach.

**Chapter 2** is focused on the identification and susceptibility profile of the *E. spinifera* clade. In **chapter 2-1**, identification was carried out using classic morphology, physiological profiles and molecular typing. Susceptibility profiles for sixteen clinical and environmental strains of *E. spinifera* were determined in **chapter 2-2**, applying different incubation temperature regimens.

Due to the long treatment needed and the often observed relapses of infections caused by black fungi combination of antimicrobial agents could represent a better approach than monotherapy. Therefore the *in vitro* combined effects of antifungal and antibiotic drugs using the checkerboard method was investigated in **chapter 2-3**.

The standardized method to evaluate the activity of antifungal agents against filamentous fungi is static because the fungi are exposed to a constant level of drug. Given the fact that, *in vivo*, the organism is exposed to fluctuating levels of antimicrobials with drug levels that fall below the MIC, we simulated the *in vivo* situation *in vitro* by removing the drug by washing and determining if any effect persisted. PAFE is described in

**chapter 2-2** for eleven clinical *E. spinifera* strains against four drugs and in **chapter 3** for different antifungal and some other antimicrobial agents against different moulds.

In **chapter 3-1**, a method to evaluate PAFE against filamentous fungi is described and the effect of amphotericin B and itraconazole was evaluated against fifteen clinical isolates of *Aspergillus* species.

Based on this method, in **chapter 3-2**, PAFE of amphotericin B and nystatin was evaluated in 30 clinical zygomycetes using two different media, RPMI-1640 and antibiotic medium 3 (AM3).

In **chapter 3-3** the activity and PAFE of chlorpromazine and trifluoroperazine was evaluated in species of *Aspergillus*, *Scedosporium* and zygomycetes.

Since prophylaxis is often given to patients at risk of developing invasive aspergillosis, in **chapter 3-4** *Aspergillus* species were pre-incubated up to eight weeks with sub-inhibitory concentrations of itraconazole, fluconazole and amphotericin B. Then, the MIC, MFC and PAFE was evaluated at each condition and was compared with that of the wild-type strains.

Finally, in **chapter 4**, a summarized conclusion of the results obtained in each chapter is given and possible future perspectives are addressed.

Chapter

2

# *Activity of antifungal agents against Exophiala spinifera*

- Molecular diversity, new species and antifungal susceptibilities in the *Exophiala spinifera* clade.
- *In vitro* activity of amphotericin B, itraconazole, terbinafine and 5-fluorocytosine against *Exophiala spinifera* and evaluation of post antifungal effects.
- In vitro activity of amphotericin B and itraconazole in combination with flucytosine, sulfadiazine and quinolones against *Exophiala spinifera.*

# Chapter 2.1

Molecular diversity, new species and antifungal susceptibilities in the *Exophiala spinifera* clade.

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# Molecular diversity, new species and antifungal susceptibilities in the *Exophiala spinifera* clade

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## Abstract

Black yeasts morphologically identified as *Exophiala cf. spinifera* were re-analyzed on the basis of morphology, nutritional physiology and ITS rDNA sequencing data.
Ribosomal small subunit (SSU) DNA sequence data confirmed that strains belonged to a clade containing the ex-type strain of *E. spinifera*. Variation in the Internal Transcribed Spacer (ITS) region of the rDNA was studied within this group. Five infraspecific groups were delineated. Phylogenetic analysis of the SSU rDNA data revealed that several of the strains analyzed were located at considerable evolutionary distance from the E. spinifera clade. ITS rDNA sequencing showed that the deviating strains could not be identified with any existing species. Two new species, *E. attenuata* and *E. nishimurae*, were therefore proposed. Antimycotic susceptibility data of the new species were compared with those obtained with members of the *E. spinifera* clade.

Keywords: *Exophiala attenuata* , *Exophiala nishimurae*, *Exophiala spinifera*, phylogeny, antifungals.

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## Introduction

Black yeasts of the genus *Exophiala* are commonly found in association with environmental substrates, including soil as well as wood and other plant materials [1]. More often, however, they are encountered as agents of human mycoses in immunocompromised or debilitated patients [2], as well as in otherwise healthy hosts. The great majority of these infections are superficial, but rarely they may be devastating, fatal systemic infections [3]. In some species a predilection for particular patient groups seems to occur [4]. The pathology of the blackyeasts and their relatives is still poorly understood.

*Exophiala spinifera* (Nielsen & Conant) McGinnis is among of the most aggressive species of the genus. Two types of mycoses caused by this taxon are apparent [5]. Adult patients reported thus far have generally had an underlying disease such as arthritis. Their lesions were of traumatic origin and remained localized with primary cutaneous or subcutaneous involvement [6,7]. In contrast, children and adolescent patients have lacked detectable constitutional disorders. Cases were disseminated and chronic, with severe secondary cutaneous lesions, and often took a fatal course after 3-6 years [3,8,9].

This divergent clinical behaviour suggests taxonomic diversity within the species. However, only a limited number of voucher strains from published case reports are still available for study. De Hoog et al. [5] analyzed the *E. spinifera* complex by sequence comparison of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). They also investigated nutritional physiology. In sequence dendrograms, *E. spinifera* was found to be flanked by a number of morphologically similar strains that were located at significant phylogenetic distance from the core group of the species. The present article aims to establish the identity of additional clinical strains, including those matching the description of *E. spinifera* sensu stricto as well as deviating strains. The molecular variability within the *E. spinifera* complex was investigated and related taxonomic investigations were also performed.

Haase et al. [10], using ribosomal small subunit (SSU) DNA phylogeny, described the *E. spinifera* complex as a statistically significantly supported monophyletic

group within the herpotrichiellaceous black yeasts. Most other species included in these authors' tree showed insuficient resolution to be recognized separately. In principle, if all strains with an *E. spinifera*-like appearance turn out to be members of a single broad clade, they are likely to share a similar ecology and evolution and thus may have similar opportunistic potential. This is suficient reason to determine the SSU position of the deviating *E. spinifera*-like strains, in addition to using ITS sequences to resolve the species level disjunctions in the group.

Since microscopic morphology alone is insufficient to allow routine laboratory identification of black yeasts and their relatives, we searched for additional key features in physiological profiles of the strains. In addition, susceptibility to six antifungal drugs was determined in order to compare the susceptibilities of deviating strains to patterns of susceptibility already recorded for *E. spinifera* [11].

## Materials and Methods

**Fungal strains.** The strains studied (Table 1) were grown on potato dextrose agar (PDA) at 24°C for 10 days.

**Morphology and physiology**. Species names are attributed on the basis of preliminary morphological identification and corrected on the basis of molecular and physiological features. Microscopic morphology on Sabouraud's glucose agar (SGA), malt extract agar (MEA) and PDA was verified before and after experimental data had been evaluated. Physiological methods applied have been described in detail by Untereiner et al. [12]. Thermotolerance was tested by incubation of freshly inoculated culture plates at 28, 37 and 40°C. All tests were performed three times in duplicate.

**DNA extraction.** About 1 $cm^2$ of mycelium was transferred to a 2 ml Eppendorf tube containing a 2:1 (w/w) mixture of silica gel and Celite (silica gel H, Merck 7736 / Kieselguhr Celite 545, Machery) and 300 µl cetyltrimethylammonium bromide (CTAB) buffer [Tris HCl, 200 mM, pH 7.5, 200 mM Na-EDTA, 82% NaCl w/v, 2% CTAB w/v]. The mycelium was ground with a micropestle (Eppendorf, Hamburg, Germany) for 1–2 min. Volume was adjusted by adding 200 µl CTAB buffer. After vigorous shaking, the sample was incubated at 65°C for 10 min. One volume (~500 µl) of chloroform was added and vortexed for 1–2 s. The samples were centrifuged at 14,000 rpm for 5 min.

After transferring the aqueous supernatant to a new Eppendorf tube, two volumes (~800 ml) ethanol 96% -20°C were added and mixed gently. Samples were incubated at -20°C for 30 min or overnight and then centrifuged for 5 min at 14,000 rpm. The pellets were rinsed twice with 500 ml ice-cold ethanol 70%. After pellets were dried at room temperature they were resuspended in 48.5 µl TE-buffer (10 mM Tris, 10 mM Na-EDTA, pH 8.0) adding 1.5 µl RNAse solution (10 mg pancreatic RNAse in 1 ml 0.01 M Naacetate, pH 5.2). The pH was adjusted by adding 100 µl 1 M Tris HCl (pH 7.4). Samples were incubated 5–30 min at 37°C and refrigerated.

**DNA amplification.** PCR was performed in 50 µl volumes of a reaction mixture containing 10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$*6H2O, 0.01% gelatin, 200 µM of each deoxynucleotide triphosphate, 50 pmol of each primer, 10–100 ng rDNA and 0.5 U Taq DNA polymerase (Amplitherm, ITK Diagnostics, Maarssen, The Netherlands).

Primers used were NS1, NS24, V9G and LS266 [2,13]. Forty amplification cycles were performed: denaturing 94°C, 30 s; annealing 58°C, 1 min; elongation 72°C, 30 s (with an initial delay of 1 min and a terminal delay of 2 min) in a GeneAmp 9600 thermocycler (Applied Biosystems, Nieuwerkerk a.d. IJssel, The Netherlands). If no amplicon was obtained the cycle was changed to 94°C, 1 min; 48°C, 1 min; 72°C, 2 min. Amplicons were cleaned using Microspin S300 HR columns (Amersham Pharmacia, Roosendaal, The Netherlands). For sequencing, PCR conditions were as follows: 25 cycles with denaturing 96°C, 10 s; annealing 50°C, 5 s; elongation 60°C, 4 s, carried out with primers ITS1 or 5 and ITS 4. For SSU sequencing, primers NS1, Oli 1, 2, 7, 10, 11, 13, 14 and NS24 were used. Base compositions of all primers used are given by De Hoog et al. [2]. DNA was precipitated with ethanol and sequenced using an ABI Prism™310 Genetic Analyzer (Applied Biosystems).

Alignment and phylogenetic analysis. Sequences obtained were adjusted using SeqMan II of Lasergene software (DNASTAR, Inc.) and aligned iteratively using Ward's averaging in the BioNumerics package (Applied Maths, Kortrijk, Belgium). Nearest neighbours were found by local Blast searches. Distance trees were based on re-aligned files using the DCSE program [14] and calculated with the Neighbor-joining method of the

TREECON package [15] with Kimura correction. Bootstrap values 490 of 100 resampled datasets are shown. The inferred topology was verified using the same data in a phylogenetic tree constructed with PAUP version 4.0b8 [16] with heuristic search option (data not shown).

**Table 1** Strains analyzed. Subspecific grouping (1–5) within *Exophiala spinifera* is mentioned in the last column. (a)–(d) = Broad ecological categories explained in text

| CBS | UTMB | ATCC | CDC | Genbank | Other number | Source | Patient | Geography | Reference | ITS |
|---|---|---|---|---|---|---|---|---|---|---|
| *Exophiala spinifera:* | | | | | | | | | | |
| | 2988 | | | | | (a) skin lesion | male 49 y | Mexico | [6] | 4 |
| 269.28 | | | | AY156960 | | (a) skin lesion | human | Germany | O. Grütz | 1 |
| 102197 | | | | | IP 98884 | (a) skin lesion | male 58 y | Senegal | [36] | 4 |
| | 1361 | | | AF549449 | 344-81 | (a) skin lesion | human | Mexico | R. Arenas | 2 |
| 356.83 | | | | AY156961 | | (a) skin lesion | human | Egypt | R.R. Saad | 1 |
| | 1184 | | | | OMH 1409-80 | (a) foot abscess | human | | J. Kane | 1 |
| | 3709 | | B-5579 | AF549444 | | (a) elbow lesion | male 6 y | U.S.A. | [43] | 1 |
| 101544 | | | B-5383 | | | (a) elbow lesion | male 6 y | U.S.A. | [43] | 1 |
| 101543 | | 56567 | B-3868 | | | (a) arm lesion | male 60 y | U.S.A. | [37] | 1 |
| 101545 | 3710 | | B-5580 | | | (a) elbow lesion | male 6 y | U.S.A. | [42] | 1 |
| | 1540 | | | | 5209 | (a) disseminated | female 5 y | Brazil | [38] | ND |
| 194.61 | | | | AY156958 | | (a) disseminated | male 7 y | India | [8] | 1 |
| | 3757 | | | AF549443 | CMM-3515 | (a) neck lymph node | | Argentina | | 2 |
| | 2643 | | | AF549448 | | (a) sputum | | | | 2 |
| 899.68T | 152 | 18218 | | AY156976 | NFC 3342, NIH 8753, DSM 1217, IHM 1767, NCPF 2358 | (a) nasal granuloma | | U.S.A. | [39] | 2 |
| | 2943 | | | AF549445 | 88-548277 | (a) sputum, AIDS | | | | 1 |
| | 2511 | | | AF549450 | 850280 | (a) lesion | cat | Australia | [40] | 3 |
| 670.76 | 760 = 820 | 26091 | B-2720 | AF549451 | IHM 1745 | (b) nest of *Anumbius annumbi* | | Uruguay | J.E. MacKinnon | 3 |
| 671.76 | 821 | 26092 | B-2721 | AY156975 | IHM 1746 | (b) nest of *Anumbius annumbi* | | Uruguay | [1] | 5 |
| 668.76 | 817 | 26088 | B-2717 | AY156973 | IHM 1610 | (b) armadillo burrow | | Uruguay | [1] | 5 |
| 669.76 | | 26090 | B-2719 | AY156974 | IHM 1740 | (c) palm tree fruit | | Uruguay | [1] | 3 |
| 101644 | | | B-5349 | AF549452 | | (c) maize kernel | | U.S.A. | J. Duvick | 2 |
| 425.92 | | | | AY156962 | | (c) heated apple juice | | Germany | R. Flörke | 1 |
| 236.93 | | | | AY156959 | | (c) heated apple juice | | Germany | R. Flörke | 1 |
| 101537 | | | | AY156970 | IFM 41846 | (c) rotten cactus | | Venezuela | K. Nishimura | 1 |
| 667.76 | 818 | 26089 | B-2718 | AY156964 | IHM 1611 | (d) fallen palm trunk | | Uruguay | [1] | 3 |
| 101533 | | | | AY156971 | IFM 41692 | (d) bark | | Venezuela | K. Nishimura | 2 |
| 110628 | | | | AY156966 | IFM 41855 | (d) bark | | Venezuela | K. Nishimura | |
| | 1236 | | | | 2503 | | | | K.J. Kwon-Chung | 3 |
| 101539 | | | | AY156969 | IFM 41856 | (e) soil | | Colombia | K. Nishimura | 3 |
| 101542 | | | | AY156967 | IFM 46126 | (e) soil | | Colombia | K. Nishimura | 5 |
| *E. attenuata:* | | | | | | | | | | |
| 101596 | | | | | IP 2133.93 | nasal granuloma | cat | France | [24] | |
| | 2905 | | | AF549446 | UTHSC 87-80 | human | | U.S.A. | | |
| 101540T | | | | | IFM 46115 | soil | | Colombia | K. Nishimura | |
| *E. nishimurae:* | | | | | | | | | | |
| 101538T | | | | AY156972 | IFM 41855 | bark | | Venezuela | K. Nishimura | |
| *E. jeanselmei:* | | | | | | | | | | |
| | 2670 = 2674 | | | AF549447 | UTHSC 86-72 | arm lesion | human | | M. Rinaldi | |
| 507.90T | | 34123 | | AY156963 | IHM 283 | mycetoma | human | Martinique | [41] | |
| 116.86 | | | | AY156965 | | mycetoma | human | Japan | [34] | |
| *C. epimyces:* | | | | | | | | | | |
| 101541 | | | | AY156968 | IFM 46117 | soil | | Brazil | K. Nishimura | |
| Unidentified: | | | | | | | | | | |
| 101534 | | | | | IFM 41698 | soil | | China | K. Nishimura | |
| 101535 | | | | | IFM 41700 | soil | | China | K. Nishimura | |

Abbreviations used: ATCC = American Type Culture Collection, Manassas, U.S.A.; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CDC = Centers for Disease Control, Atlanta, U.S.A.; CMM = Centro de Micologia Medica, Buenos Aires, Argentina; DSM = Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; IFM = Research Institute for Pathogenic Fungi, Chiba, Japan; IHM = Institute of Epidemiology and Hygiene, Montevideo, Uruguay, IP = Institut Pasteur, Paris, France; NCPF = National Collection of Pathogenic Fungi, Bristol, U.K.; NFC = Norman F. Conant Collection, Duke University, Duke, U.S.A.; NIH = National Institutes of Health, Bethesda, U.S.A.; OMH = Mycology, Laboratory Services Branch, Ontario Ministry of Health, Toronto, Canada; UTMB = University of Texas Mycology Branch, Galveston, U.S.A.

Antifungal susceptibility testing. Amphotericin B (AMB; Bristol-Myers Squibb, Woerden, The Netherlands), itraconazole (ITZ; Janssen-Cilag, Beerse, Belgium) and terbinafine (TBF; Novartis, Basel, Switzerland) were dissolved in dimethyl sulfoxide (DMSO), fluconazole (FLZ; Janssen-Cilag, Beerse, Belgium) and 5 fluorocytosine (5-FC; Hoffman La Roche, Basel, Switzerland) in water and aliquots of the stock solutions were stored at -70°C until used. Solutions were diluted in RPMI 1640 medium with L-glutamine and without bicarbonate (GIBCO BRL, Life Technologies, Woerden, The Netherlands) buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich, Steinheim, Germany). The range was 0.016–16 mg/ml for AMB, ITZ and TBF and 0.063–64 mg/ml for 5-FC and FLZ.

Isolates were subcultured on PDA for 10 days at 28°C to obtain adequate sporulation. Conidia were collected with a cotton swab and suspended in saline with 0.05% Tween 20. After the heavy particles had settled, 10 fold dilutions were made and conidial suspensions were adjusted microscopically by haemocytometer to $10^6$ conidia per ml. Viability was confirmed by plating serial dilutions on SGA culture plates. A broth microdilution method was performed according to NCCLS guidelines (M38-P [17]) using RPMI 1640 medium buffered to pH 7 with MOPS. The tests were performed in 96-well flat bottom microtitration plates (Corning, New York, NY, U.S.A) that were kept at -70°C until the day of testing. Conidial suspensions prepared as described above were diluted 1:50 in RPMI 1640 to obtain two times the desired inoculum. After the inoculation, the microtitration plates were incubated at 35°C and the MICs were read after at 72 and 96 h of incubation by a Rosys Anthos ht3 spectrophotometric reader (Anthos Labtex Instruments, Salzburg, Austria). The MIC for amphotericin B was defined as the lowest concentration of the drug that showed at least 95% reduction of growth compared with that of the growth control (MIC-0) by the following equation: [optical density (OD) 405 of wells that contained the drug / OD of the drug free well] X 100%. For ITZ, TBF, FLZ and 5-FC the MIC was defined as the lowest concentration of the drug that showed 50% growth reduction when compared with the control (MIC-2).

## Results

Sequencing of 30 strains revealed five subgroups within *E. spinifera* (Table 2, Fig. 1). Informative variation was found in 14 of 196 positions (7.1%) in ITS1 and in 10 of 175 positions (5.8%) in ITS2; the 157 positions of the 5.8S gene were identical in all strains.

**Table 2** Informative infraspecific ITS variability within *Exophiala spinifera*. Lengths of spacer domains are mentioned in brackets. Clade numbers 1–5 refer to groups distinguished in Fig. 1.

| ITS1 (196 bp) Position: | 19 | 23 | 55 | 81 | 117 | 119 | 142 | 149 | 158 | 161 | 173 | 178 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Clade: | | | | | | | | | | | | |
| 1 | CA | – | C | TT | A | C | A | – | – | A | A | A |
| 2 | CA | – | C | TT | A | C | A | – | – | A | A | C |
| 3 | T/CA | – | C | TT | T | C | A | – | A | A | A | A |
| 4 | CA | – | T | TT | A | C | A | T | – | A | A | A |
| 5 | TC | G | C | C– | A | T | C | T | – | G | G | A |

| (5.8S: 157 bp) ITS2 (175 bp) Position: | 44 | 50 | 56 | 89 | 128 | 133 | 135 | 158 |
|---|---|---|---|---|---|---|---|---|
| Clade: | | | | | | | | |
| 1 | C | C | G | C | C | C | T | CAC |
| 2 | C | C | C | C | C | C | C | CAC |
| 3 | C | C | G | C | T | A/C | T | CAC |
| 4 | A | C | A | C | T | A | T | CAC |
| 5 | C | G | G | T | C | C | C | –T |

ITS sequences of strains IFM 41698, IFM 41700, IFM 41855, IFM 46115, IFM 46117, UTMB 2670, UTMB 2674 and IP 2133.93, all morphologically identified as *E. spinifera*, were confirmed to deviate considerably from the *E. spinifera* ex-type strain, CBS 889.68. In order to establish the phylogenetic positions of these strains, SSU sequences were generated to determine the relationships of our strains with the previously established statistically supported *E. spinifera* clade [10].

In SSU sequence analysis, IFM 41698 was found to be located in the *E. spinifera* clade (data not shown), but its ITS sequence was too different from E. spinifera to allow meaningful alignment. The strain did not assimilate lactose and was weakly tolerant or intolerant to salts (5% $MgCl_2$ or NaCl; Table 3).

UTMB 2670 and 2674 were reidentified by ITS sequencing as *E. jeanselmei* (Langer.) McGinnis & A.A. Padhye (Fig. 1). IFM 41855 differed in 12 SSU sequence positions from the ex-type strain of *E. spinifera*, CBS 899.68 (data not shown). Physiologically it deviated from *E. spinifera* by being unable to assimilate meso-erythritol and D-glucuronate (Table 3).

IFM 46115 was strongly divergent. A more conservative ribosomal region, the 18S region, was sequenced. In this way, the strain was found to be located outside the *E. spinifera* clade, at a distance of 43 bases from the type strain, CBS 899.68 (data not shown). Two further strains, IP 2133.93 and UTMB 2905 had ITS sequences identical to that of IFM 46115.

The ITS sequence of IFM 46117 (=CBS 101541) was found to be nearly identical to that of *Capronia epimyces* M.E. Barr (Fig. 1); seven ITS base substitutions and two length mutations were found in otherwise highly variable regions. This affinity was confirmed by showing sequence identify in the SSU (data not shown).

**Table 3** Physiological profiles of related isolates compared with *Exophiala spinifera*

| | *Exophiala spinifera** | *E. attenuata* IFM 46115 | *E. nishimurae* IFM 41855 | *Capronia epimyces* IFM 46117 | unident. IFM 41698 | unident. IFM 41700 |
|---|---|---|---|---|---|---|
| D-Glucose | + | + | + | + | + | + |
| D-Galactose | + | + | + | + | + | + |
| L-Sorbose | + | + | + | + | + | + |
| D-Glucosamine | +,w | w | + | w | + | + |
| D-Ribose | + | + | + | + | + | + |
| D-Xylose | + | + | + | + | + | + |
| L-Arabinose | + | + | + | + | + | + |
| D-Arabinose | + | + | + | + | + | + |
| L-Rhamnose | + | + | + | + | + | − |
| Sucrose | + | + | + | + | + | − |
| Maltose | + | + | + | + | + | + |
| α,α-Trehalose | + | + | + | + | + | + |
| Methyl-α-D-Glucoside | +,w | + | w | w | + | − |
| Cellobiose | + | + | + | + | + | + |
| Salicin | + | + | + | + | + | + |
| Arbutin | v | + | + | + | + | + |
| Melibiose | +,− | + | w | + | + | − |
| Lactose | −,w | + | w | − | − | − |
| Raffinose | +,− | + | w | w | + | − |
| Melezitose | + | + | + | + | + | + |
| Inulin | −,w | − | w | + | + | − |
| Soluble starch | −,w | w | w | w | − | − |
| Glycerol | + | + | + | + | + | + |
| *meso*-Erythritol | + | + | − | − | + | + |
| Ribitol | + | + | w | w | + | + |
| Xylitol | + | + | − | w | + | + |
| L-Arabinitol | + | + | + | − | + | + |
| D-Glucitol | + | + | + | + | + | + |
| D-Mannitol | + | + | + | + | w | + |
| Galactitol | v | − | + | + | + | − |
| *myo*-Inositol | +,w | − | w | w | + | + |
| D-Gluconate | + | + | + | + | + | − |
| D-Glucuronate | + | + | − | − | − | + |
| D-Galacturonate | − | + | + | + | + | + |
| DL-Lactate | + | + | + | + | + | + |
| Succinate | +,w | + | + | + | + | − |
| Citrate | v | + | − | − | + | − |
| Methanol | − | − | − | − | − | − |
| Ethanol | + | + | + | + | − | + |
| Nitrate | + | + | + | + | + | + |
| Nitrite | + | + | + | + | + | + |
| Ethylamine | +,w | + | + | + | + | + |
| L-Lysine | +,w | + | w | + | + | + |
| Cadaverine | + | + | + | + | + | + |
| Creatine | + | + | w | + | + | + |
| Creatinine | + | + | w | w | + | + |
| 5% $MgCl_2$ | + | + | + | − | w | + |
| 10% $MgCl_2$ | +,w | + | + | − | − | + |
| 5% NaCl | +,w | + | − | − | − | + |
| 10% NaCl | −,w | − | − | − | − | − |
| 0.1% Cycloheximide | + | + | + | + | + | + |
| Mycosel (0.4%) | + | + | + | + | + | + |
| Urease | + | + | + | + | + | + |
| 30°C | + | + | + | + | + | + |
| 37°C | +,w | + | + | + | + | + |
| 40°C | −,w | − | − | − | − | − |

*Data summarized after De Hoog *et al.* [5].

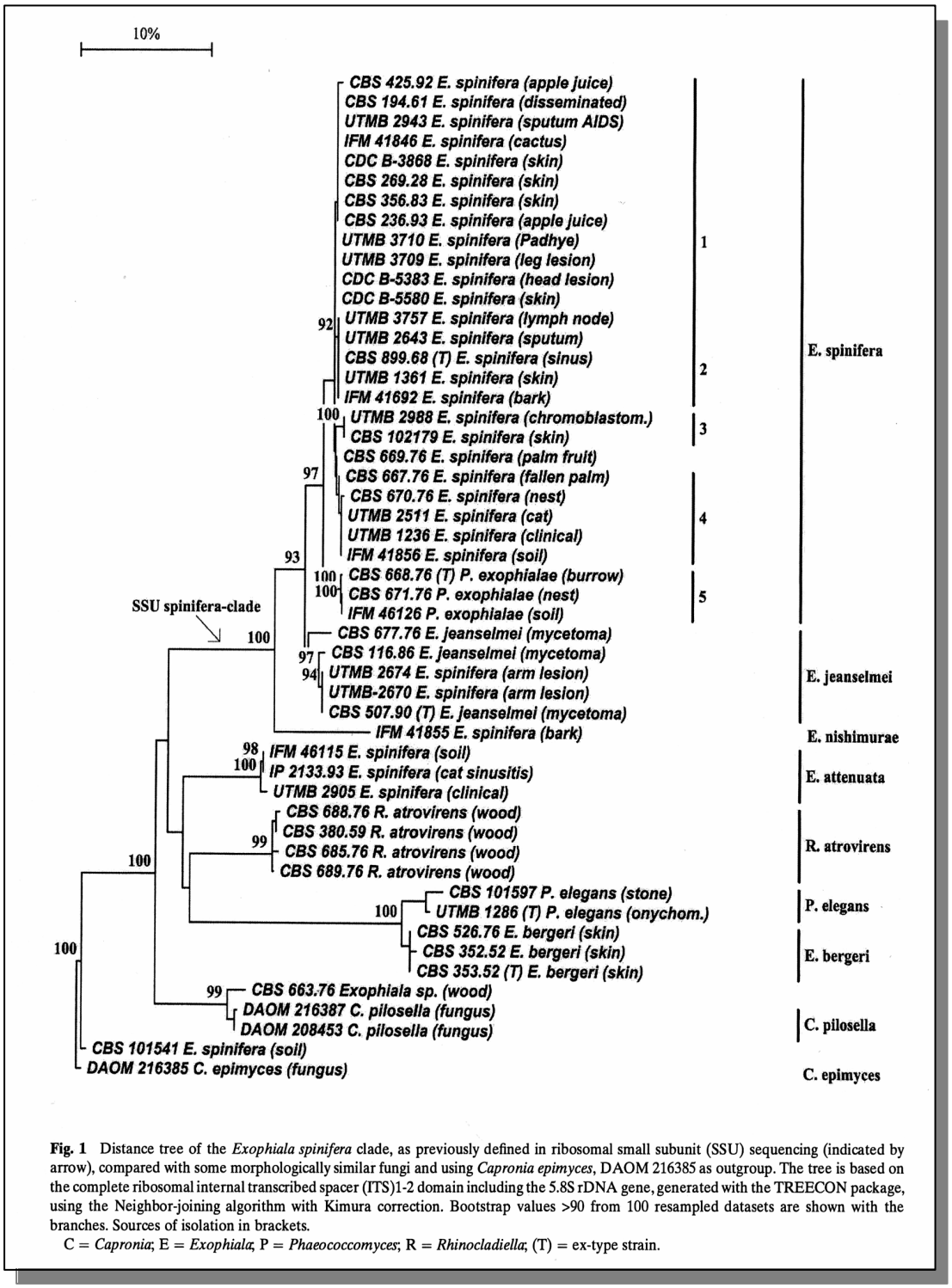


**Fig. 1** Distance tree of the *Exophiala spinifera* clade, as previously defined in ribosomal small subunit (SSU) sequencing (indicated by arrow), compared with some morphologically similar fungi and using *Capronia epimyces*, DAOM 216385 as outgroup. The tree is based on the complete ribosomal internal transcribed spacer (ITS)1-2 domain including the 5.8S rDNA gene, generated with the TREECON package, using the Neighbor-joining algorithm with Kimura correction. Bootstrap values >90 from 100 resampled datasets are shown with the branches. Sources of isolation in brackets.
C = *Capronia*; E = *Exophiala*; P = *Phaeococcomyces*; R = *Rhinocladiella*; (T) = ex-type strain.

## Discussion

The molecular taxonomy of *E. spinifera* based on ITS rDNA analysis was outlined by De Hoog et al. [5]. These authors concluded that *E. spinifera* could be recognized as a relatively well-delimited species, but that a subgroup of three strains was discernible which differed from the ex-type strain, CBS 899.68, in 15 positions. This group contained the ex-type culture of *Phaeococcomyces exophialae* de Hoog, CBS 668.76. In the present study we noted considerable infraspecific variation within *E. spinifera*; the question is whether this is taxonomically significant. The degree of variability within ITS domains of fungal species is known to differ considerably. The ITS2 subunit within ITS may be somewhat less variable than the ITS1 subunit, as was noted by Lieckfeldt & Seifert [19] in some members of the Hypocreales. Matos et al. [18], studying 48 strains of *E. dermatitidis* (Kano) de Hoog, found that variability was found in 5 of 202 bases (2.5%) in ITS1 and 2 of 209 (0.9%) in ITS2. In the Microascaceae both ITS1 and 2 showed very high, more or less equal rates of variability [20]. In contrast, in *Aureobasidium pullulans* (De Bary) Arnaud (Dothideales) ITS2 was 5 times more variable than ITS1 [21].

An explanation for these different degrees of variation is not available.

In Table 1 the strains are arranged according to their sources of isolation, which can be categorized as (a) mammalian invasive disease, (b) dwelling sites of warmblooded animals, (c) environmental surfaces rich in polysaccharides, (d) decayed plant material, and (e) soil. There is no apparent correspondence between these ecological backgrounds and the different genotypes obtained. Hence we consider *E. spinifera* to be a unit, despite its intrinsic ITS variation. No distinction of infraspecific formal entities is necessary for any of its five subgroups, including the group containing the type of Phaeococcomyces exophialae (group 5). De Hoog et al. [5] hesitated about a possible varietal status of the latter because all the strains attributed to this group were yeast-like and lacked conidiophores. However, this can be ascribed to the more general phenomenon of predominance of particular synanamorphs within individual strains of *E. spinifera* (see below).

The SSU sequence of IFM 41698, a strain originating from soil in China, was found to be located in the *E. spinifera* clade, but the large differences in ITS sequences showed that a separate species was concerned. The isolate was slow-growing and consisted of yeast-like cells and very scant hyphae with intercalary conidiogenous cells; upon receipt for our study, no structures similar to *E. spinifera* conidiophores were detected. It had a characteristic physiological profile in that it was negative for lactose assimilation and weakly tolerant or intolerant to salts. Its identity as a new species is thus confirmed, but because of its nondescript morphology we prefer to wait for additional strains before formally introducing it as a new taxon. For similar reasons, IFM 41700 was excluded from further study.

*C. epimyces*, a teleomorphic species with sequence similarity to our strain IFM 46117 (=CBS 101541), is a member of the *E. dermatitidis* clade [18], clearly phylogenetically separate from the E. spinifera clade. The physiological profile of IFM 46117 is similar to that of *C. epimyces* [12], but the former isolate is deviant in its ability to assimilate ribose and lactate. *E. spinifera* differs consistently from *C. epimyces* in its responses to seven compounds (compare De Hoog et al. [5]). *C. epimyces* is known to have an Exophiala anamorph and may exhibit an additional *Phialophora* synanamorph [25], not found in the present strain. Morphologically the *C. epimyces* anamorph is reminiscent of *Exophiala bergeri* Haase & de Hoog but differs by the frequent occurrence of large chlamydospore-like structures that might be ascoma initials. It and *E. bergeri* are physiologically distinguishable by growth responses to Lrhamnose, sucrose, melibiose, raffinose, meso-erythritol, xylitol and D-gluconate (compare Table 3 with De Hoog et al. [2, 26]). Both are able to grow at 37°C [2].

IFM 41855, originating from tree bark in Venezuela, was found to be a separate member of the *E. spinifera* clade based on SSU sequence data. It was morphologically similar to *E. jeanselmei* [5]. The strain was found to have a unique mtDNA profile [22]. Also it deviated physiologically from *E. spinifera* by being unable to assimilate meso-erythritol and D-glucuronate. In the ITS tree (Fig. 1) IFM 41855 was located clearly separate from both *E. jeanselmei* and *E. spinifera*, and was well aligned (approx. 10% ITS difference) with CBS 725.88 (data notshown). The latter strain, originating from a cerebral infection and reported as ‘‘*Exophiala* sp.’’ by Tintelnot et al. [23] will described

as a new species in a forthcoming paper (G.S. de Hoog, unpublished results). IFM 41855 is introduced below as a new species.

*Exophiala nishimurae* Vitale & de Hoog, Sp. nov (Fig.2). Coloniae ad 7 mm diam. post 10 dies, atrae; microcoloniae confluentes radiatim sulcatae et in agaro modice submersae, siccae sed textura butyrosa. Cellulae germinantes et gemmantes absentes; mycelium torulosum paucissimum. Hyphae dense aggregatae, angulis acutis ramosae, aequaliter pallide olivaceae, 2–3 µm latae, ad intervalla 12–20 µm septatae. Cellulae conidiogenae houd differentes, sessiles, laterales vel terminales ramulis insidentes, anguste ellipsoideae, zona annellata brevissima. Conidia subhyalina, ellipsoidea, 3-4 x 2.2–3.0 µm. Cellulae inflatae crassitunicatae, dilute olivaceo-brunneae, chlamydosporarum similes, ad 10 µm diam. partim formatae.

Typus CBS 101538 in herb. CBS praeservatus.

The following description is based on CBS 101538 (IFM 41855) after 10 d on SGA at 20–22°C:

Colonies attaining a diameter of 7 mm, black, consisting of confluent microcolonies leading to a soft felt with shallow radial fissures that are somewhat sunken into the agar, dry, with buttery texture. Germinating [27] and budding cells absent. Torulose mycelium nearly absent. Hyphae densely aggregated, branched at acute angles, evenly pale olivaceous, 2–3 µm wide, septate every 12–20 µm. Conidiogenous cells undifferentiated, sessile and lateral on hyphae, or terminal on short branches, narrow-ellipsoidal, producing conidia from very short annellated zones. Conidia subhyaline, ellipsoidal, 3–4 - 2.2–3.0 µm. Locally thick-walled, pale olivaceous-brown chlamydospore-like cells up to 10 mm diam present in lateral or terminal position.

Ex-type strain CBS 101538 = IFM 41855 (dried culture holotype in CBS herbarium), isolated from bark, Venezuela, K. Nishimura.

*E. nishimurae* as well as *E. bergeri* are able to grow at 37°C [2]. The latter species is known from two strains, which both were isolated from subcutaneous mycoses in humans [24, 29]. It is likely that *E. nishimurae* will also prove to be able to cause infections in humans. The low incidence of the two species may partly be caused by underdiagnosis due to the difficulty of correctly identifying black yeasts.

IFM 46115, originating from soil in Colombia, was 18S-sequenced in the present study. The sequence was compared with others in a nearly complete 18S sequence database for all known black yeasts (a portion of these data were published by Haase et al. [10]). The isolate was found to fall outside the *E. spinifera* clade, clustering in a group with low bootstrap support along with *Exophiala lecanii-corni* (Benedek & Specht)

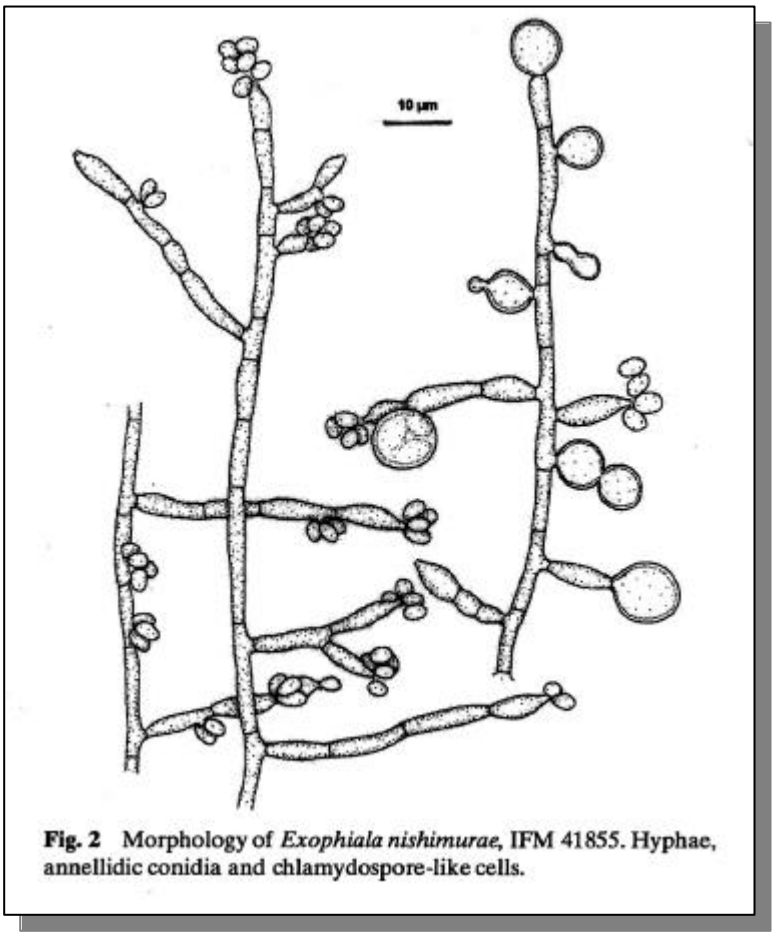


**Fig. 2** Morphology of *Exophiala nishimurae*, IFM 41855. Hyphae, annellidic conidia and chlamydospore-like cells.

Haase & De Hoog. De Hoog et al. [5] were unable to align much of the ITSdomain of IFM 46115 with that of *E. spinifera*, a problem that would be anticipated in organisms with this degree of SSU dissimilarity. These authors' published tree containing these fungi was mainly based the 5.8S gene and small parts of the ITS domain. Further investigation in the present study revealed two more strains, IP2133.93 from a lesion on a cat's nose [24] and UTMB 2905 from a human patient, with ITS sequences identical to

that of IFM 46115. These sequence data are surprising because at first sight the strains have the very characteristic morphology of *E. spinifera*, the only *Exophiala* species with large, erect and well-differentiated conidiophores. However, detailed morphological analysis of slide cultures showed that the deviating strains had conidiophores that were wide, thick- and dark-walled at the base and that tapered distinctly towards an apical region, terminating in a thin-walled, hyaline, very fragile conidiogenous cell. This cell had an inconspicuous annellated zone. In contrast, *E. spinifera* has cylindrical conidiophores with fusiform apical cells and very distinct annellated zones [2]. The atypical strains are physiologically different from *E. spinifera* in assimilating lactose and D-galacturonate but not myo-inositol (Table 3). The characters are judged sufficient to allow description of the strains as a new species, which will be introduced below.

*Exophiala attenuata* Vitale & de Hoog, sp. nov (Fig. 3). Coloniae zymatoideae, ad 12 mm diam. post 10 dies, minute cerebriformes, olivaceo-atrae, textura molli butyrosa; reversum olivaceo-atrum; coloniae hyphales ad 16 mm diam. post 10 dies, pulvinatae, in medio transverse sulcatae vel modice cerebriformes, deinde in medio diffractae et elevatae, locum aeratum sub agaro relinquentes, siccae, coactae, atro-griseae, marginem versus grisellae, reverso olivaceo-atro neque pigmentum exudantes. Cellulae gemmantes praesentes vel absentes, cellulae germinantes 7–9 x 4–7 mm; mycelium torulosum praesens vel absens. Hyphae obscurae et vulgo crassitunicatae, 2–4 m m latae, ad intervalla 12–20 µm septatae. Conidiophora erecta, ad basim 3 µm lata et crassitunicata, olivaceo-brunnea, sursum angustata et pallidiora, 70–100 µm alta, plerumque ter ad quinquies septata, saepe unum vel duos ramos vel cellulam conidiogenam ad quodque septum ferentia. Cellulae conidiogenae plerumque tenuitunicatae, subhyalinae, aculeatae vellageniformes, conidia e zona annellata brevi inconspicua proliferentes. Conidia (sub)hyalina, anguste ellipsoidea, 4–6 x 2–3 µm, deinde saepe inflata et dilute brunnea et crassitunicata. Phialides si praesentes ellipsoideae, collare terminale dilatatum e basi obscurata aperientes; phialoconidia hyalina, late ellipsoidea, minora quamannelloconidia, in capitulis densis adhaerentia.

Typus CBS 101540 in herb. CBS praeservatus.

The following description is based on CBS 101540 and CBS 101596 after 10 d on SGA at 20–22$^{o}$C:

Yeast-like colonies attaining a diameter of 12 mm, finely cerebriform, olivaceous-black, with a soft, buttery texture; reverse evenly olivaceous-black. Hyphal colonies attaining a diameter of 16 mm, cushion-shaped, evincing central transverse fissures or somewhat cerebriform, later centrally cracking, lifting up from the agar and building air spaces under the colony, dry, felty, blackish grey, dull grey towards the margin; reverse olivaceous-black. No blackish brown pigment exuded on any medium. Budding cells present or absent; germinating cells 7–9 x 4–7 µm. Torulose mycelium present or absent. Hyphae dark- and mostly thick-walled, 2–4 µm wide, septate every 12–20 µm. Conidiophores erect, 3 µmwide at the base, tapering towards the apex, basal part thick-walled, olivaceous-brown, terminal part thinwalled, hyaline; conidiophores 70–100 µm high, mostly with 3–5 transverse septa, often bearing one or two lateral branches at acute angles at each septum, dark brown near the base, becoming (sub)hyaline at the tip.
Conidiogenous cells mostly thin-walled, subhyaline, tapering or flask-shaped, producing conidia from inconspicuous annellated zones. Conidia (sub)hyaline, narrowly ellipsoidal, 4–6 x 2–3 µm, later often swelling and becoming pale brown and thick-walled. Phialides, when present, ellipsoidal, each bearing a terminal, wide collarette with darkened basis; phialoconidia hyaline, broadly ellipsoidal, somewhat smaller than annelloconidia, adhering in dense clumps.

Ex-type strain IFM 46115 = CBS 101540 (dried culture holotype in CBS herbarium), isolated from soil, Colombia; for additional strains, see Table 1.

The *E. attenuata* ITS domain deviates at 140 positions from that of *E. spinifera*. Morphologically, *E. attenuata* is distinct in having acicular conidiophores that are wide and thick-walled at the base, distinctly tapering towards the tip, with the upper half consisting of narrow, thinwalled and hyaline cells. In addition, the characteristic elongating annellated zones of *E. spinifera* are absent. *Exophiala jeanselmei* has single-celled, rocket-shaped conidiophores. There is some superficial similarity to *Phaeoacremonium* [30], particularly in the acicular conidiophore shape, but *Phaeoacremonium* is phialidic. *Kylindria* and *Xenokylindria* [31] have robust conidiophores abruptly tapering in a terminal annellated zone; their phylogenetic position probably is among the Sordariales.

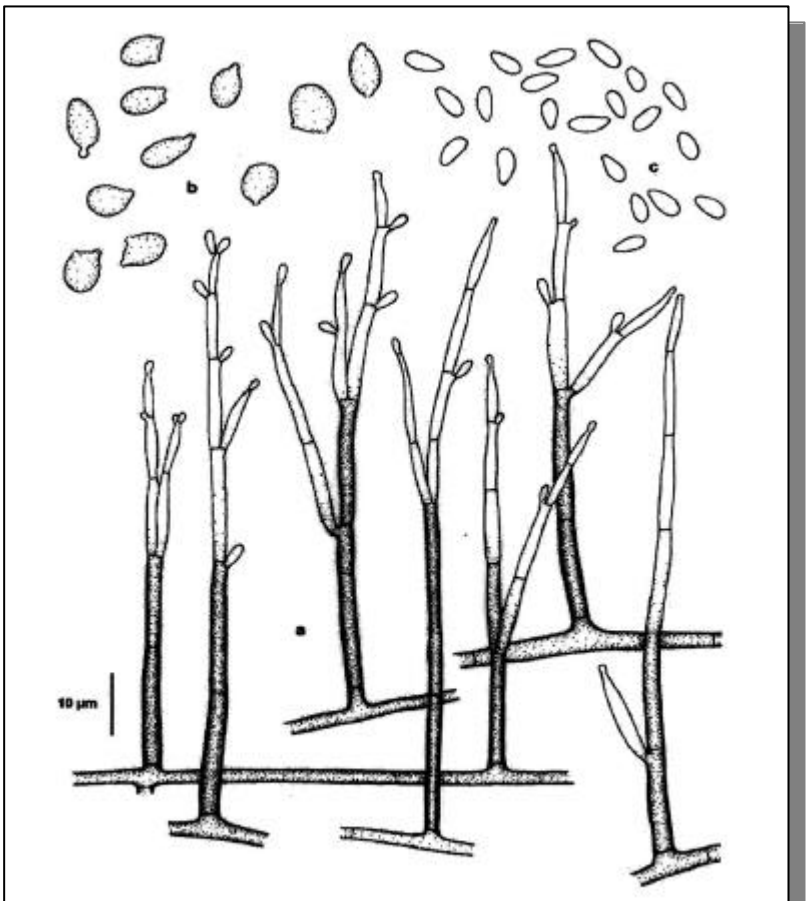


**Fig. 3** Morphology of *Exophiala attenuata*, IFM 46115. a. Conidiophores with terminal and lateral annellidic conidiogenous cells; b. Germinating cells; c. Conidia.

Two of our strains (UTMB 2905 and IFM 46115) converted into dry, filamentous growth with dark, thickwalled, strikingly curved cellular elements; the prevalent type of conidiogenesis in such cultures was phialidic rather than annellidic. Similar transition to preponderantly phialidic morphology is known in many black yeasts on nutritionally poor media [32,33]. The dry segregants were reminiscent of those described in *E. jeanselmei* as meristematic mutants [34]. In contrast, strain IP 2133.93 became more yeast-like with subculturing. This sudden preponderance of different types of growth and conidiation underlines the difficulties in morphological identification of black yeasts. Even the morphologically distinct species *E. attenuata* and *E. spinifera* can only be recognized when the annellidic anamorph is predominant.

**Table 4** Minimum inhibitory concentrations in µg/ml for the strains analyzed

| Drug | *Exophiala attenuata* UTMB 2905 | *E. attenuata* IFM 46115 | *E. nishimurae* IFM 41855 |
|---|---|---|---|
| FLZ | 8 | 16 | 32 |
| AMB | 4 | 2 | 0.25 |
| 5-FC | 8 | 4 | 0.5 |
| ITZ | 0.016 | 0.031 | 0.063 |
| VCZ | 0.031 | 0.063 | 0.5 |
| TBF | 0.125 | 0.25 | 0.25 |

The MICs of *E. attenuata* and *E. nishimurae* are summarized in Table 4; in Table 5 the geometric means and ranges are compared with those of *E. spinifera* and *P. exophialae*. ITZ, VCZ and TBF were effective against both *E. attenuata* and *E. nishimurae*. Activities against *E. spinifera* and *P. exophialae* were in the same range.

**Table 5** Geometric mean and range of MICs in µg/ml of the new species compared with *Exophiala spinifera* and *Phaeococcomyces exophialae* (number of strains tested in brackets)

| Drug | New species (3) | | *E. spinifera* (23) | | *P. exophialae* (3) | |
|---|---|---|---|---|---|---|
| | G mean | Range | G mean | Range | G mean | Range |
| FLZ | 16 | 8–32 | 55.05 | 16–128 | 101.59 | 64–128 |
| AMB | 1.26 | 0.25–4 | 0.70 | 0.125–4 | 0.40 | 0.25–0.5 |
| 5-FC | 2.52 | 0.5–8 | 3.44 | 0.125–128 | 0.79 | 0.25–2 |
| ITZ | 0.03 | 0.016–0.063 | 0.05 | 0.016–0.125 | 0.02 | 0.016–0.031 |
| VCZ | 0.10 | 0.031–0.5 | 0.30 | 0.031–1 | 0.10 | 0.031–0.25 |
| TBF | 0.20 | 0.125–0.25 | 0.26 | 0.031–2 | 0.16 | 0.125–0.25 |

FLZ showed a low degree of dose-dependent activity, *E. attenuata* being the most sensitive species. In contrast, AMB and 5-FC were least effective and showed variability among isolates. Therefore it will be appropriate to test susceptibility in any case isolates corresponding to this species prior to application of these drugs. It is apparent that in black yeasts susceptibility profiles may differ slightly with the species and may reflect its

phylogenetic position [35]. Proper identification at species level is recommended before onset of antimycotic therapy, at least in laboratories where access to rapid molecular techniques makes this practicable.

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# Chapter 2.2

*In vitro* activity of amphotericin B, itraconazole, terbinafine and 5-fluorocytosine against *Exophiala spinifera* and evaluation of post antifungal effects.

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# *In vitro* activity of amphotericin B, itraconazole, terbinafine and 5-fluorocytosine against *Exophiala spinifera* and evaluation of post antifungal effects

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Short title: Activity of antifungal drugs and PAFE in *Exophiala spinifera*

## Summary

Antifungal susceptibility profiles were determined for sixteen strains of the black yeast *Exophiala spinifera* applying different temperature regimens. Fluconazole was the least effective *in vitro*. Lowest minimal inhibitory concentration (MIC) values were found with itraconazole. The activities of antifungal agents against environmental and clinical strains were similar. Post antifungal effect (PAFE) of four drugs was determined for 11 clinical strains. PAFE was observed only for amphotericin B, with extended inhibition times seen at high drug concentrations.

Keywords: Antifungals, PAFE, black yeasts, *Exophiala*

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## Introduction

Black yeasts and their filamentous relatives in the order Chaetothyriales are found in the environment in a diversity of niches such as soil, fresh water, fungal fruiting bodies, moist tiles, surfaces of fruits, and creosote-treated wood. Their abundance is usually very limited, however, and they are therefore isolated only when selective techniques are applied. In comparison with this low density in nature, the occurrence of these fungi in clinical settings is remarkably high. Currently thirty species have been reported to be involved in mycoses of humans and other vertebrates [1]. In the past, the slow-growing black colonies of Chaetothyriales were frequently discarded as purported saprobic contaminants, but it is increasingly realized that these fungi may be the primary cause of a wide array of infections. The mycoses reported range from mild cutaneous forms to devastating systemic infections that are mostly fatal.

*Exophiala spinifera* is known as etiologic agent of disseminated infection [2], causing chronic systemic infections with disfiguring secondary cutaneous lesions in children and adolescents. Such infections are nearly always fatal [3-5]. However, about half of the strains held in collections were recovered from the environment [6]. Direct transmission from environmental sources to the symptomatic patient has as yet not been proven. Two possibilities exist. Clinical strains may be genotypically different from their environmental counterparts, e.g., in virulence, thermotolerance, ability to grow in submersion, or susceptibility to antimycotics. Alternatively, environmental strains may have genetic make-up and pathogenic potential similar to those of their clinical counterparts, and their occurrence in a habitat other than a human host may be coincidental. Clinical and environmental strains of the same species then should be treated with similar precautions. One of the aims of the present study is to investigate whether there are differences in antifungal drug susceptibility between strains from the environment and strains from proven cases of human infection.

The correlation between minimal inhibitory concentration (MIC) values obtained according to criteria of the National Committee of Clinical and Laboratory Standards [7] and the clinical response of infected patients remains problematic. MIC values,

determined in a system in which the fungus is exposed to a constant drug concentration, may be misleading with regard to the *in vivo* situation, where organisms usually experience fluctuating drug levels [8]. Using an animal model for *Aspergillus* infection, it was found that this *in vivo* fluctuation is especially important in amphotericin B therapy, but less important for itraconazole and voriconazole treatment [8].

The study of post antifungal effects (PAFE) could be helpful in determining the overall effect of antifungal drugs, since such studies simulate the in *vivo* situation which of fluctuating drug levels occur. In the present study, antifungal drug susceptibilities were studied comparing environmental and clinical strains. PAFE was also evaluated, being the first study of this effect in a member of the "black yeasts" (anamorphic Herpotrichiellaceae), exemplified by *Exophiala spinifera* strains.

## Materials and methods

***Strains and chemicals***

Eight clinical and eight environmental well documented isolates of *Exophiala spinifera* were used (Table 1). For PAFE 3 more clinical isolates were added. Drugs tested were amphotericin B (AMB) (Bristol-Myers Squibb, Woerden, The Netherlands), itraconazole (ITZ) (Janssen-Cilag, Beerse, Belgium), voriconazole (VCZ) (Pfizer Central Research, Sandwich, United Kindgdom), terbinafine (TBF) (Novartis, Basel, Switzerland), fluconazole (FCZ) (Pfizer Central Research, The Netherlands) and 5-fluorocytosine (5-FC) (ICN Pharma BV, Zoetermeer, The Netherlands). Aliquots of stock solutions were stored at –70ºC until used. All drugs with the exception of FCZ were dissolved in dimethylsulfoxide (DMSO) at concentrations of 3200 μg/ml or 3.2 mg/ml for AMB, ITZ and TBF, 12800 μg/ml for 5-FC, and 1600 μg/ml for VCZ. FCZ was dissolved in water at concentration of 25600 μg/ml. Two fold serial dilutions of the drugs were made in RPMI-1640 medium (GIFCO BRL, Woerden, The Netherlands) in order to obtain final concentrations that ranged from 0.015 to 16 μg/ml for AMB, ITZ and TBF; 0.063 to 64 μg/ml for 5-FC and 0.125 to 128 μg/ml for FCZ. RPMI 1640 medium (with L-glutamine, without bicarbonate) was buffered to pH 7.0 with 0.165 M morpholinopropansulfonic acid (MOPS) (Sigma-Aldrich, Steinheim, Germany).

**Table 1. Source and numbers of the strains tested. For detailed isolation data, see De Hoog *et al.* [6] and Vitale & De Hoog [13].**

| CBS | Other reference | Source |
|---|---|---|
| 425.92 | | Heated apple juice |
| | dH 11324 | Maize |
| 236.93 | | Apple juice |
| 669.76 | | Palm fruit |
| | IFM 41692 | Bark |
| | IFM 41856 | Soil |
| | IFM 41846 | Cactus |
| 667.76 | | Fallen palm |
| 194.61 | | Disseminated |
| 899.68 | ATCC 18218 | Nasal granuloma |
| | dH 11328 | Skin |
| | dH 11326 | Skin |
| 356.83 | | Skin |
| | dH 12309 | Chromoblastomycosis |
| | dH 11327 | Head lesion |
| 269.28 | | Skin |

**Abbreviations used: ATCC = American Type Culture Collection, Manassas, U.S.A.; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; dH = G.S. de Hoog private collection; IFM = Research Institute for Pathogenic Fungi, Chiba, Japan.**

***Susceptibility testing***

Isolates were subcultured serially at intervals of 5-7 days at 28ºC on potato dextrose agar (PDA) to obtain adequate sporulation. Conidia were collected with a cotton swab and suspended in saline with 0.5% Tween 20. After the heavy particles were allowed to settle, concentrations of conidia were determined microscopically by haemacytometer and adjusted to obtain a suspension of $10^6$ conidia/ml. Viability was confirmed by plating serial dilutions on Sabouraud glucose agar (SGA) incubated at 28ºC and determining colony counts. Inocula were prepared from conidial suspensions diluted 1:100 to a final concentration of 0.5 x $10^4$ to 4.5 x $10^4$ colony forming units (CFU) per ml.

A broth microdilution method was applied according to the NCCLS guideline M38-P [7]. Tests were performed in 96-well flat-bottom microtitration plates (Corning, Ames, MA, U.S.A.) which were kept at -70ºC until the day of testing. Conidial suspensions were diluted in RPMI 1640 to obtain two times the desired inoculum concentration. A drug-free well containing 0.01% DMSO in the medium served as the growth control. Plates were incubated at 28, 35 and 37ºC for 48, 72 and 96h. The MICs were determined using a spectrophotometric reader (Rosys Anthos ht3, Anthos Labtex, Salzburg, Austria). The relative optical densities (ODs) for each well based on measurements at 405 nm were calculated (in percent) based on the following equation: [(OD of drug-containing well – background OD)/(OD of drug-free well – background OD)] x 100%. The MIC for amphotericin B was defined as the lowest concentration of the drug that showed at least 95% reduction of growth compared with that of the growth control (MIC-0). For the remaining drugs, the MIC was defined as the lowest concentration of the drug that showed 50% reduction of growth compared with that of the growth control (MIC-2). All assays were done in duplicate.

***PAFE assay***

For PAFE 11 clinical isolates of *E. spinifera* were tested with AMB, ITZ, TBF and 5-FC applying the method adapted for use in filamentous fungi [9]. AMB, ITZ, TBF were dissolved in DMSO at initial concentrations of 400 μg/ml and 5-FC in water at an initial concentration of 3200 μg/ml. Stock solutions were stored at –70ºC until used. Then they were diluted in buffered RPMI 1640 medium. Serial dilutions of the drugs were made in

buffered RPMI 1640 at final concentrations of 4x and 1x the corresponding MIC for each strain for AMB and 5-FC, and 10x the MIC for ITZ and TBF. Control conidial suspensions were made in buffered RPMI 1640. Inocula were prepared as described above. The concentration of conidia was established microscopically by hemotocytometer Bürker-Türk chambers. Final concentration was adjusted to 4 x $10^5$ CFU/ml. Samples were incubated for 4 h with continuous shaking at 37ºC, washed three times with saline and centrifuged at 3,500 x *g* for 15 min. 98% of the supernatant was decanted and the sediment was resuspended in 10 ml buffered RPMI-1640. A procedure with two washings and removal of 90% of supernatant has been shown to reduce the antimicrobial concentration 100-fold, while with two washings and complete decanting the concentrations are reduced 10,000-fold [10]. Following this step, 100 µl of sample was diluted 10-fold in sterile water and 30 µl aliquots were plated on SGA for colony count determination, and incubated at 37ºC. The concentration of viable CFU/ml for exposed conidia was determined in order to verify the viability after drug exposure and to compare the CFU/ml both control with exposed cells. 200 µl of the resuspended suspensions were transferred to microtitration plates which were incubated at 37ºC in a computerized spectrophotometric reader (Rosys Anthos ht3, Anthos Labtex Instruments GmbH, Salzburg, Austria). Growth measured as change in turbidity at 405 nm was monitored automatically at 10 min intervals for 96 h. All assays were performed in duplicate.

***Data analysis***

Effects of drugs at different temperatures and incubation periods were analyzed for each isolate group (environmental and clinical) by ANOVA (Friedman test) and comparison between these by T-test (Mann Whitney test). P values of $<0.05$ were considered to be statistically significant.

PAFE was calculated using the point in the growth curve ($OD_0$ ) that corresponds with the first significant increase in OD as was described previously [9] by using the formula PAFE = T - C, where T was the time of the first significant increase in $OD_0$ of the exposed spores after removal of the drug and C was the time of the first significant increase in $OD_0$ of the control. Thus, PAFE was defined as the difference in time ($\Delta t$)

between exposed and controls to reach the defined point in the growth curve and was expressed in hours. The time to reach this chosen point $OD_0$ of at least 6 controls for each species was calculated and the mean, range, upper 95% confidence interval (CI) and the coefficient of variation were calculated in order to determine the reproducibility of the control curves at that point and to establish the reproducibility of the experiments. For each species the upper 95% CI of the controls was chosen as the cut-off level that distinguished between presence or absence of PAFE.

When re-growth of the exposed isolates occurred within the upper 95% CI time-frame of the controls, PAFE was considered to be absent. Alternatively, if re-growth was delayed following drug exposure and the lower 95% CI of the exposed isolates was delayed until beyond the upper 95% CI of the controls, PAFE was considered to be present. Growth curves of each exposed isolate were compared only with pooled controls from that same isolate.

## Results

The geometric means and the range of MICs obtained after 72h are given in Table 2. No significant differences were found in MICs after 72 and 96h of incubation (data not shown). The most active drug was ITZ against both environmental and clinical strains regardless of the temperature of incubation with geometric mean 0.04 and 0.06 μg/ml, respectively. VCZ (G means 0.4 and 0.2 μg/ml, respectively) and TBF (G mean 0.2 and 0.3 μg/ml, respectively) at 35ºC were also inhibitory. AMB susceptibility values varied among the isolate classes, environmental and clinical, with G mean at 35ºC of 0.3 for environmental isolates and 0.9 for clinical isolates. Differences were also seen in 5-FC values which were 1.83 for environmental and 5.66 clinical isolates. FCZ showed little effect but some strains showed MIC values of 8-16 and 32 μg/ml at all temperatures.

Statistical analysis was performed for each drug under different incubation regimens. Susceptibilities of environmental strains tested (a) after incubation periods of 72 and 96h were compared at 28, 35 and 37ºC. The same was done (b) among the clinical strains, and finally pooled data of environmental and clinical strains (c) were compared: for (a) and (b) no statistically significant differences were observed ($P>0.05$); for (c) no

statistically significant differences were found between incubation periods, but a temperature-related difference was observed with AMB ($P<0.05$) between environmental and clinical strains.

Table 2. MICs of environmental and clinical strains at different temperatures after 72 h.

**Gm: geometric mean.**

| Drugs | Environmental | | | | | | Clinical | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 28ºC | | 35ºC | | 37ºC | | 28ºC | | 35ºC | | 37ºC | |
| | Gm | Range | Gm | Range | Gm | Range | Gm | Range | Gm | Range | Gm | Range |
| AMB | 0.77 | 0.125-4 | 0.35 | 0.125-0.5 | 0.59 | 0.125-2 | 1.30 | 0.25-4 | 0.92 | 0.5-2 | 1.09 | 0.5-4 |
| FCZ | 26.91 | 16-64 | 41.50 | 16-128 | 38.05 | 16-64 | 32.00 | 8-64 | 45.25 | 16-128 | 38.05 | 8->128 |
| VCZ | 0.25 | 0.125-0.5 | 0.46 | 0.125-4 | 0.25 | 0.063-0.5 | 0.28 | 0.031-1 | 0.27 | 0.031-0.5 | 0.25 | 0.063-0.5 |
| TBF | 0.12 | 0.031-0.5 | 0.25 | 0.031-2 | 0.30 | 0.031-1 | 0.63 | 0.5-1 | 0.30 | 0.031-1 | 0.35 | 0.063-1 |
| 5-FC | 3.17 | 1-8 | 1.83 | 0.125-64 | 1.83 | 0.25-16 | 2.69 | 0.25-16 | 5.66 | 0.5-64 | 5.19 | 1-16 |
| ITZ | 0.02 | 0.016-0.031 | 0.04 | 0.016-0.125 | 0.05 | 0.016-0.125 | 0.07 | 0.031-0.125 | 0.06 | 0.031-0.125 | 0.05 | 0.016-0.125 |

Eleven clinical isolates were selected for PAFE experiments. The MIC range were from 0.5 to 2 μg/ml for AMB, from 0.031 to 0.125 μg/ml for ITZ, from 0.063 to 0.5 μg/ml for TBF and from 0.5 to 64 μg/ml for 5-FC. When PAFE was present the growth curve of the exposed conidia shifted to the right compared with that of the control (Fig. 1). After 4 h of incubation at 4x MIC all strains displayed significant PAFE with AMB with a mean of 16.2 h and a range of 9.7 to 19 h. An example of PAFE observed in strain dH 12309 is shown in Fig. 1. For 1x MIC, 7 out of 11 strains tested showed PAFE, with a mean of 9.8 h and a range of 1.6 to 16 h (Table 3). For the other drugs PAFE was not observed (data no shown). The growth curve after exposure to ITZ for strain dH 12309 is given in Fig. 2 where PAFE was not observed at 4 and 10 times the MIC.

Table 3. PAFEs induced by exposure of ***Exophiala spinifera*** to amphotericin B at concentrations of 4 and 1 times the MIC, after 4 h of exposure using $OD_0$ criteria.

| Strain | Control (U 95% CI) | 4x MIC (L 95% CI) | PAFE (h) | 1x MIC (L 95% CI) | PAFE ( h) |
|---|---|---|---|---|---|
| 12309 | 35.6 | 47.9 | 18.4* | 36.1 | 7.7* |
| 194.61 | 40.6 | 43.4 | 19.0* | 43.9 | 16.0* |
| 12308 | 55.7 | 62.0 | 10.7* | 54.5 | 6.2 |
| 12301 | 56.7 | 57.4 | 9.7* | 54.1 | 8.9 |
| 356.83 | 39.0 | 47.4 | 19.0* | 42.8 | 13.5* |
| 12304 | 46.3 | 57.9 | 19.0* | 49.6 | 14.5* |
| 11328 | 42.3 | 49.9 | 18.6* | 49.0 | 13.1* |
| 899.68 | 57.7 | 63.9 | 16.4* | 59.3 | 10.6* |
| 11327 | 12.2 | 25.3 | 16.5* | 10.5 | 10.2 |
| 12312 | 18.1 | 34.7 | 13.0* | 18.7 | 6.0* |
| 269.28 | 24.8 | 39.3 | 18.0* | 13.3 | 1.6 |

U 95% CI: Upper 95% confidence interval

L 95% CI: Lower 95% confidence interval

*: statistically significant PAFE

Fig. 1. PAFE of 18.4 h observed in strain dH 12309 after exposure to amphotericin B for 4 h at 4 times the MIC.

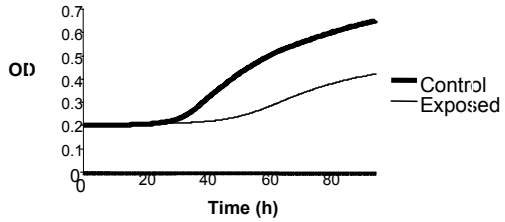
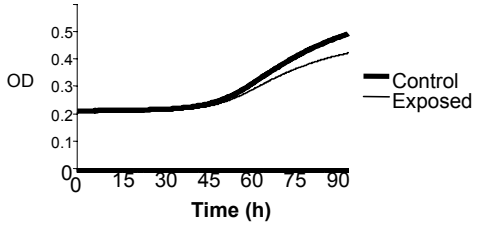



Fig. 2. PAFE absent from strain dH 12309 after exposure to itraconazole for 4 h at 4 times the MIC. The same graph was obtained after exposure to drug at 10 times the MIC.




## Discussion

Our antimycotic susceptibility data for AMB and VCZ are similar to those given by McGinnis & Pasarell [11], but we obtained higher susceptibility values for ITZ. Our ITZ, TBF, VCZ and AMB MIC values matched those of Meletiadis et al. [12].

Since *Exophiala spinifera* is encountered in soil and in association with plant material such as maize ears and fruit juices , it is presumably a saprotrophic fungus. However, about half of the strains collected to date were obtained as agents of skin and soft-tissue infections or of fatal, systemic infections [6]. Traumatic cutaneous or subcutaneous infections have mainly been observed in adults, while systemic cases for which no portal of entry has been defined, have occurred only in children and

adolescents. This remarkable ecological and clinical spectrum of *E. spinifera* raises the possibility that some strains may be more tolerant than others of higher temperatures. Our results, however, show that susceptibilities to antifungal agents generally were not influenced by temperature. The only exception was a small but statistically significant difference in activity of AMB at different temperatures (table 2). This deviation was based on a single difference of two dilution steps.

Recently we described a new species, *E. attenuata*, which is morphologically similar to *E. spinifera* and which as yet has not been reported to cause infection in humans [13]. Whether it has an opportunistic potential to humans similar to that of *E. spinifera* is not known. The close phylogenetic kinship of the two fungi suggests this potential. The etiologic agent of a case in a cat ascribed to *E. spinifera* by Chermette et al.[14] was later recognized to be *E. attenuata* by Vitale & De Hoog [13]. The significance of phylogeny for predicting the clinical significance of fungal species was stressed earlier by De Hoog [15] and McGinnis & Pasarell [11]. De Hoog [16] used phylogenetic relationships with known human pathogens as one of the criteria to assign fungal species to a particular BioSafety Level. In view of this predicted opportunistic potential we included environmental *E. spinifera* isolates in our study.

Therapeutic measures used in cases of *E. spinifera* infection have been highly diverse, including surgical excision, systemic use of antifungals, or a combination of both. Relapses have frequently been observed, and therefore long-term antimycotic therapy seems mandatory. The optimal treatment is still in dispute [17], the efficacy of applied antifungal therapy regimens is variable. Rajam et al. [3], Lacaz et al. [18], Dai et al.[4] Dieye et al. [19], Wang et al. [20], Mirza et al. [21], Campos et al.[22], Padhye et al.[5] and Lacroix et al. [23] refrained from use of any therapy, while Nielsen & Conant [2], and Gold et al.[24] used surgery alone. A chromoblastomycosis-like infection reported by Barba et al. [25] was successfully treated with 200 mg ITZ daily for 12 months. The isolate involved had an *in vitro* MIC value of 0.031 µg/ml. Padhye et al. [26] in treating a similar case caused by strain CDC B-5383 used cryosurgery followed by KTZ (up to 400 mg/d), 5-FC and FCZ, all without success. The *in vitro* data showed that the strain had an MIC of 64 µg/ml for FCZ, 2 µg/ml for 5-FC and 0.125 µg/ml for ITZ. Clinical improvement was obtained with the combination of ITZ and 5-FC, but the

patient later relapsed. A subcutaneous lesion caused by isolate CDC B-3868 was treated by Padhye et al. [27] using KTZ monotherapy. The dose was raised from 200 mg/d to 400 mg/d during treatment. After 6 weeks, growth of the isolate was again obtained from the lesion, and hence the therapy was changed to KTZ (600 mg/d) plus 5-FC (2 g/d). The lesion resolved after 1 month. Our present data showed a 5-FC MIC value of 4 µg/ml for the same strain; hence the healing that occurred should be ascribed to KTZ. Sharkey et al. [28] described cases of phaeohyphomycoses by members of various dematiaceous genera where treatment with amphotericin B, ketoconazole or miconazole appeared inadequate. Among these cases was an infection in a joint due to *E spinifera.* A total dose of 450 mg amphotericin B was given within a 1 month which led to improvement, although subsequent cultures remained positive after azole therapy. Ketoconazole was given at 100 mg/day during 8 months, subsequently 400 mg/day for four more months, but progression was observed. Further, itraconazole with a daily dose of 100 mg was administered, but total remission after 8 months was observed. The *in vitro* MIC for this strain was 0.15 µg/ml. Kotylo et al. [29] administered KTZ 200 mg twice daily to their patient, but the lesion did not improve. They then performed surgery followed by ITZ therapy of 50 mg/day for 6 weeks, then 100 mg/day for 5 weeks. This treatment was successful. Negroni et al. [30] documented a patient with a skin lesion and adenopathy who was treated with ITZ 5 g/day and 5-FC 200 mg/day for 8 months. Improvement was noted, but two years later relapse occurred. The same was observed with ITZ 200 mg/day and 5-FC 6 g/day for 4 months, even after the ITZ dosage was raised to 400 mg/day. AMB and liposomal AMB were then started but discontinued due to serious adverse effects. Thereafter, 400 mg/day ITZ, 6 g/day 5-FC and 300 mg/day ranitidine were given for 8 months, leading to remission. The *in vitro* MIC values for this strain were 0.031 µg/ml for ITZ and 4 µg/ml for 5-FC. Treatment of two cases in cats [14, 31] was unsuccessful. It should be noted, that the causative agent in the case reported by Chermette et al.[14] has been re-identified as *E. attenuata* Vitale et al [13]; the strain of Kettlewell et al. [31] is not known to be preserved.

*In vivo* antimycotic data for *E. spinifera* are scant. Most existing studies are based on older, less effective compounds. The overview given above clearly illustrates the persistent nature of infections caused by *E. spinifera*. Relapse is common despite

prolonged treatment, and poor resolution is frequently obtained despite relatively promising MIC values for the drug used. Correspondence of *in vitro* data to *in vivo* efficacy seems to be low.

Post antibiotic effect (PAE), i.e., persisting suppression of growth of a microbe after limited exposure to an antibiotic compound, may simulate the situation *in vivo* better than standard MIC testing does, since the organism is exposed only temporarily to an inhibitory drug level. The PAE effect was first reported with bacteria [10]. It was found to be dependent on several factors such as concentration of the drug, exposure time, inoculum, and type of medium. Later, similar phenomena were observed in fungi and referred to as Post Antifungal Effect (PAFE) [9, 32]. In our study we found that PAFE was dependent on concentration. This is slightly different from data reported in *Aspergillus* [9], with *Exophiala* longer values of PAFE being obtained. *Candida* species also displayed relatively long PAFEs when exposed to amphotericin B at concentrations above the MIC, and relatively short PAFEs after exposure to sub-MIC drug levels [32]. The inability of itraconazole to induce PAFE in *Exophiala* is in accordance with observations reported for *Aspergillus* and *Candida* where no measurable PAFE was observed following exposure to azoles [9, 32, 33]. The absence of PAFE mediated by these drugs may be related to the different mechanisms by which the drugs act. AMB acts directly by binding to sterols in the fungal cell and altering membrane permeability, which leads to cell death, whereas azoles or allylamines interfere with the biosynthesis pathway of ergosterol. The exposure times applied in PAFE testing may be too short to allow these drugs to exert a significant action. PAFE was not observed when *E. spinifera* was tested with 5-FC. PAFE was induced with 5-FC PAFE in *Candida* [34]. The discrepancy is perhaps explained by the fact that these authors used YNB media rather than RPMI-1640. YNB was found as more nutritious media provided highest growth compared with RPMI-1640 [35]. The action of the drug is dependent in the fungi growth, so might be possible that the media influence in the presence of PAFE.

PAFE is an alternative *in vitro* susceptibility assay that may provide additional information on the interaction of antifungal agent and fungus. *In vivo* experiments are needed to evaluate the importance of this phenomenon as a method to determine antifungal therapeutic regimens.

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# Chapter 2.3

In vitro activity of amphotericin B and itraconazole in combination with flucytosine, sulfadiazine and quinolones against *Exophiala spinifera.*

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# In vitro activity of amphotericin B and itraconazole in combination with flucytosine, sulfadiazine and quinolones against *Exophiala spinifera*

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## Abstract

The combined effects of antifungal and antibiotic drugs against *Exophiala spinifera* were evaluated in vitro by the chequerboard method, calculated as a fractional inhibitory concentration (FIC) index. Amphotericin B was combined with flucytosine and ciprofloxacin, whereas itraconazole was combined with ciprofloxacin, levofloxacin, lomefloxacin and sulfadiazine. Synergic effects were observed for the combinations of itraconazole with ciprofloxacin and levofloxacin, and amphotericin B with ciprofloxacin and flucytosine. No antagonism was observed for any combination tested.

**Keywords**: *Exophiala*, black yeasts, quinolones, drug combinations

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Black yeast-like fungi are increasingly being recognized as potential human pathogens. Clinical pictures include either subcutaneous or systemic mycetoma, chromoblastomycosis and phaeohyphomycosis.[1]

As model species we selected *Exophiala spinifera*. Although this is a very rare agent of disease in humans, it is one of the most aggressive species of black yeast, potentially causing disseminated infections with fatal outcome in children and adolescents. In adults, in contrast, infections are mostly localized or subcutaneous lesions.[2]

The optimal therapy for black yeast infections is controversial. Surgical excision, antifungal drug monotherapy or drug combinations have been used, but prolonged treatment is needed because relapses often occur. Consequently, new approaches to treatment are overdue.

Quinolones are potent inhibitors of DNA gyrase and are extensively used in clinical practice for bacterial infections.[3]

Sulphonamides act as competitive antagonists of p-aminobenzoic acid (PABA), which is an integral component of the structure of folic acid. Decreased folic acid synthesis results n a decrease in nucleotides with subsequent growth inhibition.[4] Both types of drug have favourable pharmacokinetic profiles in cerebrospinal fluid and bone.[4]

In one report, a nodule on a finger was treated with surgical excision and three antifungals, but after a month another lesion appeared in the left arm with no healing of the finger lesion. In this case, treatment with co-trimoxazole was initiated on diagnosis of a *Nocardia* superinfection, and after 11 days both lesions had improved. This suggests that sulphonamides might be effective in treating this infection.[5]

Quinolones have a broad spectrum of activity as inhibitors of DNA gyrase, type 2 topoisomerase, which is present in prokaryotes and eukaryotes. The presence of high levels of topoisomerases I and II has been reported in pathogenic fungi.[6] Although they are inapplicable as sole antifungal agents, quinolones augment the activity of amphotericin B and azoles.[7,8] For example, in a murine model study of candidiasis, similar survival rates were demonstrated in mice treated with fluconazole alone 80 mg/kg/day and in those treated with fluconazole (40 mg/kg/day) and ciprofloxacin.[8]

The aim of the present study was to investigate the in vitro activity of quinolones and sulfadiazine, either alone or in combination with amphotericin B or itraconazole, as well as the combination of amphotericin B and flucytosine against *E. spinifera* strains.

Thus, for this study eight clinical and two environmental well-documented isolates of *E. spinifera* were used (Table 1).

**Table 1.** Numbers, MIC data and source of the strains tested [2,10]

| | | MIC (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CBS | Other reference | AMB | ITZ | 5-FC | CIP | LOM | LEV | SDZ | Source |
| 425.92 | | 1 | 0.031 | 4 | >16 | >12 | >20 | >160 | heated apple juice |
| 236.93 | | 0.5 | 0.031 | 8 | >16 | >12 | >20 | >160 | apple juice |
| 194.61 | | 1 | 0.063 | 16 | >16 | >12 | >20 | >160 | disseminated |
| 899.68 | ATCC 18218 | 0.25 | 0.125 | 8 | >16 | >12 | >20 | >160 | nasal granuloma |
| | dH 11328 | 0.5 | 0.031 | 32 | >16 | >12 | >20 | >160 | skin |
| | dH 11326 | 2 | 0.063 | 4 | >16 | >12 | >20 | >160 | skin |
| 356.83 | | 1 | 0.125 | 4 | >16 | >12 | >20 | >160 | skin |
| | dH 12309 | 2 | 0.031 | 16 | >16 | >12 | >20 | >160 | chromoblastomycosis |
| | dH 11327 | 1 | 0.125 | 2 | >16 | >12 | >20 | >160 | head lesion |
| 269.28 | | 1 | 0.063 | 2 | >16 | >12 | >20 | >160 | skin |

ATCC, American Type Culture Collection, Manassas, VA, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; dH, G. S. de Hoog private collection; AMB, amphotericin B; ITZ, itraconazole; 5-FC, flucytosine; CIP, ciprofloxacin; LOM, lomefloxacin; LEV, levofloxacin; SDZ, sulfadiazine.

Drugs tested were amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands), itraconazole (Janssen-Cilag, Beerse, Belgium), flucytosine (ICN Pharma BV, Zoetermeer, The Netherlands), ciprofloxacin (Bayer AG, Leverkusen, Germany), lomefloxacin (Searle Nederland, Maarssen, The Netherlands), levofloxacin (Hoechst Pharma, Amsterdam, The Netherlands) and sulfadiazine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All drugs were dissolved in dimethylsulphoxide (DMSO), with the exception of flucytosine and ciprofloxacin, which were dissolved in water.

Two-fold serial dilutions of the drugs were made in RPMI-1640 medium (Gibco BRL, Woerden, The Netherlands) to obtain final concentrations that ranged from: 0.015 to 8 mg/L for amphotericin B; 0.02 to 1 mg/L for itraconazole; 0.25 to 128 mg/L for flucytosine; 0.125 to 8 mg/L for ciprofloxacin; 0.09 to 6 mg/L for lomefloxacin; 0.15 to

10 mg/L for levofloxacin; and 2.5 to 160 mg/L for sulfadiazine. RPMI-1640 medium (with L-glutamine, without bicarbonate) was buffered to pH 7.0 with 0.165 M MOPS (Sigma-Aldrich, Steinheim, Germany).

Tests were performed in 96-well flat-bottom microtitration plates (Corning, Ames, MA, USA), which were kept at −70°C until the day of testing. The MIC of each drug alone was tested by a broth microdilution method, according to NCCLS guidelines (M38-P)[9] that provide for testing of polyenes, flucytosine and azole antifungal agents against filamentous fungi.

Conidial suspensions were diluted in RPMI-1640 to obtain twice the desired inoculum concentration. A drug-free well containing 0.01% DMSO in the medium served as the growth control for the drugs dissolved in this solvent. Plates were incubated at 35°C for 72 h. The MICs were determined by spectrophotometry. The relative optical densities (ODs) for each well based on measurements at 405 nm were calculated (as a percentage) based on the following equation: [(OD of drug-containing well – background OD)/(OD of drug-free well – background OD)] × 100%. The MIC of each drug alone was defined as the lowest concentration of the drug that showed at least 95% reduction of growth compared with that of the growth control (MIC-0) for amphotericin B, quinolones and sulfadiazine, and for flucytosine and itraconazole as the lowest concentration of the drug that showed 50% reduction of growth compared with that of the growth control (MIC-2).

A two-dimensional, two-agent broth microdilution chequerboard method was used to study the interaction between the drugs. For all the combinations tested, MIC endpoints considered were MIC-0 when amphotericin B was combined and MIC-2 when itraconazole was combined with the corresponding drug. The FICs of both drugs used in combination were calculated and added to obtain the FIC indices. The FIC index was calculated as follows: (MIC of drug A + drug B/MIC of drug A) + (MIC of drug A + drug B/MIC of drug B).

Drug interactions were defined as synergic if the FIC index was =0.5, antagonistic if the FIC index was >4 and noninteractive between 0.5 and 4.

The MIC of amphotericin B ranged from 0.25 to 2 mg/L; that of itraconazole from 0.031 to 0.125 mg/L; that of flucytosine from 2 to 32 mg/L. For sulfadiazine and quinolones no

activity was found with the drug alone (Table 1). Amphotericin B combined with flucytosine showed a synergic effect for five strains and no interaction for four. For amphotericin B plus ciprofloxacin, a synergic effect was observed against five strains and no interaction for two (Table 2).

Itraconazole combined with levofloxacin, lomefloxacin and ciprofloxacin showed synergic activity against seven, four and six strains, respectively. No interaction when combined with itraconazole was observed with six strains for lomefloxacin, four strains for ciprofloxacin and three strains for levofloxacin. When itraconazole was combined with sulfadiazine, synergic activity was observed against five strains and no interaction against three strains (Table 2). No antagonistic effect was observed in any combination.

**Table 2.** Amphotericin B and itraconazole in combination with quinolones, flucytosine or sulfadiazine

| | Amphotericin B | | | | Itraconazole | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ciprofloxacin | | flucytosine | | ciprofloxacin | | levofloxacin | | lomefloxacin | | sulfadiazine | |
| Strains[2,10] | FICI | INT | FICI | INT | FICI | INT | FICI | INT | FICI | INT | FICI | INT |
| 356.83 | ND | ND | 0.37 | SYN | 1.06 | NI | 1.00 | NI | 0.75 | NI | 1.00 | NI |
| 899.68 | 0.18 | SYN | ND | ND | 0.28 | SYN | 0.25 | SYN | 0.75 | NI | 0.31 | SYN |
| 11326 | 0.37 | SYN | 0.31 | SYN | 0.078 | SYN | 0.015 | SYN | 0.09 | SYN | 1.00 | NI |
| 194.61 | 1.00 | NI | 0.37 | SYN | 0.5 | SYN | 0.13 | SYN | 0.09 | SYN | 0.13 | SYN |
| 11327 | 0.25 | SYN | 0.75 | NI | 0.31 | SYN | 0.25 | SYN | 1.00 | NI | 0.13 | SYN |
| 425.92 | 1.00 | NI | 0.75 | NI | 1.00 | NI | 1.00 | NI | 0.62 | NI | 1.00 | NI |
| 269.28 | 0.25 | SYN | 0.37 | SYN | 0.09 | SYN | 0.02 | SYN | 0.09 | SYN | 0.13 | SYN |
| 12309 | 0.51 | NI | 0.75 | NI | 0.62 | NI | 0.13 | SYN | 0.75 | NI | ND | ND |
| 236.93 | 0.5 | SYN | 0.31 | SYN | 0.5 | SYN | 0.07 | SYN | 0.13 | SYN | 0.13 | SYN |
| 11328 | 0.75 | NI | 0.75 | NI | 0.62 | NI | 1.00 | NI | 1.5 | NI | ND | ND |

FICI, fractional inhibitory concentration index; SYN, synergic; NI, no interaction; INT, interpretation; ND, not determined.

Only very few in vitro and animal studies have been carried out with this group of fungi. One study, on a central nervous system phaeohyphomycosis in mice infected with three different genera of black fungi. *Ochroconis constricta* showed good correlation in vitro–in vivo with amphotericin B, although high doses of amphotericin B were needed. For *Cladophialophora bantiana*, flucytosine was the most effective, but no single drug achieved full recovery. This drug was also the most effective against *Exophiala dermatitidis*.[11]

Quinolones and sulfadiazine were selected for this study because of their favourable distribution in the body and their ability to enhance antifungal activity when used in combination.

Flucytosine was selected as a classic drug that possesses activity against black fungi. Our study indicates that for some isolates, quinolones augmented the activity either of itraconazole or amphotericin B when combined, since synergic effects were sometimes observed and antagonism not demonstrated for any combination tested. The concentrations tested can be achieved in vivo. Enhanced activity of antifungal agents combined with these classes of antimicrobials has also been found for *Candida* species, although occasionally discrepancies were observed between in vitro and in vivo data.8,10

In our study, we found synergic interaction when sulfadiazine were combined with itraconazole. No effect was observed in E. spinifera when the drug was used alone. The blood levels that can be reached in vivo (30–60 mg/L)4 exceed the MIC of the combination of sulfadiazine and itraconazole.

Synergic effects between sulphonamides and azoles were also found in *Candida* in which synergy was observed between ketoconazole and co-trimoxazole.[13]

In summary, the results presented here strongly suggest that quinolones or sulphonamides enhance the antifungal activity of drugs currently used for some isolates of *E. spinifera*. This provides potential alternative therapeutic options in infections from dematiaceous fungi. More investigations are needed to confirm these observations.

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Chapter

3

# *Post-drug-exposure effects in moulds*

- Method for measuring post antifungal effect in *Aspergillus* species
- Evaluation of post antifungal effect (PAFE) of amphotericin B and nystatin using two different media in 30 Zygomycetes
- Activity and post antifungal effect of chlorpromazine and trifluopherazine against *Aspergillus*, *Scedosporium* and Zygomycetes.
- Evaluation of minimal inhibitory concentration, minimal fungicidal concentration and post antifungal effect (PAFE) of *Aspergillus fumigatus,* after exposure to subinhibitory concentrations of itraconazole, fluconazole and amphotericin B.

# Chapter 3.1

## Method for measuring post antifungal effect in *Aspergillus* species.

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# Method for Measuring Postantifungal Effect in *Aspergillus* Species

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## Abstract

An in vitro method for determination of post-antifungal effect (PAFE) in moulds was developed by using three isolates each of *Aspergillus fumigatus, A. flavus, A. terreus, A. nidulans,* and *A. ustus.* MICs of amphotericin B and itraconazole were determined by using National Committee for Clinical Laboratory Standards guidelines (M38-P). The inoculum was prepared in RPMI 1640 broth buffered with MOPS (morpholinepropanesulfonic acid) at pH 7.0, and conidia were exposed to amphotericin B and itraconazole at concentrations of 4, 1, and 0.25 times the MIC, each for 4, 2, and 1 h at 37°C. The same procedure was followed for controls with drug-free medium. Following exposure, the conidia were washed three times in saline and the numbers of CFU per milliliter were determined. Exposed and control conidia were then inoculated into microtitration plates and incubated at 37°C for 48 h in a spectrophotometer reader. The optical density (OD) was measured automatically at 10-min intervals, resulting in growth curves. PAFE was quantified by comparing three arbitrary points in the control growth curve, the first increase of OD and the points when 20 and 50% of the maximal growth were reached, with the growth curve of drug-exposed conidia. Amphotericin B induced PAFE in *A. fumigatus* at four times the MIC after 2 and 4 h of exposure ranging

from 1.83 to 6.00 h and 9.33 to 10.80 h, respectively. Significantly shorter PAFEs or lack of PAFE was observed for *A. terreus, A. ustus,* and *A. nidulans*. Itraconazole did not induce measurable PAFE in the *Aspergillus* isolates at any concentration or exposure time tested. Further studies are warranted to investigate the implications of PAFE in relation to clinical efficacy and dosing frequency.

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## Introduction

*Aspergillus* species are documented as some of the most prevalent airborne molds, and the frequency of mycoses due to these fungi is rising worldwide, especially invasive infections that occur in immunocompromised patients, such as cancer patients, bone marrow transplant recipients, solid-organ transplant recipients (13), and human immunodeficiency virus-infected patients (18).

*Aspergillus fumigatus* and *Aspergillus flavus* are encountered most frequently, although other species have been documented to cause disease. *Aspergillus terreus* is an increasing cause of invasive infection in neutropenic patients and *Aspergillus nidulans* in patients with chronic granulomatous disease (22). Although amphotericin B is considered the drug of choice for treatment of invasive aspergillosis, the clinical response is limited, especially in infections caused by the latter two species. Alternatively, itraconazole is a triazole antifungal drug with good in vitro activity against *Aspergillus* and has been used successfully as first-line treatment in patients with invasive disease (2, 6).

Methods for in vitro susceptibility testing of molds have been proposed only recently by the National Committee for Clinical Laboratory Standards (NCCLS) (19), but the correlation between MIC and clinical response remains unclear, especially for amphotericin B. With the exception of *A. terreus*, MICs for clinical *Aspergillus* isolates of amphotericin B are normally below 1 μg/ml, and the distribution range of MICs is narrow. A correlation between treatment failure and MIC could not be demonstrated in an animal model of invasive aspergillosis (24). Only for itraconazole (7) and voriconazole was a good correlation found between MIC and response in animal models.

One explanation of discrepancies between MIC and clinical efficacy is post-drug-exposure effects. For instance, the success of intermittent dosing regimens for some antimicrobial agents has been attributed to delay in regrowth of microorganisms after drug concentrations in the tissues have fallen below the MIC. This so-called postantibiotic effect (PAE) was first described for bacteria (20). Post-drug-exposure effects in filamentous fungi have not been studied, mainly because of technical problems such as the nonhomogeneous growth of molds in liquid media. The aim of this study was to develop a system for studying postantifungal effects (PAFE) in filamentous fungi and to evaluate the PAFE of amphotericin B and itraconazole against *Aspergillus* species.

## Materials and Methods

**Isolates**. Fifteen clinical strains from our private collection were evaluated: *A. fumigatus* AZN VO2-31, AZN VO2-32, and AZN VO2-33; *A. terreus* AZN 5914, AZN 2868, and AZN 515; *Aspergillus ustus* AZN 943, AZN 677, and AZN 678; *A. nidulans* AZN 8033, AZN 8950, and AZN 8958; and *A. flavus* AZN 2865, AZN 4094, and AZN 284. The strains were cultured from respiratory secretions (12), the external ear (2), and cerebrospinal fluid (1).

**Antifungal agents**. Amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands) and itraconazole (Janssen-Cilag, Beerse, Belgium) were utilized for MIC determinations and PAFE studies. The drugs were dissolved in dimethyl sulfoxide (DMSO) and aliquots of the stock solution were stored at -70°C until used. Then they were diluted in RPMI 1640 medium (with L-glutamine and without bicarbonate) (GIBCO BRL, Life Technologies, Woerden, The Netherlands) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Antifungal susceptibility testing. The isolates were passaged twice at an interval of 5 to 7 days at 28°C by subculturing onto Sabouraud glucose agar (SGA) to obtain adequate sporulation. Conidia were collected with a cotton swab and suspended in saline with 0.05% Tween 20. After the heavy particles had been allowed to settle, the turbidity of supernatants was measured spectrophotometrically (Spectronic 20D; Milton Roy, Rochester, N.Y.) at 530 nm, and transmission was adjusted to 80 to 82%, corresponding to 0.5 x $10^6$ to 4.5 x $10^6$ CFU/ml. The viability was confirmed by plating serial dilutions onto SGA plates.

For inoculum preparation the conidial suspensions were diluted 1:100 in order to obtain a final inoculum of 0.5 x $10^4$ to 4.5 x $10^4$ CFU/ml. A broth microdilution method was performed according to NCCLS guidelines (M38-P) (19) using RPMI 1640 medium buffered to pH 7.0 with 0.165 M MOPS. Amphotericin B and itraconazole were dissolved in DMSO at concentrations of 3,200 μg/ml. Twofold serial dilutions of the drugs were made in RPMI 1640 medium in order to obtain final concentrations that

ranged from 0.015 to 16 μg/ml for both drugs. A drug-free well containing 0.01% DMSO in the medium served as the growth control. The tests were performed in 96-well flat-bottom microtitration plates (Corning Incorporated, New York, N.Y.), which were kept at -70°C until the day of testing. Conidial suspensions prepared as described above were diluted 1:50 in RPMI 1640 to obtain twice the desired inoculum. After the inoculation, the microtitration plates were incubated at 35°C for 48 h. The MICs were read by spectrophotometric reader (Rosys Anthos ht3; Anthos Labtex Instruments GmbH, Salzburg, Austria). Background optical density (OD) was determined by spectrophotometric measurement of noninoculated wells processed in the same way as the inoculated wells. The relative ODs for each well based on measurements at 405 nm were calculated (in percentages) with the following formula: [(OD of drug-containing well - background OD)/(OD of drug-free well - background OD)] x 100. The MIC of amphotericin B was defined as the lowest concentration of the drug that resulted in at least 95% reduction of growth compared with the growth control, and that of itraconazole was defined as the lowest concentration of the drug that resulted in 50% of reduction of growth compared with the growth control.

**PAFE assay**. The method used for determining PAFE in molds was based on methods used for bacteria and yeasts, with some modifications (4, 10, 12). Amphotericin B and itraconazole were dissolved in DMSO at initial concentrations of 400 μg/ml, and aliquots of the stock solution were stored at -70°C until used. Then they were diluted 50 times in RPMI 1640 medium (with L-glutamine without bicarbonate) buffered to pH 7.0 with 0.165 M MOPS. Serial dilutions of the drugs were made in RPMI 1640 in order to obtain final concentrations of 4, 1, and 0.25 times the corresponding MIC of each drug for each strain. Control conidial suspensions were made in RPMI 1640. The isolates were passaged twice at an interval of 5 to 7 days at 28°C by subculturing onto SGA to obtain adequate sporulation. Conidia were collected with a cotton swab and suspended in saline with 0.5% Tween 20. After the heavy particles were allowed to settle, the supernatant was transferred to another tube and vortexed for 10 s, and 10- and 100-fold dilutions were made. The concentration of conidia was established microscopically by Burker Turk hemocytometer chambers. Then the concentration was adjusted to obtain 4 x 105 conidia/ml. One milliliter of this suspension was added to tubes containing 9 ml of RPMI

1640 alone (control) or with amphotericin B or itraconazole at the concentrations mentioned above, resulting in a final volume of 10 ml. The hydrophobic nature of Aspergillus species made it necessary to add 0.5% Tween 20 to the media in order to keep the conidia in the solution during washing. The final inoculum therefore was 4 x 104 CFU/ml. Following this procedure, each strain was incubated for either 4, 2, or 1 h with continuous shaking at 37°C.

After incubation, the conidia were washed with saline 0.5% Tween 20, and centrifuged at 3,500 x g during 15 min. After three wash cycles, 98% of the supernatant was completely decanted and the pellets were resuspended in a final volume of 10 ml RPMI 1640 with 0.05% Tween 20. The procedure with two washings and removal of 90% of supernatant has been shown to reduce the antimicrobial concentration by 100-fold and with complete decanting, two washings can reduce concentrations as much as 10,000-fold (4). Following this step, 100 µl of sample was diluted 10-fold in sterile water, and 30-µl aliquots were plated onto SGA plates for colony count determination and incubated at 37°C for 24 and 48 h. The concentration of viable CFU per milliliter for drug-exposed conidia was determined in order to verify the concentration of viable conidia after drug exposure and to allow adjustment of the inoculum, if necessary, to match that of controls. From the resuspended suspension, 200 µl was placed in microtitration plates which were incubated at 37°C in a computerized spectrophotometric reader (Rosys Anthos ht3; Anthos Labtex Instruments GmbH). Growth was automatically monitored in terms of change in turbidity at 405 nm at 10-min intervals for 48 h. All assays were performed in duplicate.

In order to correlate OD changes with the morphology of the *Aspergillus* species, the microscopic morphology was examined in microtitration plates by a reverse microscope at different time points for each species for the control and the drug-exposed strains. Conidia were counted, and the percent germination was estimated in duplicate at 0, 4, 8, 12, 16, 24, and 36 h.

**Data analysis**. Three points in the growth curve were considered to quantify the PAFE: (i) the first significant increase in the OD (repeatable increase in OD for three consecutive measurements; $OD_0$) (16); (ii) the point where the OD reached 20% of the maximum of the growth curve at 48 h ($OD_{20}$); and (iii) the point where the OD reached

50% of the maximum of the growth curve at 48 h ($OD_{50}$). The PAFE was quantified by using the formula T - C, where T was the time of the first significant increase in $OD_0$ of the drug-exposed conidia after removal of the drug and C was the time of the first significant increase in $OD_0$ of the control. For $OD_{20}$ and $OD_{50}$, T was defined as the time required for the relative OD of the drug-exposed conidial suspension to reach the absorbance level calculated by the formula FC x ($OD_{max}$ - $OD_{min}$) + $OD_{min}$ after removal of the drug and C was the time required for the relative OD of the drug-free control conidial suspension to reach the same absorbance level calculated by the same formula, where ODmax is the maximum OD reached, $OD_{min}$ is the baseline OD level reached, and FC is a variable of 0.2 and 0.5 that gives a point at 20% and 50% of the maximum of the curve, respectively. Thus, PAFE was defined as the difference in the time required to reach the defined point in the growth curves of drug-exposed conidia and controls and was expressed in hours.

The time to reach these chosen arbitrary points ($OD_0$, $OD_{20}$, and $OD_{50}$) of at least eight controls for each species was determined, and the mean, range, upper 95% confidence interval (CI), and coefficient of variation (CV) were calculated in order to determine the reproducibility of the control curves at each point and to establish the reproducibility of the experiments.

For each chosen point ($OD_0$, $OD_{20}$, and $OD_{50}$) and for each species the upper 95% CI of the controls and the lower 95% CI of the drug-exposed isolates were chosen as the cutoff that distinguished between presence and absence of PAFE. When regrowth of the drug-exposed isolates occurred within the upper 95% CI time frame of the controls, PAFE was considered absent. Alternatively, if regrowth was delayed following drug exposure and the lower 95% CI of the drug-exposed isolates was delayed until beyond the upper 95% CI of the controls, PAFE was considered present (Fig. 1). Growth curves of each drug-exposed isolate were compared only with pooled controls from that same isolate.

The inter- and intraspecies variation of PAFE were analyzed with all the raw data by analysis of variance using Tukey-Kramer multiple-comparison tests. P values of $<0.05$ were considered statistically significant.

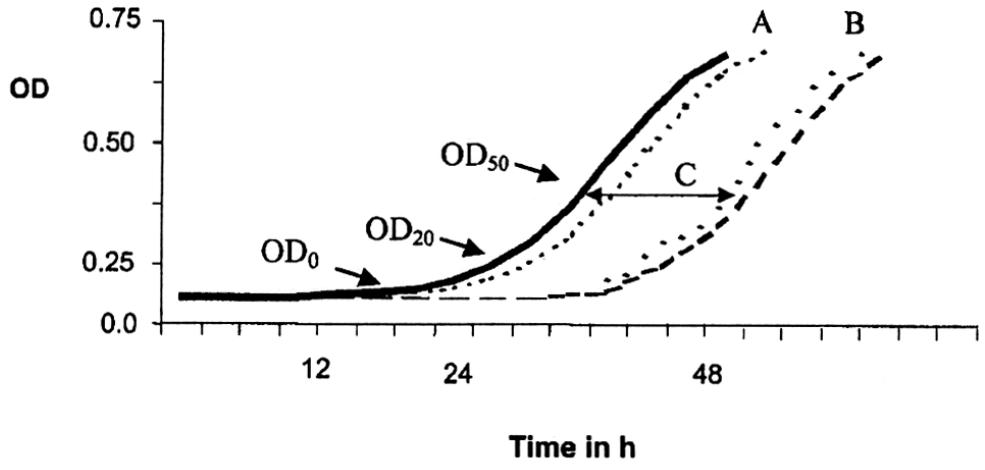


FIG. 1. Growth curve of control (—) and drug-exposed (– – –) molds showing PAFE. The mold was grown in RPMI 1640 medium incubated at 37°C during 48 h. Following drug exposure, the curve was shifted to the right when PAFE is present. PAFE was quantified by using $OD_0$, $OD_{20}$, and $OD_{50}$. The time needed to reach each criterion for the control was subtracted from that for the drug-exposed conidia (C). PAFE was considered to be present when the upper 95% CI of the control growth curve (A) did not overlap with the lower 95% CI of the drug-exposed-conidia growth curve (B).

## Results

For all *Aspergillus* isolates tested the amphotericin B MIC was 1 μg/ml and that of itraconazole ranged between 0.125 and 2 μg/ml (*A. fumigatus*, 0.125 to 0.5 μg/ml; *A. flavus*, 0.125 to 0.25 μg/ml; *A. terreus*, 0.125 μg/ml; *A. nidulans*, 0.125 to 0.25 μg/ml; and *A. ustus*, 2 μg/ml).

**Inoculum**. Based on the inoculum preparation procedure of the NCCLS M38-P guideline, the range of numbers of viable CFU per milliliter recovered in the colony counts was too great to achieve reproducible growth curves. Therefore, a more stringent range was used based on inoculum preparation by hemocytometer. By this method, a limited range of $2 \times 10^4$ to $4 \times 10^4$ viable CFU/ml was achieved. Exposure of conidia to amphotericin B and itraconazole for 1 to 4 h had no effect on the viability of the

*Aspergillus* species based on subculture of serial twofold dilutions (data not shown). Therefore adjustment of the inoculum following drug exposure to match that of the controls was not necessary.

**Microscopic morphology**. Microscopic examination of the conidia before and after drug exposure showed that germination did not occur within the maximal exposure period of 4 h for any species except *A. flavus*, where germ tubes were visible within 4 h of incubation. At 8 h, germination had not occurred for any species except *A. flavus*. For *A. fumigatus* and *A. ustus,* germination of conidia occurred after 12 h for 95% of the controls and drug-exposed conidia in strains where PAFE was not present. When PAFE was present, germination of conidia was further delayed. *A. terreus* and *A. nidulans* germinated after 16 and 20 h, respectively. After 36 h, mycelia were observed for all the species for both controls and drug-exposed conidia.

There were no microscopic differences between drug-exposed conidia and controls. During incubation, the morphology of the hyphae and the frequency of branching appeared to be similar for both drug-exposed and non-drug-exposed strains. The only difference between drug-exposed and non-drug-exposed strains was the time at which germination commenced.

**PAFE assay**. The shapes of the growth curves were different depending on the species tested; however, they were reproducible among the replicates. For the same species, the shapes of the control and the drug-exposed strains were identical. When PAFE was present, the growth curve of the drug-exposed conidia was shifted to the right compared with that of the control. Examples of growth curves for *A. fumigatus* and *A. ustus* are shown in Fig. 2. A sharp increase in OD was observed for *A. ustus* after 30 h of incubation which was found to be related to the formation of crystals in the well (Fig. 2C and D).

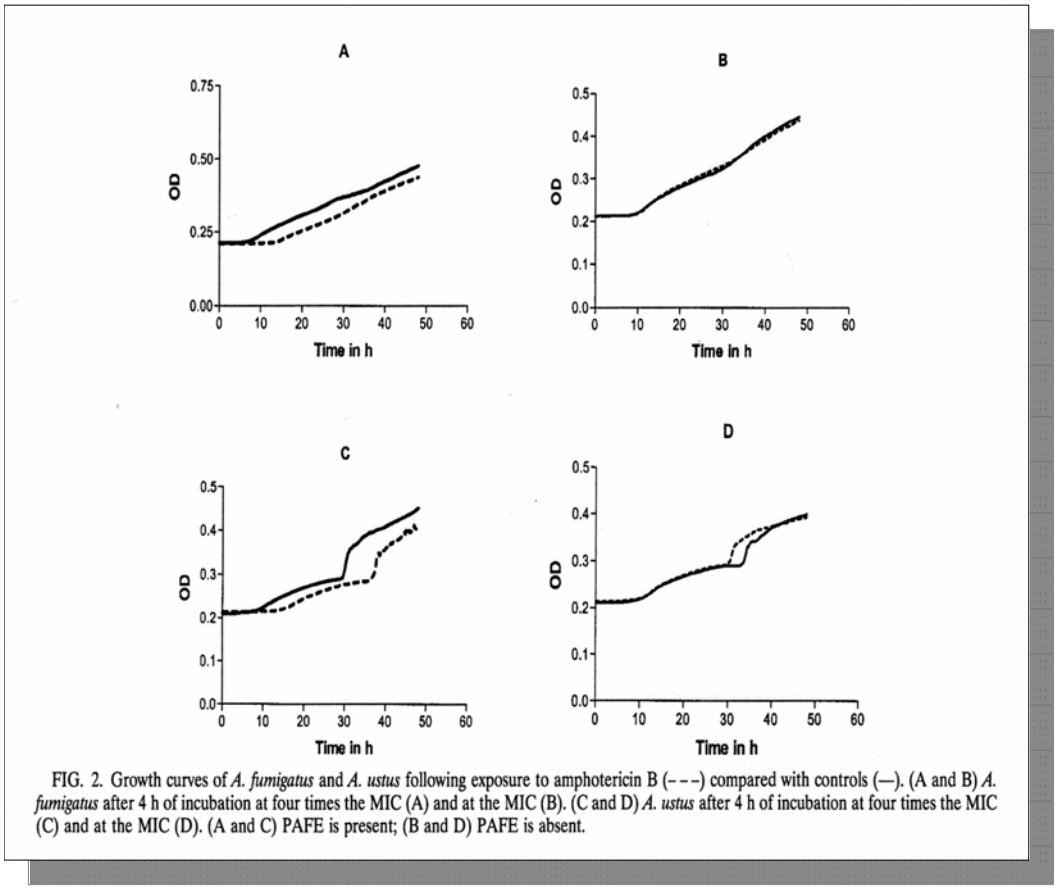


FIG. 2. Growth curves of *A. fumigatus* and *A. ustus* following exposure to amphotericin B (– – –) compared with controls (—). (A and B) *A. fumigatus* after 4 h of incubation at four times the MIC (A) and at the MIC (B). (C and D) *A. ustus* after 4 h of incubation at four times the MIC (C) and at the MIC (D). (A and C) PAFE is present; (B and D) PAFE is absent.

The variability in OD for each of the chosen points for all tested species was calculated by analyzing 180 growth curves in total. In general the variability, expressed as the CV, was not significantly different for the three criteria analyzed, although the CV was the lowest for $OD_0$ (Table 1). When four replicates were used for both control and drug-exposed isolates, the CV was lower than 13% for $OD_0$ and lower than 25% for $OD_{20}$ and $OD_{50}$.

TABLE 1. Variability in time of one *Aspergillus* strain of each species to reach the three arbitrary growth curve criteria[a]

| Strain | Criterion | 4-h incubation | | 2-h incubation | |
|---|---|---|---|---|---|
| | | Mean time (range)[b] | Upper 95% CI (CV) | Mean time (range)[b] | Upper 95% CI (CV) |
| *A. fumigatus* AZN $VO_2$-33 | $OD_0$ | 8.1 (7.5–9.6) | 9.8 (12.7) | 8.9 (8.2–10.6) | 10.7 (12.4) |
| | $OD_{20}$ | 11.7 (11.1–12.6) | 12.9 (6.6) | 11.7 (11.1–12.6) | 14.4 (4.7) |
| | $OD_{50}$ | 24.4 (22.5–26.5) | 27.1 (6.7) | 24.3 (22.5–26.5) | 27.1 (6.7) |
| *A. terreus* AZN 5914 | $OD_0$ | 12.4 (12–12.7) | 13.1 (5.8) | 15 (14.2–15.7) | 16.4 (5.8) |
| | $OD_{20}$ | 21.7 (16.6–25.8) | 29.3 (22.0) | 24.2 (18.2–29.7) | 33.8 (25.1) |
| | $OD_{50}$ | 28.9 (24.1–34.0) | 36.6 (16.6) | 30.2 (25.3–34.7) | 38.3 (16.8) |
| *A. ustus* AZN 677 | $OD_0$ | 12.7 (12–133.3) | 13.9 (6.1) | 8.5 (7.7–9.3) | 10 (10.7) |
| | $OD_{20}$ | 18.6 (14.7–24.9) | 25.9 (24.7) | 18 (15.6–24.3) | 24.7 (23.3) |
| | $OD_{50}$ | 29.3 (20.9–35.3) | 40.7 (24.3) | 32.1 (23.8–38.2) | 43.4 (22.2) |
| *A. nidulans* AZN 8950 | $OD_0$ | 27.9 (27.2–28.7) | 29.3 (3.1) | 26.5 (25–28) | 29.3 (6.5) |
| | $OD_{20}$ | 25.4 (20–29.7) | 31.9 (16.1) | 26.8 (10.9–38.7) | 45.3 (43.2) |
| | $OD_{50}$ | 34.7 (27.3–42.1) | 44.7 (18.2) | 38.2 (31.1–44.7) | 48.4 (16.7) |
| *A. flavus* AZN 4094 | $OD_0$ | 7.5 (6.2–9.9) | 12.7 (27.6) | 11 (10.3–11.4) | 12.5 (5.5) |
| | $OD_{20}$ | 14 (10.6–17.6) | 19.6 (25.3) | 18.8 (13.8–21.9) | 24.8 (20.1) |
| | $OD_{50}$ | 25.3 (17.5–29) | 33.7 (21) | 25.2 (18.8–28.1) | 32.2 (17.3) |

[a] Strains were incubated for 2 or 4 hours to serve as controls for the drug-exposed isolates.
[b] In hours.

**Amphotericin B**. In general, PAFE was not observed after 1 h of exposure to amphotericin B at any concentration for all the species and for any of the three criteria used (data not shown). The magnitude of the PAFEs induced by amphotericin B at the chosen points in the growth curve ($OD_0$, $OD_{20}$, and $OD_{50}$) after 2 and 4 h of exposure and at four times the corresponding MIC and at the MIC in RPMI 1640 broth are shown in Table 2.

*A. fumigatus*. By the $OD_0$ criterion, the PAFE induced by amphotericin B was longer for *A. fumigatus* than for the other *Aspergillus* species, with mean PAFEs of 9.9 and 4.0 h after 4 h of exposure at four times the MIC and at the MIC, respectively, and 4.0 h after 2 h of exposure to four times the MIC. Also, at concentrations of amphotericin B of four times the MIC and equal to the MIC, after 4 h PAFE was observed for *A. fumigatus*, with means of 8.9 and 3.6 h, respectively, using the $OD_{20}$ criterion. At $OD_{50}$ the *A. fumigatus* isolates also displayed PAFE, with means of 5.3 h after 4 h of exposure and four times the MIC and 2.7 h at the MIC. Amphotericin B induced PAFE against *A. fumigatus* after 2 h of incubation at four times the MIC, with a mean of 4.0 h using the $OD_0$ criterion.

TABLE 2. PAFEs induced by exposure of *Aspergillus* species to amphotericin B

| Strain | PAFE (h) obtained with indicated criterion, exposure time, and drug concn[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $OD_0$ | | | | $OD_{20}$ | | | | $OD_{50}$ | | | |
| | 4 h | | 2 h | | 4 h | | 2 h | | 4 h | | 2 h | |
| | 4× | 1× | 4× | 1× | 4× | 1× | 4× | 1× | 4× | 1× | 4× | 1× |
| *A. fumigatus* | | | | | | | | | | | | |
| AZN $VO_2$-31 | 9.3[b] | 3.6[b] | 1.8[b] | 0.8 | 10.5[b] | 5.6[b] | 2.5 | 0.9 | 4.2[b] | 2.8[b] | 2.5 | 0.9 |
| AZN $VO_2$-32 | 9.7[b] | 3.3[b] | 6.0[b] | 2.2[b] | 8.9[b] | 1.5 | 5.4[b] | 2.5[b] | 5.3[b] | 3.7[b] | 1.9 | 1.8 |
| AZN $VO_2$-33 | 10.8[b] | 5.2[b] | 4.3[b] | 2.7[b] | 7.1[b] | 3.7[b] | 6.5[b] | 1.7[b] | 6.5[b] | 1.6 | 4.4[b] | 0.1 |
| *A. terreus* | | | | | | | | | | | | |
| AZN 5914 | 1.2 | 0.5 | 0.7 | 0.5 | 1.2 | 0.7 | 0.9 | 0.4 | 0.4 | 0.3 | 0.7 | 0.5 |
| AZN 515 | 2.1[b] | 0.4 | 0.2 | 0.1 | 4.2[b] | 2.3[b] | 0.8 | 0.6 | 4.3[b] | 2.1[b] | 0.4 | 0.6 |
| AZN 2868 | 4.3[b] | 2.1[b] | 1.1[b] | 0.4[b] | 1.2[b] | 0.8[b] | 0.7[b] | 0.2 | 1.4[b] | 1.5[b] | 0.6 | 0.7 |
| *A. ustus* | | | | | | | | | | | | |
| AZN 677 | 4.2[b] | 2.3[b] | 1.3[b] | 0.2[b] | 2.9 | 1.9 | 1.5 | 0.3 | 3.2 | 2.3 | 3.3 | 0.3 |
| AZN 678 | 5.8[b] | 1.8[b] | 1.2[b] | 0.5[b] | 3.1 | 2.1 | 1.2 | 0.1 | 4.8[b] | 2.8[b] | 0.1 | 0.3 |
| AZN 943 | 1.8 | 1.2 | 0.5 | 0.5 | 5.2 | 2.1 | 1.8 | 0.8 | 2.9 | 1.5 | 2.9 | 1.3 |
| *A. nidulans* | | | | | | | | | | | | |
| AZN 8950 | 0.3 | −0.2 | 0.2 | 0.5 | 0.5 | 0.1 | 0.8 | 0.8 | 0.1 | 0.1 | 2.9 | 1.2 |
| AZN 8033 | 0.8[b] | 0.1[b] | 0.2[b] | 2.2[b] | 1.5 | 3.1 | 4.9 | 0.9 | 4.6[b] | 1.8[b] | 2.0 | 0.5 |
| AZN 8958 | 0.8[b] | 0.7[b] | 4.7 | 0.1 | 2.4 | 1.7 | 0.9 | 0.7 | 2.1 | 4.7 | 0.8 | 0.6 |
| *A. flavus* | | | | | | | | | | | | |
| AZN 4094 | 1.7 | 0.1 | −2.5 | −3.2 | 2.1 | 0.1 | −2.4 | 2.6 | 0.1 | 0.2 | 0.7 | 1.6 |
| AZN 2865 | 3.5 | −0.7 | 0.2 | 0.8 | 2.9 | 0.9 | −4.5 | 1.7[b] | 1.2 | 0.1 | 0.1 | 3.3 |
| AZN 284 | 3.5[b] | 1.0 | 0.2 | 1.8 | 2.8 | −0.8 | −3.0 | 1.4[b] | 0.1 | 0.1 | 0.1 | 0.1 |

[a] 4×, 4 times the MIC; 1×, MIC.
[b] PAFE was significant.

Other *Aspergillus* species. For *A. terreus* and *A. nidulans*, after 4 h of exposure to concentrations of amphotericin B of four times the MIC, the mean PAFEs were 2.5 and 0.7 h at $OD_0$. Amphotericin B induced PAFE against *A. ustus* at concentrations of four times the MIC and equal to the MIC after 4 and 2 h of exposure, with mean PAFEs of 3.9, 1.8, 1.0, and 0.4 h, respectively, using the $OD_0$ criterion. The results for *A. flavus* were inconsistent and did not allow conclusions to be drawn.

At $OD_0$, PAFE was induced by amphotericin B after 4 h of exposure for all three *A. fumigatus* isolates and for two of three strains of *A. ustus, A. terreus*, and *A. nidulans.* After 2 h of exposure, PAFE was observed only for *A. fumigatus* for all the chosen criteria (Table 2). A statistically significantly longer PAFE was induced by amphotericin B after 4 h of exposure at four times the MIC for *A. fumigatus* than for the other *Aspergillus* species ($P < 0.05$). For A. fumigatus, significantly longer PAFEs were found after 4 h of exposure to amphotericin B at the MIC compared with *A. terreus*, after 2 h of

exposure at four times the MIC compared with *A. nidulans*, and after 2 h of exposure at the MIC compared with *A. terreus* and *A. ustus* ($P < 0.05$). No statistically significant intraspecies variation was observed.

**Itraconazole**. Itraconazole failed to induce a PAFE at any concentration tested and for any exposure time. Even exposure to itraconazole concentrations as high as 10, 20, and 50 times the MIC failed to induce a PAFE (data not shown).

## Discussion

**PAFE assay**. In this study a method that allows the quantification and study of post-antifungal-drug-exposure effects in molds was developed. Since the use of viability counts to monitor microbial growth kinetics following drug removal is not feasible with molds due to their nonhomogeneous growth, OD measurements were used. Although the growth curves thus obtained allow quantification of PAFE, microscopic examination of the fungal growth in the wells of the microtitration plates is of great importance. Post-drug-exposure effects other than growth suppression could have an impact on the OD measured. For instance, alteration of the frequency of hyphal branching could result in changes in the OD. However, careful microscopic examination of the molds at different times postexposure revealed no differences in morphology compared with unexposed controls. The reliability of measurement of fungal growth using this spectrophotometric system has been described before, and even small changes in morphology can be detected (16).

It has been demonstrated that *Aspergillus* conidia germinate when incubated at 37°C in RPMI 1640 after 6 h or more (15, 16), with exception only of *A. flavus*, which germinates within 4 h. Therefore, a maximum exposure period of 4 h was chosen. The exposure of Aspergillus conidia to amphotericin B did not affect the viability of the conidia at any of the concentrations or incubation times used. Consequently, adjustment of the inoculum to match that of the controls was not necessary.

The criterion used to define PAFE is arbitrary, although most workers have used the difference between time required for the control and the drug-exposed bacteria or yeasts to increase 1 $\log^{10}$ compared with the initial CFU count (4, 10) or, if a spectrophotometric procedure was used, time to reach a 0.05 absorbance level (9). In the present study, PAFE

was not evaluated with CFU counts because of the nonhomogeneous growth of molds. Therefore, we used a spectrophotometric procedure that was originally developed to study growth characteristics of molds (16). Since viability counts were not performed, several arbitrary chosen criteria were analyzed to calculate the PAFE, namely, $OD_0$, $OD_{20}$, and $OD_{50}$. The variability between replicates of control isolates with these three criteria was not significantly different. Furthermore, for each criterion, only small differences in PAFE were observed, especially for *A. fumigatus* and *A. terreus*. However, using $OD_0$ to quantify PAFE has several advantages. First, the variability among the controls was lower when $OD_0$ was used than with the other two criteria. When four replicates were used, the CV was below 13% for both drug-exposed and control strains with $OD_0$. Second, $OD_0$ is reached earlier than the other criteria, leaving less impact of variables such as production of metabolites, crystals, or cellular debris that might interfere with the OD reading (12). Third, in order to calculate $OD_{20}$ and $OD_{50}$, the OD that corresponds to maximal growth is required, thereby prolonging the time before results become available, especially in slow-growing fungi. RPMI 1640 medium was chosen since it is recommended by the NCCLS for MIC determination of conidium-forming fungi. However, it has been shown that RPMI 1640 poorly supports the growth of *Aspergillus* species (16). The optimal growth curve, which contains a lag phase, a log phase, and a plateau, is not obtained when *Aspergillus* species are cultured in RPMI 1640, but a linear curve was found with the same spectrophotometric system (16). Maximal growth of *Aspergillus* is not reached within 99 h, and therefore we defined maximal growth as the OD that was reached after 48 h of incubation. Alternative media that support the growth of *Aspergillus* more adequately could be useful in this respect, although further studies are needed.

**PAFE**. PAEs have been found to be dependent on several factors, such as the concentration of the antibiotic agent, the time during which the bacteria were exposed to the drug, and the characteristics of the drug used to induce the effect (20). PAFE also appears to depend on drug, concentration, and duration of exposure, as was described for *Candida* species (10). In the present study, the PAFE displayed by amphotericin B showed dependence on the concentration and the exposure period. This is partly consistent with a previous published study with *Candida,* where longer PAFEs were

found following exposure to amphotericin B at concentrations above the MIC compared with shorter PAFEs following exposure to sub-MIC drug concentrations (10). The echinocandin caspofungin (MK-0991) also induced a PAFE against *Candida*, and the magnitude of the effect depended on the concentration of the drug. Both amphotericin B and caspofungin exhibit fungicidal activity in vitro against *Candida* (10, 21, 23).

**Amphotericin B**. Overall, the magnitude of the PAFE induced by amphotericin B in vitro was the greatest against *A. fumigatus*. The range found in our study was similar to that found in a previous study with Candida species, where the PAFE ranged between 0.5 and 10 h (9, 11). However, significantly shorter PAFEs were observed for *A. terreus* and *A. nidulans*, where only exposure to high concentrations of amphotericin B could induce a PAFE. Invasive aspergillosis caused by *A. terreus* and *A. nidulans* is often clinically unresponsive to treatment with amphotericin B (5, 22). The short PAFE or lack of PAFE we found in *A. terreus* and *A. nidulans* isolates could contribute to the poor clinical response.

**Itraconazole**. In our study, exposure to the triazole drug itraconazole did not induce PAFE in any of the *Aspergillus* species tested. Even exposure of *Aspergillus* isolates to a concentration of 50 times the MIC for 4 h was not sufficient to induce PAFE. The inability of itraconazole to induce PAFE in *Aspergillus* is in accordance with observations reported for *Candida*, where no measurable PAFE was observed following exposure to fluconazole (10, 17, 23). The lack of PAFE induced by azoles could be due to the exposure period being too short to cause sufficient damage to the mold. However, longer exposure periods could not be investigated with our system, since germination of the control strains occurred after 6 h of incubation.

**Implications**. Two characteristics determine the time course of antimicrobial activity: the effect of increasing drug concentrations on the extent of killing of the microorganism and the presence or absence of postexposure effects. Our results indicate that post-antifungal-drug-exposure effects occur in *Aspergillus*. The consequences of PAFE of amphotericin B in several *Aspergillus* species for the dosing regimen of the drug remain unclear. Due to the pharmacokinetics of amphotericin B, the concentrations in plasma do not normally exceed 2 μg/ml (14), and after leaving the circulation, the drug slowly accumulates in some tissue compartments (3). Consequently, the concentrations of amphotericin B in the

blood do not accurately reflect the concentrations in tissues (14). The concentration in mouse kidneys can be up to four times greater than the level in serum, and it may appear that antifungal activity persists despite little to no measurable drug in serum (8). The MICs for strains tested in this study were 1 μg/ml, indicating that four times the MIC is a concentration that is achievable in vivo.

Given the fact that amphotericin B is administered once daily, our results suggest that growth suppression is achieved for a significant period for *A. fumigatus* depending on the concentration at the site of infection. However, a larger collection of isolates should be tested and in vivo models are required to confirm our findings. In a murine model of invasive candidiasis, the pharmacodynamics of amphotericin B were best predicted by the peak serum level/MIC ratio (1). Furthermore, high infrequent doses of amphotericin B were as effective as lower, more frequently administered doses in treating invasive candidiasis (1). Since PAFEs of amphotericin B for *A. fumigatus* were similar to those observed with Candida albicans, infrequent dosing might be a useful strategy in the treatment of invasive aspergillosis due to *A. fumigatus*.

In conclusion, we have developed an assay that allows the study of PAFE of different antifungal agents in *Aspergillus* species and other molds. Amphotericin B induced PAFE in *A. fumigatus*, but in *A. terreus* and *A. nidulans* short PAFEs or a lack of PAFE was observed. However, further studies are warranted, including in vivo experiments, to study the impact of PAFE in *Aspergillus* on dosing regimens of amphotericin B.

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# Chapter 3.2

Evaluation of post antifungal effect (PAFE) of amphotericin B and nystatin using two different media in 30 Zygomycetes.

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# Evaluation of the Post Antifungal Effect (PAFE) of Amphotericin B and Nystatin using two different media in 30 Zygomycetes

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Running title: Post antifungal effect in Zygomycetes

## Abstract

The evaluation of post antifungal effect (PAFE) of amphotericin B and nystatin against 30 clinical Zygomycetes were performed using two different media. PAFE is a suppression of fungal growth after limited drug exposure. The MIC of both drugs was determined using NCCLS M38-P guidelines. A spectrophotometric method was used to determine PAFE in vitro. Spores were exposed to amphotericin B and nystatin in RPMI-1640 or AM3 at concentrations of 4x and 1x the MIC for 4h for *Absidia* sp. and at 1x and 0.5x the MIC for 1h for the other strains. Drugs were eliminated by washing. Exposed and control spores were cultured in microtiter wells and incubated for 48h. PAFE was calculated as T-C (Dt) between the control and the exposure fungi. The first increase in optical density ($OD_0$) was used to calculate PAFE and was considered significant when the value of the lower 95%CI of the exposed strain was > upper 95%CI of the control. MIC range in RPMI-1640: 0.06-4 mg/L for amphotericin B and 0.5-8 mg/L for nystatin;

in AM3: 0.06-2 mg/L for amphotericin B and 0.5-4 mg/L for nystatin. Killing was not observed at the concentration and exposure time used. In RPMI-1640, for amphotericin B the rank order for PAFE was *A. corymbifera* (5.6h) > *R. oryzae* (5.2h) > *Mucor* sp (3.5h) > *R. microsporus* (3h) and for nystatin the rank order was Mucor sp (5.8h) > *R. oryzae* (3.3h) > *A. corymbifera* (2.9h) > *R. microsporus* (1.7h). PAFE was not induced in *Rhizomucor* sp. PAFE was dependent on drug concentration.

**Keywords**: PAFE, Zygomycetes, amphotericin B, nystatin

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## Introduction

Organisms of the class Zygomycetes were first noted to cause disease in humans in publications from the nineteenth century. The first description of a case of disseminated disease was in a cancer patient and caused by *Absidia corymbifera* [1]. The main clinical syndromes in human disease are sinusitis, rhinocerebral, pulmonary, cutaneous, subcutaneous, gastrointestinal and disseminated zygomycosis. Other disease states occur with a much lower frequency. Risk factors for developing invasive zygomycosis include diabetes with ketoacidosis, neutropenia, transplant or patients with leukemia who receive treatment with corticosteroids. The most common causative organisms are *Rhizopus*, *Absidia* followed by *Mucor* and *Rhizomucor*[2,3].

Treatment of zygomycosis requires surgical intervention, antifungal therapy and resolution of the underlying inmunocompromised condition. Amphotericin B remains the first-line choice for treatment of infections due to Zygomycetes. However clinical response is poor, especially in patients with disseminated disease [2]. Triazoles and allylamines exhibit partly in vitro activity against this class of fungi [4] but these agents are not used clinically. Nystatin exhibits also good in vitro activity but no clinical data are available to confirm their in vivo activity.

Post drug exposure effects are important to understand and optimalize drug efficacy in vivo. We have developed an in vitro model that enables the study of post antifungal effects (PAFE) in filamentous fungi [5]. The aim of the present study was to evaluate the PAFE induced by the polyenes amphotericin B and nystatin against Zygomycetes and to compare the PAFE in two different media.

## Materials and methods

**Isolates**. 30 strains from our private collection were evaluated: *Absidia corymbifera* (AZN 24, AZN 319, AZN 911, AZN 1184, AZN 2134, AZN 2543, AZN 3113, AZN 3114, AZN 4095, AZN 6429); *Rhizopus oryzae* (AZN 593, AZN 1523, AZN 3440, AZN5618, AZN 6142, AZN 6373, AZN 1925); *Rhizopus microsporus* (AZN 23, AZN

190, AZN 410, AZN 1185, AZN 5005, AZN 5816); *Rhizomucor miehei* AZN 4839; *Rhizomucor pusillus* AZN 22; *Mucor hiemalis* (AZN 21, AZN 175, AZN 1379) and *Mucor rouxii* AZN 1183.

Antifungal agents. Amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands) and Nystatin (Gist-Brocades, Delft, The Netherlands) were utilized for MIC determinations and PAFE studies. The drugs were dissolved in dimethyl sulfoxide (DMSO) and aliquots of the stock solution were stored at −70ºC until use. Then, they were diluted in RPMI-1640 medium (with L-glutamine, without bicarbonate) (GIFCO BRL, Life Technologies, Woerden, The Netherlands) and Antibiotic Medium 3 (AM3) (BBL/Becton Dickinson, Cockeysville, Md.), both buffered to pH 7.0 with 0.165 M morpholinopropansulfonic acid (MOPS) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

**Antifungal susceptibility testing**. The isolates were passaged twice at an interval of 5-7 days at 28ºC by subculturing onto Sabouraud glucose agar (SGA) to obtain adequate sporulation. Spores were collected with a cotton swab and suspended in saline with 0.01% Tween 80%. The resulting spore suspensions were counted with a haemocytometer and diluted in RPMI-1640 or AM3 1:100 in order to obtain a final inoculum of 1-5 x 104 spores per ml. The viability was confirmed by plating serial dilutions onto SGA plates.

Amphotericin B and nystatin were dissolved in DMSO at concentrations of 3200 mg/L. Two fold serial dilutions of the drugs were made in RPMI-1640 and AM3 medium in order to obtain final concentrations that ranged from 0.015 to 16 mg/L of both drugs. A drug-free well containing 0.01% DMSO in the medium served as the growth control. The tests were performed in 96-well flat bottom microtitration plates (Corning Incorporated, N.Y., USA) which were kept at -70ºC until the day of testing. After the inoculation, the microtitration plates were incubated at 35ºC for 48 h. The MICs were read by spectrophotometric reader (Rosys Anthos ht3, Anthos Labtex Instruments GmbH, Salzburg, Austria). Background optical density (OD) was measured spectrophotometrically in non inoculated wells processed in the same way as the inoculated wells. The relative ODs for each well based on measurements at 405 nm were calculated (in percentage) based on the following equation: [(OD of drug containing well

– background OD)/(OD of drug-free well – background OD)] x 100%. The MIC for both drugs was defined as the lowest concentration of the drug that showed at least 95 % reduction of growth compared with that of the growth control (MIC-0).

**PAFE assay**. The method used for determining PAFE was recently described for *Aspergillus* spp 5. Amphotericin B and nystatin were dissolved in DMSO at initial concentrations of 400 mg/L and aliquots of the stock solution were stored at –70ºC until used. Then they were diluted 50 times in RPMI-1640 (with L-glutamine without bicarbonate) or AM3 medium buffered to pH 7.0 with 0.165 M MOPS. Serial dilutions of the drugs were made in both media in order to obtain final concentrations of 4, 1 and 0.5 times the corresponding MIC. Control spore suspensions were made in RPMI-1640 and AM3 without drug. The isolates were passaged twice at an interval of 5-7 days at 28ºC by subculturing onto SGA to obtain adequate sporulation. Spores were collected with a cotton swab and suspended in saline with 0.01% Tween 80. After the heavy particles were allowed to settle, the supernatant was transferred to another tube, vortexed for 10 sec, and 10 and 100 times dilutions were made. The concentration of spores was established microscopically by haemocytometer Burker Turk chambers. Then, the concentration was adjusted to obtain 4 x $10^5$ spores/ml. One milliliter of this suspension was added to tubes containing 9 ml of RPMI-1640 or AM3 alone (control) or with amphotericin B or nystatin in concentrations mentioned above resulting in a final volume of 10 ml. The final inoculum therefore was 4 x $10^4$ CFU/ml. Following this procedure, each strain was incubated for 4 or 1 h with continuous shaking at 37ºC.

After incubation the spores were washed with saline plus 0.01% Tween 80 and centrifuged at 3,500 x g during 15 min. After three wash cycles, 98% of the supernatant was completely decanted and the pellets were resuspended in a final volume of 10 ml RPMI-1640 or AM3 with 0.01% Tween 80. Following this step, 100 ml of sample was diluted 10-fold in sterile water and 30 ml aliquots were plated onto SGA plates for colony count determination, and incubated at 37ºC for 24 h. The concentration of viable CFU/ml for exposed spores was determined in order to verify the concentration of viable spores post drug exposure and to allow adjustment of the inoculum, if necessary, to match that of controls. From the resuspended suspension, 200 ml was placed in microtitration plates and incubated at 37ºC in a computerized spectrophotometric reader (Rosys Anthos ht3).

Any growth was automatically monitored in terms of change in turbidity at 405 nm, at 10 min intervals for 48 h. All assays were performed in duplicate.

**Data analysis**. The repetitive OD measurements for each well resulted in the growth curve. PAFE was determined by comparing the growth curve of the exposed spores with that of the controls. $OD_0$ was used to calculate PAFE as was previously described[5], by using the formula PAFE = T - C, where T was the time of the first significant increase in OD0 of the exposed spores after removal of the drug and C was the time of the first significant increase in $OD_0$ of the control. Thus, PAFE was defined as the difference in time (Dt) between exposed and controls to reach the defined point in the growth curve and was expressed in hours. The time to reach this chosen point, $OD_0$ , of at least 8 controls for each species was calculated and the mean, range, upper 95% confidence interval (CI) and the coefficient of variance were calculated in order to determine the reproducibility of the control curves at that point and to establish the reproducibility of the experiments. For each species the upper 95% CI of the controls was chosen as the cut-off level that distinguished between presence or absence of PAFE. When re-growth of the exposed isolates occurred within the upper 95% CI time-frame of the controls, PAFE was considered to be absent. Alternatively, if re-growth was delayed following drug exposure and the lower 95% CI of the exposed isolates was delayed until beyond the upper 95% CI of the controls, PAFE was considered to be present. Growth curves of each exposed isolate were compared only with pooled controls from that same isolate.

Comparison of PAFE between both media and both drugs were analyzed by analysis of variance for repeated observations followed by Bonferroni's Multiple Comparison Tests. P values of $< 0.05$ were considered statistically significant.

## Results

The MIC ranges determined in RPMI-1640 were: 0.063-4 mg/L for amphotericin B (*A. corymbifera* 0.125-0.25 mg/L; *R. oryzae* 0.5-4 mg/ml; *R. microsporus* 0.5-2 mg/L; *R. miehei* 0.063 mg/L; *R. pusillus* 0.125 mg/L; *M. hiemalis* 0.063-0.25 mg/L and *M. rouxii* 0.063 mg/L) and 0.5-8 mg/L for nystatin (*A. corymbifera* 0.5-1 mg/L; *R. oryzae* 2-8

mg/L; *R. microsporus* 2-8 mg/L; *R. miehei* 0.5 mg/L; *R. pusillus* 1 mg/L; *M. hiemalis* 0.5 mg/L and *M. rouxii* 0.5 mg/L).

For AM3 the MICs of amphotericin B ranged from 0.063 to 2 mg/L (*A. corymbifera* 0.125-1 mg/L; *R. oryzae* 0.5-2 mg/L; *R. microsporus* 0.25-2 mg/L; *R. miehei* 0.063 mg/L; *R. pusillus* 0.125 mg/L; *M. hiemalis* 0.25-0.5 mg/L and *M. rouxii* 0.063 mg/L) and for nystatin from 0.5 to 4 mg/L (*A. corymbifera* 0.5-2 mg/L; *R. oryzae* 1-4 mg/L; *R. microsporus* 1-2 mg/L; *R. miehei* 0.5 mg/L ; *R. pusillus* 1 mg/L; *M. hiemalis* 0.5-1 mg/L and *M. rouxii* 0.5 mg/L).

**Viability of the spores**. Exposure of spores to the used concentrations of amphotericin B and nystatin for 1 to 4 h had no effect on the viability of the *A. corymbifera* isolates, but for the other genera tested both drugs induced fungicidal activity after 4 or 1h of incubation at a concentration of 4 times the MIC (Figure 1). Therefore, PAFE was determined for 1 h exposure at 1 and 0.5 times the corresponding MIC for these genera. In these conditions killing was not observed for the concentration and exposure time chosen based on subculture of serial two-fold dilutions (data not shown).

Inoculum. By using a haemocytometer chamber a limited range of 2 to 4 x $10^4$ viable CFU/ml was achieved.

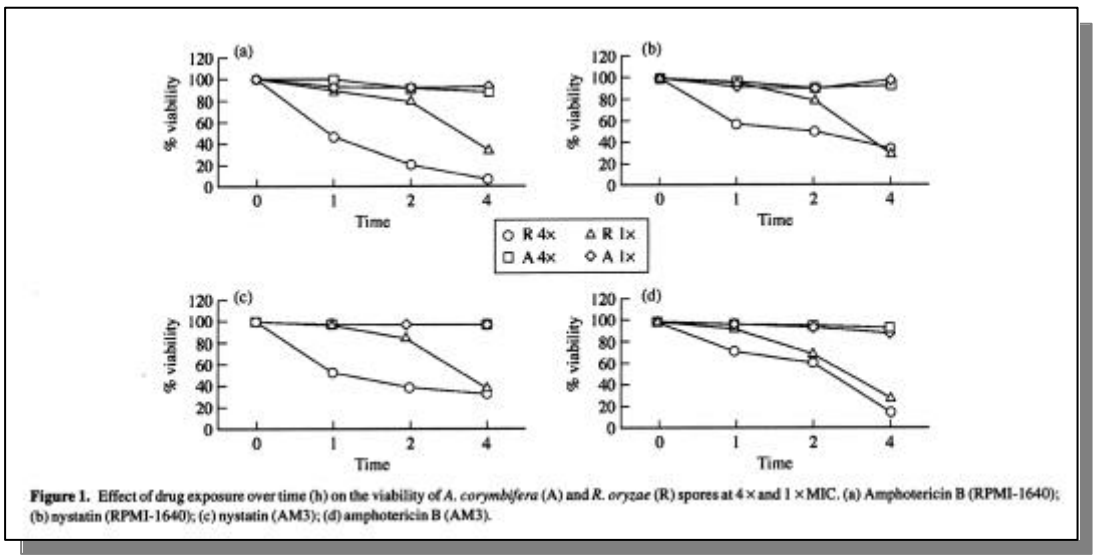


**Figure 1.** Effect of drug exposure over time (h) on the viability of *A. corymbifera* (A) and *R. oryzae* (R) spores at 4 × and 1 × MIC. (a) Amphotericin B (RPMI-1640); (b) nystatin (RPMI-1640); (c) nystatin (AM3); (d) amphotericin B (AM3).

**PAFE assay**. All growth curves were reproducible among the replicates. For the same species the shape of the growth curve of the control was identical to that of the exposed strains. When PAFE was present the growth curve of the exposed spores was shifted to the right compared with that of the control. Examples of growth curves for *A. corymbifera* and *R. oryzae* are shown in Fig. 2.

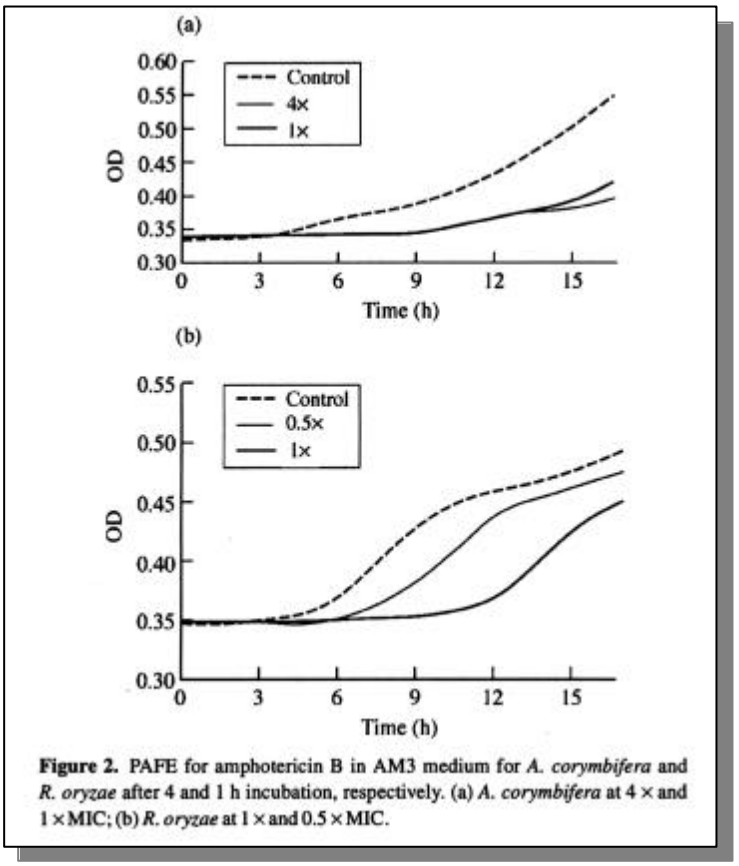


**Figure 2.** PAFE for amphotericin B in AM3 medium for *A. corymbifera* and *R. oryzae* after 4 and 1 h incubation, respectively. (a) *A. corymbifera* at 4 × and 1 × MIC; (b) *R. oryzae* at 1 × and 0.5 × MIC.

**Morphology**. Microscopic examination of the spores before and after drug exposure showed that germination did not occur within the maximal exposure period of 4 h for Absidia and 1 h for the other species in both media. In RPMI-1640 even after 5 h of incubation germination had not occured for control or exposed spores for any of the genera. However in AM3 spores of *Rhizopus* started to germinate after 3 h, and after 5 h 100% of spores were germinated. For *Absidia* 80% of spores had germinated after 5 h of incubation. Germination of controls and drug exposed spores occurred at the same time

and to the same extent in those strains where PAFE was not present. When PAFE was present, germination of spores was further delayed.

Microscopic examination of the moulds at different time intervals post-drug exposure revealed that increase of OD correlated with the development of hyphae as described previously [6]. No differences in morphology were noted between exposed and non-exposed fungi.

The variability in OD, expressed as coefficient of variation (CV) among the control and exposed isolates was lower than 13%.

PAFE was induced by both drugs in both media for all strains tested with the exception of *Rhizomucor* spp. (table 1). PAFE was induced by amphotericin B after 4 or 1h of exposure for the 10 *A. corymbifera* and for the 7 *R. oryzae* strains for all the conditions tested. (Table 1).

Overall, higher concentrations of drug induced longer PAFEs. However, the extent of PAFE differed between amphotericin B and nystatin for the various species.

In general, amphotericin B induced longer PAFE compared with nystatin: 4.6 h for amphotericin B versus 2.9 h for nystatin in RPMI-1640 ($p < 0.05$), and 4.3 h versus 3 h for amphotericin B and nystatin, respectively, in AM3 ($p < 0.05$). No statistical significant difference was observed for amphotericin B and nystatin between both media ($p > 0.05$), with the exception of low concentration of nystatin. (Table 2).

**Comparision between different genera:** all *A. corymbifera* strains displayed PAFE for both drugs at 4 and 1x times the MIC after 4 h exposure in the same medium. However for nystatin longer PAFEs in AM3 for both concentrations was seen compared with RPMI-1640 (Table 1). PAFE was not induced by amphotericin B or nystatin after 1 h exposure (data not shown). For *Rhizopus* spp. no differences in PAFE values were seen when both drugs were compared. Both drugs tended to induce longer PAFE in *R. oryzae* compared with *R. microsporum*. (Table 1). For *Mucor* spp. nystatin tended to induce longer PAFE values than with amphotericin B in the same medium. This observation was only found for this genus. Both, amphotericin B and nystatin failed to induce PAFE in *Rhizomucor* strains.

**Sub MIC exposure:** *A. corymbifera* did not display any PAFE in contrast to the other genera where most showed significant PAFE values. For amphotericin B, *Rhizopus* spp

and *Mucor* spp displayed PAFE in RPMI-1640, but in AM3 only *Mucor* spp did not show the effect.

For nystatin *Rhizopus* spp and *Mucor* spp displayed PAFE in RPMI-1640 and in AM3 only *R. microsporus* did not show the effect.

**Table 1.** PAFEs induced by exposure of different Zygomycetes to amphotericin B and nystatin in RPMI-1640 and AM3

| | | | | PAFE in RPMI | | | PAFE in AM3 | | |
|---|---|---|---|---|---|---|---|---|---|
| Strain | Cc[a] | Exposure[b] | Drug | mean[c] | range | N/TN[d] | mean | range | N/TN[d] |
| *A. corymbifera* | 4× | 4 | AMB | 5.6 | 1.9–9.1 | 10/10 | 5.9 | 5.2–6.9 | 10/10 |
| (*n* = 10) | 1× | 4 | AMB | 4.2 | 1.4–5.5 | 10/10 | 5.3 | 4.3–6 | 10/10 |
| | 4× | 4 | Nys | 2.9 | 2.5–4 | 9/10 | 5.5 | 4.2–6.9 | 10/10 |
| | 1× | 4 | Nys | 0.8 | 0.1–1.2 | 0/10 | 4.9 | 3.5–6 | 10/10 |
| *R. oryzae* | 1× | 1 | AMB | 5.2 | 3.1–7.5 | 7/7 | 4.3 | 2.4–5.9 | 7/7 |
| (*n* = 7) | 0.5× | 1 | AMB | 3.9 | 2–6.1 | 7/7 | 2.2 | 1–3.7 | 7/7 |
| | 1× | 1 | Nys | 3.3 | 2.4–4.6 | 7/7 | 2.6 | 1.6–3.7 | 5/7 |
| | 0.5× | 1 | Nys | 2.7 | 2–3.5 | 7/7 | 1.5 | 0.5–2.2 | 3/7 |
| *R. microsporus* | 1× | 1 | AMB | 3 | 0.8–8.5 | 6/7 | 2.9 | –0.1–9.4 | 3/7 |
| (*n* = 7) | 0.5× | 1 | AMB | 1 | 0.3–3.4 | 3/7 | 1 | –0.2–3.9 | 3/7 |
| | 1× | 1 | Nys | 1.7 | 0.7–5.4 | 3/7 | 0.2 | –0.35–0.9 | 0/7 |
| | 0.5× | 1 | Nys | 0.7 | 0.2–1.2 | 1/7 | 0.1 | –0.2–0.3 | 0/7 |
| *Mucor* spp. | 1× | 1 | AMB | 3.5 | 2.1–6.9 | 3/4 | 2.2 | 0.9–3.4 | 3/4 |
| (*n* = 4) | 0.5× | 1 | AMB | 2.1 | 1.1–3.6 | 2/4 | 1 | –0.1–1.7 | 0/4 |
| | 1× | 1 | Nys | 5.8 | 4–8.3 | 3/4 | 4.8 | 2.9–6.4 | 3/4 |
| | 0.5× | 1 | Nys | 2.9 | 2.1–3.8 | 2/4 | 3 | 2.2–3.9 | 2/4 |
| *Rhizomucor* spp. | 1× | 1 | AMB | 0.5 | –0.1–1 | 0/2 | 0.2 | 0.1–0.2 | 0/2 |
| (*n* = 2) | 0.5× | 1 | AMB | –0.7 | –0.2–1.2 | 0/2 | 0 | 0–0.1 | 0/2 |
| | 1× | 1 | Nys | 1 | 0.7–1.4 | 0/2 | 0 | 0 | 0/2 |
| | 0.5× | 1 | Nys | –0.4 | –1.2–0.5 | 0/2 | –0.2 | –0.3–0 | 0/2 |

[a]Concentration of the drug expressed in 4 ×, 1 × and 0.5 × MIC.
[b]Exposure period in hours.
[c]PAFE is expressed in hours.
[d]N/TN, number of strains that displayed significant PAFE/total number of strains tested.

**Table 2.** PAFEs for all the strains ($n = 30$) comparing amphotericin versus nystatin and RPMI-1640 versus AM3

| | | PAFE in RPMI | | PAFE in AM3 | | |
|---|---|---|---|---|---|---|
| Concentration | Drug | mean | range | mean | range | Statistical analysis[c] |
| Hc[a] | AMB | 4.6 | (–0.1–9.1) | 4.3 | (–0.1–9.4) | $P > 0.05$ |
| Hc | Nys | 2.9 | (0.7–8.3) | 3.0 | (0–6.9) | $P > 0.05$ |
| Lc[b] | AMB | 2.7 | (–0.2–6.1) | 2.4 | (0–6) | $P > 0.05$ |
| Lc | Nys | 1 | (–1.2–3.8) | 2.2 | (–0.2–6) | $P < 0.05$ |

Overall ($n = 30$).
[a]Higher concentration evaluated corresponded to 4 × MIC for *A. corymbifera* and 1 × MIC for the other genera.
[b]Lower concentration evaluated corresponded to 1 × MIC for *A. corymbifera* and 0.5 × MIC for the other genera.
[c]Statistical analysis of variance for repeated observations followed by Bonferroni's Multiple Comparison.

## Discussion

The method to quantify post antifungal drug exposure in Zygomycetes was based on the method recently described for *Aspergillus* species using a spectrophotometric procedure and $OD_0$ (first increase in OD) as criterion to calculate PAFE [5]. The reliability of measurement of fungal growth using this spectrophotometric system has been described previously and even small changes in morphology can be detected. Furthermore the system was validated previously for *Rhizopus* [6].

The exposure of *Absidia* spores to amphotericin B or nystatin did not affect the viability of the spores at any of the concentrations or incubation periods tested. This finding was similar to that observed for *Aspergillus* species, where viability was not affected by exposure of the conidia to amphotericin B [5]. However, exposure of the other genera of zygomycetes at concentrations above the MIC, resulted in significant decrease in viability indicating rapid fungicidal activity. Rapid fungicidal activity was observed previously for *Candida* at concentrations of amphotericin B above the MIC [7].

Post antibiotic effects have been found to be dependent of several factors such as the concentration of the antibiotic, exposure time, media used and pH[8]. In Zygomycetes, PAFE appears also to depend on the class of drugs, concentration and duration of exposure, as was previously described for *Candida* and *Aspergillus* species [5,9].

The nutrient medium is a major factor that influences the results of in vitro susceptibility tests 10. RPMI-1640 has been evaluated extensively for in vitro susceptibility testing of yeasts and moulds and has been shown to give reproducible results [11-13]. AM3 has been shown to discriminate better between *Candida* strains susceptible and resistant to ampothericin B [14], although there are no data that show that this is also the case for zygomycetes. Since batch-to-batch variation was described for the in vitro testing of antifungal agents with AM3, a single batch was used in the present study. In our evaluation PAFE was observed for both media, indicating that the effect was not dependent on the media used.

In general, PAFE was similar in RPMI-1640 and AM3 for both drugs with the exception of *A. corymbifera* for which nystatin tended to induce longer PAFE in AM3 than in RPMI-1640.

Amphotericin B and nystatin belong to the polyenes which have a broad fungicidal spectrum in vitro. Due to problems of solubilization and toxicity, parenteral administration of nystatin is not use for systemic treatment [15]. However the incorporation of this drug in liposomes reduced toxicity and preserved antifungal activity [16]. Intravenous liposomal nystatin studied in rabbits displayed nonlinear pharmacokinetics, potentially therapeutic peak plasma concentrations and substantial penetration in tissues. After multiple dosing over 15 days the maximum concentrations in mg/g (mean) were: in lung (72.84); liver (41.26); spleen (46.57); kidney (22.85) with a plasma concentration of 34.74 mg/L [17].

When studying the relationship between drug concentration and antimicrobial effect, the time course of antifungal activity of polyenes is characterised by concentration-dependent killing, i.e. enhanced microbial killing by increasing drug levels, and long PAFE [18]. Although this relationship was studied with conventional amphotericin B, it has been demonstrated with antibacterial drugs that specific pharmacodynamic parameters predictive of activity varies for different drug classes but not for drugs within a class [18].

This was confirmed in the present study where amphotericin B and nystatine, both polyenes, showed comparable PAFE characteristics against zygomycetes. It can be assumed that the same is true for lipid-formulations of amphotericin B since the active compound is amphotericin B.

The interpretation of our results can only be meaningful if drug levels that induce PAFE in vitro are within the range achievable in humans. Although serum concentrations of conventional amphotericin B are generally below 2 mg/l, higher levels have been found at the site of infection [19,20]. The association between dosing of conventional amphotericin B and treatment effect is best described by the pharmacodynamic parameter peak level / MIC, indicating that the peak concentration achieved in the tissues is a major factor in relation to efficacy [18,21]. The drug levels of the lipid formulations of amphotericin B are higher than those of conventional amphotericin B, although the pharmacodynamic properties of the individual lipid-formulations differ significantly [22]. Also for some isolates even sub-MIC concentrations induced PAFE.

The strains tested in this study had a maximum MIC value of 8 mg/ml for nystatin and 4mg/ml for amphotericin B, indicating that testing the PAFE by using 4 times the MIC, the maximum concentration is 32 or 16 mg/L respectively, values that are achievable in tissues.

For both drugs PAFE was observed, however amphotericin B displayed longer values compared with nystatin.

PAFE displayed by amphotericin B and nystatin were concentration dependent. This is consistent with previous studies with *Aspergillus* and *Candida* where longer PAFEs were found following exposure to amphotericin B or nystatin at higher concentrations [5,7,9].

The mean PAFE values found in our study for all zygomycetes were similar to those described for *Aspergillus* species previously tested although amphotericin B induced longer PAFE against *A. fumigatus* (mean PAFE= 9.94 h) [5]. For nystatin similar PAFEs were found for *Candida albicans* (mean= 6.85 h) whereas *C.* non-*albicans* species presented longer PAFE [23].

In conclusion, amphotericin B and nystatin induced PAFE in Zygomycetes but lower PAFE values were observed with nystatin. In general the used media did not significantly influence the effect. Determination of PAFE could be useful as an in vitro tool together

with the in vitro susceptibility testing to give a better understanding of the activity of antifungal agents. There are not available data of in vivo studies of PAFE for Zygomycetes that could support any theory about the importance of this effect as predictor of therapy failure . For this reason , further studies are warranted, including in vivo experiments, to study the impact of PAFE in Zygomycetes on dosing regimens of amphotericin B or nystatin and the usefulness of this assay to assist in predicting clinical outcome.

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# Chapter 3.3

Activity and post antifungal effect of chlorpromazine and trifluopherazine against *Aspergillus*, *Scedosporium* and Zygomycetes.

*Submitted for publication*

# Activity and Post Antifungal Effect of Chlorpromazine and Trifluopherazine against *Aspergillus, Scedosporium* and Zygomycetes.

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## Abstract

The in vitro activity of two phenothiazine compounds, chlorpromazine and trifluopherazine were evaluated against *Aspergillus* species, Zygomycetes and *Scedosporium* species. Both drugs inhibited the growth of all species tested at concentrations of 16 to 64 µg/ml. Growth inhibition was also observed after exposure and removal of the drug (post-antifungal effect, PAFE). For *Aspergillus* species the mean PAFE was 3.7 h and 4.7 h; for zygomycetes, 3.1 h and 3.4 h; for *Scedosporium*, 4.3 h and 5.3 h for chlorpromazine and trifluoroperazine, respectively. This study shows that these phenothiazine compunds are moderately active against a broad range of filamentous fungal pathogens.

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## Introduction

Opportunistic fungi have emerged during the past decade as important causes of morbidity and mortality in immunocompromised patients. The spectrum of moulds that cause invasive fungal infections is evolving from predominantly *Aspergillus* to a variety of molds including zygomycetes and *Scedosporium* (26). *Aspergillus* species remain the most common infectious cause of pneumonia in hematopoietic stem cell transplant recipients. Zygomycetes cause aggressive infections especially in patients with diabetic ketoacidosis, neutropenia or in those receiving corticosteroids. An increasing number of members of the class of zygomycetes are reported to cause lethal infections, despite aggressive medical and surgical interventions (15, 26). *Scedosporium* species have evolved into important agents causing disseminated infections, mainly in neutropenic patients and often treatment with antifungals agents has been unsuccesfull (7). Both *Scedosporium apiospermum* and *S. prolificans* are intrinsically resistant to many antifungal agents (7, 20).

Although voriconazole was shown to be superior to amphotericin B and other licenced antifungal therapy for the primary treatment of invasive aspergillosis, the response rates remain low. The outcome of treatment of invasive zygomycosis and scedosporiosis remains poor even with the use of lipid-formulations of amphotericin B (4, 6, 23). The poor outcome of treatment of invasive mould infections is primarily due to host factors such as the underlying condition. Furthermore, delay in obtaining a diagnosis and correct identification of the infecting mould is believed to contribute to the poor outcome. Finally, the susceptibility of the infecting strain is important with respect to the response. However, the efficacy of treatment is as low as 50% even when the infecting strain is susceptible to the drug administered. Given this situation the potential of other compounds should be investigated for their antifungal activity, especially when they interact with a target that differs from known targets of conventional antifungal drugs.

Phenothiazine compounds as chlorpromazine and trifluopherazine are antipsychotic agents that inhibit calmodulin leading to multiple cellular effects including modification

of membranes, alteration of cyclic nucleotide metabolism and intercalation into DNA (17, 19).

Calmodulin, a $Ca^{2+}$ binding protein, was suggested in genetic studies as essential for growth of fungi (16). Calmodulin has been reported to be essential for cell proliferation in fungi including *Saccharomyces cerevisiae, Schizosaccharomyces pombe*, *Cryptococcus neoformans, Candida albicans* and *Aspergillus nidulans* (14, 16, 21). Phenothiazine compounds have been reported to exhibit antibacterial and antifungal activity in vitro and in vivo (2, 3, 5, 8, 24, 27, 28). The aim of this study was to determine whether phenothiazines possess in vitro activity against difficult-to-treat filamentous fungi.

## Materials and methods

**Isolates**. 84 strains from our private collection were evaluated: *A. fumigatus* (AZN 58, AZN 59, AZN 60, AZN 62, AZN 63, AZN 64, AZN 65, AZN 5161, AZN 7820, AZN 8248, AZN 9339, AZN 9370, AZN 5241, AZN 5242, AZN 7720, AZN 7722, AZN 9360, AZN 9362, AZN 9372, AZN 267); *A. ustus* (AZN 677, AZN 678, AZN 741, AZN 924, AZN 2725, AZN 6989, AZN 943, AZN 7463, AZN 9420); *A. terreus* (AZN 142, AZN 286, AZN 299, AZN 515, AZN 2868, AZN 5914, AZN 8764, AZN 9152, AZN 4061, AZN 7320); *A. corymbifera* (AZN 24, AZN 319, AZN 911, AZN 1184, AZN 2134, AZN 2543, AZN 3113, AZN 3114, AZN 4095, AZN 6429); *R. oryzae* (AZN 593, AZN 1523, AZN 3440, AZN5618, AZN 6142, AZN 6373, AZN 1925); *R. microsporus* (AZN 23, AZN 190, AZN 410, AZN 1185, AZN 5005, AZN 5816); *S. apiospermum* (AZN 409, AZN 631, AZN 1711, AZN 2036, AZN 2420, AZN 6476, AZN 7110, AZN 9604, AZN 4-59, AZN 7-20) and *S. prolificans* (AZN 7307, AZN 7889, AZN 7891, AZN 7892, AZN 7893, AZN 7894, AZN 7895, AZN 7897, AZN 7898, AZN 7900).

**Antifungal agents**. Chlorpromazine and trifluopherazine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were utilized for MIC determinations and post antifungal effect (PAFE) studies. The drugs were dissolved in water and aliquots of the stock solution were stored at −70 ºC until use. Then, they were diluted in RPMI 1640 medium (with L-glutamine, without bicarbonate) (GIFCO BRL, Life Technologies, Woerden, The Netherlands) buffered to pH 7.0 with 0.165 M morpholinopropansulfonic acid (MOPS) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

**Antifungal susceptibility testing**. **Antifungal susceptibility testing.** The test was performed following the NCCLS guidelines (22). The isolates were passaged twice at an interval of 5-7 days at 35ºC by subculturing onto Sabouraud glucose agar (SGA) to obtain adequate sporulation. Spores were collected with a cotton swab and suspended in saline with 0.01% Tween 20 or Tween 80. The resulting conidia or spore suspensions were counted with a haemocytometer and diluted in RPMI-1640 1:100 in order to obtain a final inoculum of 1-5 x $10^4$ conidia or spores per ml. The viability was confirmed by plating serial dilutions onto SGA plates.

Chlorpromazine and trifluopherazine were dissolved in water at concentrations of 10,200 mg/L. Two fold serial dilutions of the drugs were made in RPMI-1640 in order to obtain final concentrations that ranged from 0.5 to 256 mg/L of both drugs. A drug-free well containing 0.01% water in the medium served as the growth control. The tests were performed in 96-well flat bottom microtitration plates (Corning Incorporated, N.Y., USA) which were kept at -70ºC until the day of testing. After the inoculation, the microtitration plates were incubated at 35ºC for 24 to 72 h. The MICs were read by spectrophotometric reading (Rosys Anthos ht3, Anthos Labtex Instruments GmbH, Salzburg, Austria). Background optical density (OD) was measured spectrophotometrically in non-inoculated wells processed in the same way as the inoculated wells. The relative ODs for each well based on measurements at 405 nm were calculated (in percentage) based on the following equation: [(OD of drug containing well – background OD)/(OD of drug-free well – background OD)] x 100%. The MIC for both drugs was defined as the lowest concentration of the drug that showed at least 95 % reduction of growth compared with that of the growth control (MIC-0). The susceptibility was performed in duplicate.

**PAFE assay.** The method used for determining PAFE was described for *Aspergillus* spp. (25). Chlorpromazine and trifluopherazine were dissolved in water to obtain an initial concentration of 16,000 mg/L and aliquots of the stock solution were stored at –70ºC until used. The day that the assay was done, the drugs were diluted in water to obtain a dilution of 5,100 mg/L. Serial dilutions of the drugs were made in RPMI-1640 with MOPS in order to obtain final concentrations of 2 and 1 times the corresponding MIC. Control conidia or spore suspensions were made in RPMI-1640 without drug. The isolates were passaged twice at an interval of 5-7 days at 35ºC by subculturing onto SGA to obtain adequate sporulation. Conidia or spores were collected with a cotton swab and suspended in saline with 0.01% Tween 20 or 80. Tween 20 was used for Aspergillus and Scedosporium species and Tween 80 for Zygomycetes. After the heavy particles were allowed to settle, the supernatant was transferred to another tube, vortexed for 10 sec, and 10 and 100 times dilutions were made. The concentration of conidia or spores was established microscopically by hematocymeter Burker Turk chambers. Then, the concentration was adjusted to obtain 4 x 105 conidia or spores/ml. One milliliter of this suspension was added to tubes containing 9 ml of RPMI-1640 alone (control) or

chlorpromazine or trifluopherazine in concentrations mentioned above resulting in a final volume of 10 ml. The final inoculum therefore was 4 x $10^4$ CFU/ml. Following this procedure, the incubation was for 1 up to 6 h with continuous shaking at 37ºC according to the corresponding strain.

After incubation, the conidia or spores were washed with saline plus 0.5 % Tween 20 or 80 and centrifuged at 3,500 x g during 15 min. After three wash cycles, 98% of the supernatant was completely decanted and the pellets were resuspended in a final volume of 10 ml RPMI-1640 with 0.01% Tween 20 or 80. Following this step, 100 ml of sample was diluted 10-fold in sterile water and 30 ml aliquots were plated onto SGA plates for colony count determination, and incubated at 37ºC for 24 up 72 h. The concentration of viable CFU/ml for exposed conidia or spores was determined in order to verify the concentration of viable conidia or spores post drug exposure compared with the controls. From the resuspended suspension, 200 ml was placed in microtitration plates and incubated at 37ºC in a computerized spectrophotometric reader (Rosys Anthos ht3). Any growth was automatically monitored in terms of change in turbidity at 405 nm, at 10 min intervals for 48 h to 72 h. All assays were performed in duplicate.

**Data analysis**. The repetitive OD measurements for each well resulted in the growth curve. PAFE was determined by comparing the growth curve of the exposed spores with that of the controls. $OD_0$ was used to calculate PAFE as was previously described [21], by using the formula PAFE = T - C, where T was the time of the first significant increase in $OD_0$ of the exposed conidia or spores after removal of the drug and C was the time of the first significant increase in $OD_0$ of the control. Thus, PAFE was defined as the difference in time (Dt) between exposed and controls to reach the defined point in the growth curve and was expressed in hours. The time to reach this chosen point, $OD_0$ , of at least 8 controls for each species was calculated and the mean, range, upper 95% confidence interval (CI) and the coefficient of variance were calculated in order to determine the reproducibility of the control curves at that point and to establish the reproducibility of the experiments. For each species the upper 95% CI of the controls was chosen as the cut-off level that distinguished between presence or absence of PAFE. When re-growth of the exposed isolates occurred within the upper 95% CI time-frame of the controls, PAFE was considered to be absent. Alternatively, if re-growth was delayed following drug

exposure and the lower 95% CI of the exposed isolates was delayed until beyond the upper 95% CI of the controls, PAFE was considered to be present. Growth curves of each exposed isolate were compared only with pooled controls from that same isolate.

Comparison of PAFE between both drugs en each group for both concentrations were analyzed by analysis of variance for repeated observations followed by Bonferroni's Multiple Comparison Tests. P values of $< 0.05$ were considered statistically significant.

## Results

Chlorpromazine and trifluopherazine were active in vitro against all moulds tested. (Table 1). The geometric mean (GM) in mg/L of chlorpromazine were: *A fumigatus*: 42.2; *A. ustus*: 64; *A. terreus*: 64; *A. corymbifera*: 59.7; *R. oryzae*: 32; *R. microsporum*: 32; *S. apiospermum*: 48.5 and *S. prolificans*: 34.3.

The GM, in mg/L, of trifluopherazine were: *A fumigatus*: 34.3; *A. ustus*: 32; *A. terreus*: 34.3; *A. corymbifera*: 34.3; *R. oryzae*: 16; *R. microsporum*: 16; *S. apiospermum*: 39.4; and *S. prolificans*: 32.

Viability of the conidia or spores. Exposure of conidia to the used concentrations of chlorpromazine and trifluopherazine for 6 h had no effect on the viability of the *Aspergillus* and *Scedosporium* species. For Zygomycetes exposure of spores to the used concentrations of chlorpromazine and trifluoperazina for 4 h had no effect on the viability of *A. corymbifera* but for the *Rhizopus* species tested both drugs reduced the concentration of viable spores by more than 80 % compared with the control. Therefore, PAFE was determined for 1 h exposure at 2 and 1 times the corresponding MIC for *Rhizopus* species. In these conditions viability was not affected based on subculture of serial two-fold dilutions (data not shown).

Inoculum. By using a haemocytometer chamber a limited range of 2 to 4 x $10^4$ viable CFU/ml was achieved.

**PAFE assay**. All growth curves were reproducible among the replicates although they were different depending on the strain evaluated. The shape of the curves was identical for both drugs.

Table 1. The in vitro activity of chlorpromazine and trifluopherazine against 84 filamentous fungi.

| Fungus (n= number of isolates) | $MIC_{90}$ mg/L (range) | |
|---|---|---|
| | Chlorpromazine | Trifluopherazine |
| *Aspergillus fumigatus* (n=20) | 64 (32-64) | 32 (32-64) |
| *Aspergillus terreus* (n=10) | 64 (64) | 32 (32-64) |
| *Aspergillus ustus* (n=10) | 64 (64) | 32 (32) |
| *Absidia corymbifera* (n=10) | 64 (32-64) | 32 (32-64) |
| *Rhizopus oryzae* (n=7) | 32 (32) | 16 (16) |
| *Rhizopus microsporum* (n=7) | 32 (32) | 16 (16) |
| *S. apiospermum* (n=10) | 64 (32-64) | 64 (32-64) |
| *S. prolificans* (n=10) | 64 (32-64) | 32 (32) |

$MIC_{90}$: minimal inhibitory concentration that inhibited 90% of the isolates tested.

For the same species the shape of the growth curve of the control was identical to that of the exposed strains. When PAFE was present the growth curve of the exposed spores was shifted to the right compared to that of the control. Examples of growth curves are shown in Fig. 1.

Microscopic morphology. Microscopic examination of the conidia or spores before and after drug exposure showed that germination did not occur within the maximal exposure period in each case. Germination of controls and drug exposed conidia or spores occurred

at the same time and to the same extent in those strains where PAFE was not present. When PAFE was present, germination of the exposed conidia or spores was further delayed.

Table 2. PAFEs induced by chlorpromazine against 84 filamentous fungi.

| Fungus (n= number of isolates) | PAFE (h) | | | |
|---|---|---|---|---|
| | Cc* | Mean | Range | N/TN |
| *A.fumigatus* ( n=20) | 2x | 3 | 0.9-5.8 | 15/20 |
| | 1x | 0.2 | -0.5-0.8 | 0/20 |
| *A. terreus* (n=10) | 2x | 3.2 | 1.1-5.3 | 6/10 |
| | 1x | 0.2 | -1.2 | 2/10 |
| *A. ustus* (n=10) | 2x | 5.5 | 4-7.3 | 10/10 |
| | 1x | 1.3 | -1.2-4.2 | 2/10 |
| Overall: *Aspergillus* species | 2x | 3.7 | 0.9-7.3 | 31/40 |
| (n=40) | 1x | 0.4 | -2.1-4.2 | 4/40 |
| *A.corymbifera* (n=10) | 2x | 4.2 | 3.1-5 | 10/10 |
| | 1x | 1.8 | 0.5-2.5 | 8/10 |
| *R.oryzae* (n=7) | 2x | 2.5 | 1.3-3.8 | 7/7 |
| | 1x | 0.3 | 0.1-0.6 | 0/7 |
| *R. microsporus* (n=7) | 2x | 2 | 1.1-3 | 7/7 |
| | 1x | 0.3 | -0.03-0.9 | 0/7 |
| Overall: Zygomycetes | 2x | 3.1 | 1.1-5 | 24/24 |
| (n=24) | 1x | 0.9 | -0.03-2.5 | 8/24 |
| *S. apiospermun* (n=10) | 2x | 4.5 | 0.5-10.7 | 8/10 |
| | 1x | 1.3 | 0.4-2.1 | 1/10 |
| *S. prolificans* (n=10) | 2x | 4.1 | 2.5-7.4 | 10/10 |
| | 1x | 0.9 | 0.4-2.1 | 0/10 |
| Overall: *Scedosporium species* | 2x | 4.3 | 0.5-10.7 | 18/20 |
| | 1x | 1.1 | -1.8-3.5 | 1/20 |
| (n= 20) | | | | |

*: concentration of the drug tested. 2 times (2x) and 1 time (1x) the corresponding MIC.
N/TN: number of strains for which trifluopherazine displayed significant PAFE/ total number of strains tested considering the 95% CI.

Comparison amongst all the strains. In general, PAFE was induced by both drugs and was influenced by concentration. The highest concentration resulted in the longest PAFE in all the strains tested. (Table 2-3).

Overall, the mean and the range of the PAFE induced by chlorpromazine at 1 and 2 times the MIC were 0.7 h (-2.1-4.2) and 3.6 h (0.5-10.7), respectively, compared with 0.5 h (-2.3-3.9) and 4.5 h (-3.2-10) for trifluopherazine. At 2 times the MIC, 87 % (73/84) of all strains showed PAFE for chlorpromazine and 93 % (78/84) for trifluopherazine.

Comparison between *Aspergillus* species. PAFE was not observed for *A. fumigatus* at 1 time the MIC for both drugs. However, for these species at 2 times the MIC, chlorpromazine induced PAFE against 75% of the strains (mean PAFE 3h) and trifluopherazine against 85% of the strains (mean PAFE 4h). (Table 2-3).Chlorpromazine induced PAFE against 60% of *A. terreus* and *A. ustus* strains and trifluopherazine against 100% of the strains at 2 times the MIC. For these species chlorpromazine induced PAFE at 1 time the MIC for only 2 of the 10 strains tested. (Table 2-3).

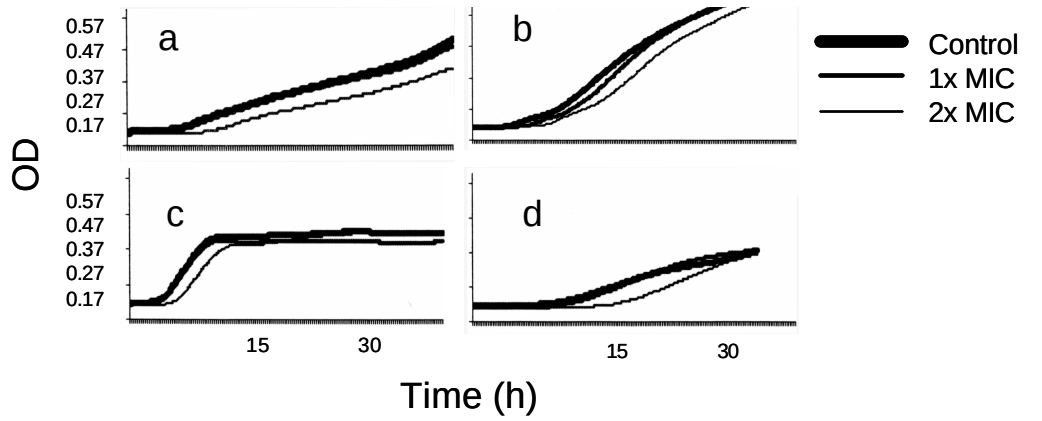
Overall, for *Aspergillus* species, chlorpromazine induced PAFE at 2 times the MIC for 76% of the strains and trifluopherazine for 92%. (Table 2-3).

Both drugs induced the longest PAFE at 2 times the MIC for *A. ustus* with statistical significant difference compared with *A. fumigatus* and *A. terreus* ($p<0.05$). (Table 2-3).

The PAFE induced by trifluopherazine at 2 times the MIC was significantly longer than by chlorpromazine ($p<0.05$). (Table 2-3).

Comparison between different genera of Zygomycetes. PAFE was observed for all the *A. corymbifera* strains at 2 times the MIC with a mean of 4.2 h for chlorpromazine and 4.5 h for trifluopherazine. For *Rhizopus* spp. no differences in PAFE values were seen between both drugs.

Figure 1. Growth curves of 4 filamentous fungi exposed to trifluopherazine at 1 and 2 times the MIC values




a: *A. fumigatus* b: *A. corymbifera*
c: *R. oryzae* d: *S. prolificans*

Chlorpromazine induced PAFE for *R. oryzae* and *R. microsporus* at 2 times the MIC, (mean PAFE 2.5 h and 2 h respectively), whereas for trifluopherazine the mean PAFE values were 2.5 h and 2.8 h, respectively. No PAFE was observed after exposure of both species to either drug at a concentration equivalent to the MIC. (Table 2-3).

Overall, for Zygomycetes, chlorpromazine and trifluopherazine induced PAFE at 2 times the MIC for 100% of the strains. (Table 2-3).

Table 3. PAFEs induced by trifluopherazine against 84 filamentous fungi.

| Fungus (n= number of isolates) | Cc* | PAFE (h) Mean | Range | N/TN |
|---|---|---|---|---|
| *A.fumigatus* ( n=20) | 2x | 4 | 0.3-6.6 | 17/20 |
| | 1x | -0.1 | -1.2-0.9 | 0/20 |
| *A. terreus* (n=10) | 2x | 4.5 | 1-8.6 | 10/10 |
| | 1x | 0.7 | -1.4-3.1 | 2/10 |
| *A. ustus* (n=10) | 2x | 6.5 | 2.9-10 | 10/10 |
| | 1x | -0.07 | -2.3-2.3 | 0/10 |
| Overall: *Aspergillus* species | 2x | 4.7 | 0.3-10 | 37/40 |
| (n=40) | 1x | 0.1 | -2.3-3.1 | 2/40 |
| *A.corymbifera* (n=10) | 2x | 4.5 | 3.5-6 | 10/10 |
| | 1x | 1.4 | 0.1-2.3 | 6/10 |
| *R.oryzae* (n=7) | 2x | 2.5 | 1.2-3.6 | 7/7 |
| | 1x | 0.6 | 0-1.3 | 0/7 |
| *R. microsporus* (n=7) | 2x | 2.8 | 2-3.4 | 7/7 |
| | 1x | 0.8 | 0.1-1 | 0/7 |
| Overall: Zygomycetes | 2x | 3.4 | 1.2-6 | 24/24 |
| (n=24) | 1x | 1 | -0.03-2.3 | 7/24 |
| *S. apiospermun* (n=10) | 2x | 4.7 | -3.2-9.8 | 7/10 |
| | 1x | 1.1 | -1.6-3.9 | 1/10 |
| *S. prolificans* (n=10) | 2x | 5.9 | 3-9.7 | 10/10 |
| | 1x | 0.4 | -2.3-2.2 | 0/10 |
| Overall: *Scedosporium* | 2x | 5.3 | -3.2-9.8 | 17/20 |
| *species* | 1x | 0.7 | -2.3-3.9 | 1/20 |
| (n= 20) | | | | |

*: concentration of the drug tested. 2 times (2x) and 1 time (1x) the corresponding MIC.
N/TN: number of strains for which trifluopherazine displayed significant PAFE/ total number of strains tested considering the 95% CI.

Both drugs induced the longest PAFE at 2 times the MIC , with a mean PAFE value of 3.6 h and 4.5 h for chlorpromazine and trifluopherazine, respectively. Thus statistical significant difference between both drugs was observed ($p<0.05$). (Table 2-3).

Comparison between *S. apiospermum* and *S. prolificans*. The mean PAFE value for chlorpromazine at 2 times the MIC was 4.5 h for *S. apiospermum* and 4.1 h for *S. prolificans* and for trifluopherazine was 4.7 h and 5.9 h, respectively. (Table 2-3). For only one strain of *S. apiospermum* of the 20 tested both drugs induced PAFE at 1 time the MIC, whereas at 2 times the MIC, for 90% of the strains the effect was observed with

mean PAFE values of 4.3 h and 5.3 h for chlorpromazine and trifluopherazine, respectively. No statistical significant difference was found between both drugs ($p>0.05$). (Table 2-3). Overall, for these species, chlorpromazine induced PAFE at 2 times the MIC for 90% of the strains and trifluopherazine for 85%. (Table 2-3).

## Discussion

In this study, chlorpromazine and trifluopherazine were shown to be moderately active against *Aspergillus*, zygomycetes and *Scedosporium* at concentrations ranging from 16 to 64 µg/ml. These concentrations are beyond the achievable plasma concentrations in humans, that vary between 0.5 and 1 µg/ml. Although the plasma concentrations of these drugs is low, they may be concentrated to as much as 70 to 100 fold the plasma level in tissues and macrophages (2, 12). In autopsy studies concentrations of phenothiazines of up to 190 µg/g were found in the liver (13). The in vitro activity observed is comparable with that shown for *Candida* species (10 to 40 µg/ml) (8, 28) and *C. neoformans* (40 µg/ml) (27). Activity at similar concentrations was also reported against *Staphylococcus aureus* (35 µg/ml), *Acinetobacter* species (40 µg/ml), *Streptococcus faecalis* (60 µg/ml) and *Escherichia coli* (80 µg/ml) (1). In a murine model of invasive candidosis the survival of infected control mice with *C. albicans* was 14% while for mice receiving daily injections of 4.2 mg/kg of body weight of trifluopherazine, 85.7% survived. Similar results were obtained in a *C. neoformans* in vivo model in which the survival increased from 10% to 70% for mice receiving daily injections of 7 mg/kg of body weight of trifluopherazine (8).

The mechanism of action of these drugs against fungi is unknown, although they induce multiple effects in eukaryotic cells including modification of membranes, alteration of cyclic nucleotid metabolism and action on calmodulin (8, 17, 19). Since the presence of calmodulin in *Candida* was reported, this could act as target for these drugs (14). *A. nidulans* contains genes encoding for $Ca^{2+}$ binding proteins that are essential for growth (16). Alternatively, phenothiazines were found to be modulators of membrane drug transporters (multidrug efflux pumps), which might be an alternative mechanism of

action. However, the exact mode of action in the fungal pathogens tested in our study remains to be established.

Besides inhibition of growth, exposure of *Aspergillus*, zygomycetes and *Scedosporium* to the phenotiazines resulted in a concentration-dependent post antifungal effect. In previous studies with *Aspergillus* and *Candida* using this and other in vitro models, PAFE was observed only for polyenes and not for other antifungal agents including azoles and allylamines (9-11, 25). Amphotericin B induced longer PAFE than the antipsychotic drugs against *A. fumigatus*; 9.9 h for amphotericin B compared with 3.7 h for chlorpromazine and 4.7 h for trifluopherazine. However, for *A. terreus* and *A. ustus* similar PAFE values were observed for amphotericin B and chlorpromazine and trifluopherazine (25). The phenothiazines are the first drugs to induce PAFE against *Scedosporium*.

The role of phenothiazines for the treatment of patients with fungal infection is unknown, given the high number of side-effects which suggests non-specific toxicity against eukaryotic cells. This is supported by the fact that a large variety of micoorganisms, both prokaryotic and eukaryotic, are inhibited by similar concentrations of drug. However, the profile of effects induced by the drugs is greatly influenced by geometric stereoisomerisim of the compounds. The *cis* form of the compounds possess both neuroleptic and antifungal activity, while the *trans* form shows antifungal activity but no neuroleptic effects (18).

Given the geometric stereoisomerism, the pharmacokinetic properties of the drugs with high drug levels in brain tissue, and the potent post antifungal effect in difficult-to-treat moulds, phenothiazines might be an interesting drug to further explore for its potential for treatment of invasive fungal infection either alone or in combination with antifungal agents. For this, the mechanism of action in fungi needs to be identified.

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# Chapter 3.4

Evaluation of minimal inhibitory concentration, minimal fungicidal concentration and post antifungal effect (PAFE) of *Aspergillus fumigatus* after exposure to subinhibitory concentrations of itraconazole, fluconazole and amphotericin B.

*Submitted for publication*

# Evaluation of Minimal Inhibitory Concentration, Minimal Fungicidal Concentration and Post Antifungal Effect (PAFE) of *Aspergillus fumigatus* After Exposure to Subinhibitory Concentrations of Itraconazole, Fluconazole and Amphotericin B.

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## Abstract

We evaluated the minimal inhibitory concentration (MIC), minimal fungicidal concentration (MFC) and post antifungal effect (PAFE) of amphotericin B (AMB), itraconazole (ITZ) and fluconazole (FCZ), in nine clinical strains of *Aspergillus fumigatus* of which three were ITZ susceptible (ISS), three ITZ resistant (IRS) and three ITZ mutants (IRM). The evaluation was carried out by pre-incubating all strains at sub-inhibitory concentrations of ITZ, AMB and FCZ, and by comparing the results with those from the wild-type strains.

The MICs and MFCs of AMB were similar for the pre and wild-type strains. The MICs for ITZ were similar after 1 week of pre-incubation although the MFCs for the ISS

increased. However, after 8 weeks of pre-incubation the MICs of the ISS also increased. When the strains were pre-incubated with FCZ, the MICs of ITZ for the non and pre-incubated strains were similar whereas the MFCs were higher for the pre-incubated strains. Preincubation with AMB did not alter the MICs and MFCs of ITZ.

For the wild-type strains, PAFE was induced by AMB at 4, 1 and 0.5 times the corresponding MIC but not by ITZ or FCZ. After pre-incubation longer PAFEs were observed with the exception of those pre-incubated with ITZ during 1 week at 1 and 0.5 times the MIC. In all cases, PAFE was concentration dependent.

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## Introduction

Invasive fungal infections are a common cause of morbidity and mortality in immunocompromised patients such as neutropenic patients or hematopoietic stem cell transplant recipients (2). AMB remains a first line drug for treatment of fungal infections but in certain high risk patient groups establishing prophylactic strategies is vital to prevent invasive infections. The use of AMB as prophylactic agent is limited due to a lack of gastrointestinal absorption and its side effects (3). FCZ is well absorbed and has a favorable toxicity profile but has a narrow spectrum of activity excluding several non-*albicans Candida* species and moulds (8). ITZ has a broad spectrum of activity and is frequently used for prophylaxis of invasive fungal infections (3, 7, 20, 21). However, attenuation of the effectiveness of AMB after ITZ prophylaxis has been reported (3, 7, 24). It has been postulated that azoles inhibit the biosynthesis of ergosterol resulting in its depletion thereby reducing the target for AMB. Thus, subsequent exposure of fungi to azoles and AMB would result in an antagonistic interaction. This effect was described for *Candida* species and *Aspergillus* species in vitro and in vivo (9, 10, 18, 22). In contrast, administration of AMB followed by azoles was reported to be effective (1, 12, 19) and administration of AMB and azoles simultaneously appears not to result in significant antagonism between the drugs.

Since patients at high risk for fungal infections commonly receive either FCZ or ITZ prophylaxis and switch to an AMB formulation when suspected for invasive fungal infection, we sought to simulate this situation in vitro. *Aspergillus fumigatus* strains with variable susceptibility to ITZ were selected including susceptible strains, laboratory resistant mutants and clinical resistant strains. The strains were pre-incubated with the azoles ITZ and FCZ, and with the polyene AMB during a period of up to eight weeks. The activity of AMB, ITZ and FCZ against the wild-type and pre-incubated strains was determined using several methods including minimal inhibitory concentration (MIC), minimal fungicidal lethal concentration (MFC) and post antifungal effect (PAFE).

## Materials and methods

**Isolates.** Nine *A. fumigatus* strains from our private collection were evaluated. ITZ susceptible (ISS) were AZN 9339, AZN 5161, AZN 7820. These isolates were used to create laboratory resistant mutants to ITZ (IRM): AZN 5161, AZN 9339, AZN 7820 and ITZ resistant strains (IRS) that were clinical strains obtained from different patients with high MIC values of ITZ (AZN 7722, AZN 58, AZG 7).

**Antifungal agents.** AMB (Bristol-Myers Squibb, Woerden, The Netherlands), ITZ (Janssen-Cilag, Beerse, Belgium) and FCZ (Pfizer, Capelle aan den IJssel, The Netherlands) were utilized for all in vitro studies. AMB and ITZ were dissolved in dimethyl sulfoxide (DMSO) and FCZ in water. Aliquots of the stock solution were stored at –70 ºC until use. The stock solutions were diluted in RPMI-1640 medium (with L-glutamine without bicarbonate) (GIBCO BRL, Life Technologies, Woerden, The Netherlands) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

**ITZ resistant mutants.** In order to obtain resistant mutants, the wild-type strains were maintained on potato dextrose agar for 6 days at 35ºC and the conidia were collected to prepare a suspension. The concentration of conidia was established microscopically by hematocymeter Burker Turk chambers. Approximately $5 \times 10^6$ conidia per plate were spread on potato dextrose agar plates containing 8 µg/ml of ITZ. The plates were incubated for 6 days at 35ºC and then were subcultured following the same procedure once a week during 6 months. The in vitro activity of ITZ was determined over time using the NCCLS M-38A guideline (16).

**Pre-incubation of the strains.** All *A. fumigatus* strains were preincubated at subinhibitory concentrations of the drugs in plates containing solid media. The plates were prepared as follows: RPMI-1640 was prepared at double concentration (2x) and sterilized by filtration. Agar-agar was prepared at double concentration (2x) and autoclaved. Then, both media were mixed and when the temperature was appropriate, the drugs were added.

The final drug concentrations in the media were: ITZ 0.06 µg/ml for ISS and 1µg/ml for IRS; 0.12 µg/ml for AMB and 8 µg/ml for FCZ for all strains. Each strain was then

subcultured once a week in these plates containing antifungals during a total period of 8 weeks.

**Antifungal susceptibility testing.** The MICs of AMB, ITZ and FCZ were determined according to the NCCLS guideline (16). The wild-type isolates were passaged twice at an interval of 5-7 days at 28ºC by subculturing onto Sabouraud glucose agar (SGA) to obtain adequate sporulation. The susceptibility of the pre-incubated strains was determined after one and eight weeks of pre-incubation of the wild-type strains with each drug. Conidia were collected with a cotton swab and suspended in saline with 0.05% Tween 20%. After the heavy particles were allowed to settle, the turbidity of supernatants was measured spectrophotometrically (Spectronic 20D; Milton, Roy, Rochester, N.Y., USA) at 530 nm and transmission was adjusted to 80-82% corresponding to 0.5 to 4.5 x $10^6$ CFU/ml. The viability was confirmed by plating serial dilutions onto SGA plates.

For inoculum preparation the conidia suspensions were diluted 1:100 in order to obtain a final inoculum of 0.5 to 4.5 x $10^4$ CFU/ml. A broth microdilution method was performed according to the NCCLS guideline (16) using RPMI-1640 medium buffered to pH 7.0 with 0.165 M MOPS. AMB and ITZ were dissolved in DMSO and FCZ in water at a concentration of 3,200 µg/ml. Two fold serial dilutions of the drugs were made in RPMI-1640 medium in order to obtain final concentrations of 0.25 to 64 µg/ml for FCZ and from 0.015 to 16 µg/ml for the other drugs. A drug-free well containing 0.01% DMSO served as the growth control for ITZ and AMB. The tests were performed in 96-well flat bottom microtitration plates (Corning Incorporated, N.Y., USA) which were kept at -70ºC until the day of testing. Conidia suspensions, prepared as described above, were diluted 1:50 in RPMI-1640 to obtain two times the desired inoculum. After inoculation, the microtitration plates were incubated at 35ºC for 48 h. The MICs were read by spectrophotometric reader (Rosys Anthos ht3, Anthos Labtex Instruments GmbH, Salzburg, Austria). Background optical density (OD) was determined by spectrophotometric measurement of noninoculated wells processed in the same way as the inoculated wells. The relative ODs for each well based on measurements at 405 nm were calculated (in percentage) based on the following equation: [(OD of drug containing well – background OD)/(OD of drug-free well – background OD)] x 100%. The MIC for AMB was defined as the lowest concentration of the drug that showed at least 95 %

reduction of growth compared with that of the growth control (MIC-0) and for ITZ the MIC was defined as the lowest concentration of the drug that showed 50% of reduction of growth compared with that of the growth control (MIC-2). Off scale MICs or MFCs were converted to the next highest concentration.

**Minimal fungicidal concentration (MFC).** The MFC was determined for each drug with the wild-type and the pre-incubated strains by streaking 100 µl from each well that showed complete inhibition of growth onto SDA plates. The plates were incubated at 35ºC. The MFC was defined as the lowest drug concentration at which growth of less than two colonies was observed, which corresponds to 99.9% killing.

**PAFE assay.** The method used for determining PAFE was recently described for *Aspergillus* spp (23). PAFE was evaluated for all the drugs in different conditions: a) for the wild-type, b) for ISS, IRM and IRS incubated for one week, c) for ISS, IRM and IRS incubated for eight weeks. AMB and ITZ were dissolved in DMSO at initial concentrations of 400 µg/ml whereas FCZ was dissolved in water and aliquots of the stock solution were stored at –70 ºC until used. Then they were diluted 50 times in RPMI-1640 (with L-glutamine without bicarbonate) buffered to pH 7.0 with 0.165 M MOPS. Serial dilutions of the drugs were made in order to obtain final concentrations of 4, 1 and 0.5 times the corresponding MIC. Control spore suspensions were made in RPMI-1640 without drug. The non-exposed isolates were passaged twice at an interval of 5-7 days at 28ºC by subculturing onto SGA to obtain adequate sporulation. For the pre-incubated strains, the conidia were taken directly from the plates with RPMI-1640 agar with the corresponding drug. Conidia were collected with a cotton swab and suspended in saline with 0.01% Tween 20. After the heavy particles were allowed to settle, the supernatant was transferred to another tube, vortexed for 10 sec, and 10 and 100 times dilutions were made. The concentration of conidia was established microscopically by haemotocymeter Burker Turk chambers. Then, the concentration was adjusted to obtain 4 x $10^5$ conidia/ml. One milliliter of this suspension was added to tubes containing 9 ml of RPMI-1640 alone (control) or with AMB, ITZ or FCZ in concentrations mentioned above resulting in a final volume of 10 ml. The final inoculum therefore was 4 x $10^4$ CFU/ml. Following this procedure, each strain was exposed for 4 h with continuous shaking at 37 ºC.

After incubation the conidia were washed with saline plus 0.01% Tween 20 and centrifuged at 3,500 x *g* during 15 min. After three wash cycles, 98% of the supernatant was completely decanted and the pellets were resuspended in a final volume of 10 ml RPMI-1640 with 0.01% Tween 20. Following this step, 100 µl of sample was diluted 10-fold in sterile water and 30 µl aliquots were plated onto SGA plates for CFU determination, and incubated at 37ºC for 48 h. The concentration of viable CFU/ml for exposed conidia was determined in order to verify the concentration of viable conidia post drug exposure and to allow adjustment of the inoculum, if necessary, to match that of controls. From the resuspended suspension, 200 µl was placed in microtitration plates and incubated at 37ºC in a computerized spectrophotometric reader (Rosys Anthos ht3). Any growth was automatically monitored in terms of change in turbidity at 405 nm, at 10 min intervals for 48 h. All assays were performed in duplicate.

**Data analysis.** The repetitive OD measurements for each well resulted in a growth curve. PAFE was determined by comparing the growth curve of the exposed conidia with that of the controls. $OD_0$ was used to calculate PAFE as was previously described (23), by using the formula PAFE = T - C, where T was the time of the first significant increase in $OD_0$ of the exposed conidia after removal of the drug and C was the time of the first significant increase in $OD_0$ of the control. Thus, PAFE was defined as the difference in time ($\Delta t$) between exposed and controls to reach the defined point in the growth curve and was expressed in hours. The time to reach this chosen point, $OD_0$, of at least 8 controls for each species was calculated and the mean, range, upper 95% confidence interval (CI) and the coefficient of variance were calculated in order to determine the reproducibility of the control curves at that point and to establish the reproducibility of the experiments. For each species the upper 95% CI of the controls was chosen as the cut-off level that distinguished between presence or absence of PAFE. When re-growth of the exposed isolates occurred within the upper 95% CI time frame of the controls, PAFE was considered to be absent. Alternatively, if re-growth was delayed following drug exposure and the lower 95% CI of the exposed isolates was delayed until beyond the upper 95% CI of the controls, PAFE was considered to be present. Growth curves of each exposed isolate were compared only with pooled controls from that same isolate.

Comparison of PAFE between non and pre-incubated strains of ISS, IRM and IRS were analyzed by analysis of variance for repeated observations followed by Bonferroni's Multiple Comparison Tests. *P* values of < 0.05 were consideredstatistically significant.

## Results

In this study 9 *A. fumigatus* strains were studied characterized by three ITZ resistance-profiles: ITZ susceptible (ISS), ITZ resistant (IRS) and ITZ resistant mutants (IRM). Each of these strains were exposed to 6 pre-incubation conditions, including exposure to ITZ for 1 and 8 weeks; FCZ for 1 and 8 weeks and AMB for 1 and 8 weeks. For each of these strains and conditions three parameters were studied namely MIC, MFC and PAFE.

**Inoculum.** By using a haemocytometer chamber a limited range of 2 to 4 x $10^4$ viable CFU/ml was achieved. This stringent range was chosen to achieve reproducible growth curves.

**Viability:** Exposure of conidia to the used concentrations of antifungal agents for 4 h had no effect on the viability in any condition tested for all the strains. In these conditions viability was not affected based on subculture of serial two-fold dilutions (data not shown).

**Microscopic morphology.** Microscopic examination of the conidia before and after drug exposure showed that germination did not occur within the maximal exposure period of 4 h for all species. The germination of 95% of the controls and drug-exposed conidia commenced after 12 h when PAFE was absent as was demonstrated before.

For the strains where PAFE was observed no differences in morphology was seen, the only difference was the time at which the germination started.

Table 1. MIC and MFC of amphotericin B for all the *A. fumigatus* non-preincubated and incubated for 1 or 8 weeks with amphotericin B (AMB), itraconazole (ITZ) or fluconazole (FCZ).

| | Amphotericin B MIC/MFC (µg/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Strain number-profile<br>Pre-incubation condition | 9339 ISS | 5161 ISS | 7820 ISS | 9339 IRM | 5161 IRM | 7820 IRM | AZG-7 IRS | 58 IRS | 7722 IRS |
| Wild-type | 1/4 | 0.5/2 | 0.5/2 | 1/4 | 0.5/4 | 1/4 | 0.5/2 | 0.5/2 | 1/2 |
| 1 week ITZ | 1/2 | 1/4 | 1/2 | 1/4 | 0.5/4 | 1/4 | 1/2 | 1/2 | 1/2 |
| 8 weeks ITZ | 2/4 | 1/2 | 2/4 | 1/4 | 1/8 | 2/4 | 1/2 | 1/2 | 1/2 |
| 1 week AMB | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | 2/8 |
| 8 weeks AMB | 0.5/2 | 0.5/1 | 1/2 | 1/2 | 0.5/1 | 1/2 | 0.5/1 | 0.5/1 | 0.5/2 |
| 1 week FCZ | 1/4 | 1/4 | 2/4 | 2/4 | 2/4 | 2/4 | 2/2 | 2/4 | 1/2 |
| 8 weeks FCZ | 1/4 | 1/2 | 1/2 | 1/2 | 1/2 | 1/4 | 1/2 | 1/2 | 1/2 |

ISS: itraconazole susceptible strains
IRS: itraconazole resistant strains
IRM: itraconazole resistant mutants strains

**MIC determination.** The MIC of AMB of the tested *A. fumigatus* isolates is shown in table 1. Pre-incubation with AMB, ITZ and FCZ for 1 or 8 weeks did not affect the MIC of AMB.

The MIC of ITZ is shown in table 2. The ISS had low MICs of ITZ while the IRS and IRM had high MICs. Pre-incubation of the ISS with ITZ for 1 week had no effect on the MIC, while after 8 weeks of pre-incubation the MIC had risen with 2 to 6 dilution steps. Pre-incubation with AMB and FCZ had no effect on the MIC of ITZ. The MIC of the IRS and IRM strains was not affected by pre-incubation with AMB, ITZ and FCZ. For FCZ all the strains showed MICs > 64 µg/ml.

**MFC determination.** The MFCs of AMB ranged from 4 to 8 µg/ml for the wild-type strains. Pre-incubation of the ISS strains with AMB, ITZ or FCZ resulted in MFC values ranging from 1 to > 16 µg/ml, but no differences of ≥ 2 dilution steps were found between the wild-type and the pre-incubated strains for the same isolate. The highest MFC values were observed when the strains were preincubated with FCZ, with values ranging from 4 to > 16 µg/ml. In general no differences in MFC between any of the pre-incubated strains was observed (Table 1 and 2).

For the IRS and IRM the MFC was not determined.

Table 2. MIC and MFC of itraconazole for all the *A. fumigatus* non-preincubated and incubated for 1 or 8 weeks with amphotericin B (AMB), itraconazole (ITZ) or fluconazole (FCZ).

| | Itraconazole MIC/MFC (μg/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Strain number-profile<br>Pre-incubation condition | 9339 ISS | 5161 ISS | 7820 ISS | 9339 IRM | 5161 IRM | 7820 IRM | AZG-7 IRS | 58 IRS | 7722 IRS |
| Wild-type | 0.25/8 | 0.12/8 | 0.12/4 | >16/ND | 1/8 | 16/ND | >16/ND | 16/ND | 16/ND |
| 1 week ITZ | 0.5/>16 | 0.25/>16 | 0.063/>16 | >16/ND | >16/ND | 16/ND | >16/ND | 16/ND | 16/ND |
| 8 weeks ITZ | 16/>16 | >16/ND | 2/8 | >16/ND | >16/ND | 16/ND | >16/ND | 16/ND | 16/ND |
| 1 week AMB | 0.25/1 | 0.25/2 | 0.5/4 | >16/ND | >16/ND | >16/ND | >16/ND | 16/ND | 16/ND |
| 8 weeks AMB | 0.25/4 | 0.25/>16 | 0.5/8 | >16/ND | >16/ND | >16/ND | >16/ND | >16/ND | >16/ND |
| 1 week FCZ | 0.25/>16 | 0.25/>16 | 0.5/>16 | >16/ND | >16/ND | >16/ND | >16/ND | >16/ND | >16/ND |
| 8 weeks FCZ | 0.25/>16 | 0.25/>16 | 0.25/>16 | >16/ND | >16/ND | >16/ND | >16/ND | >16/ND | >16/ND |

ISS: itraconazole susceptible strains
IRS: itraconazole resistant strains
IRM: itraconazole resistant mutants strains
N.D.: no determined

In general, PAFEs induced by AMB were always longer in the strains that had been pre-incubated with either AMB, ITZ or FCZ compared with the wild-type strains. However, AMB failed to induce PAFE in all strains that had been exposed to ITZ for 1 week at a concentration of 1 and 0.5 times the corresponding MIC.

**PAFE assay.** The shape of the growth curves of the control and the exposed strains were identical and reproducible among the replicates. When PAFE was present the growth curve of the exposed conidia was shifted to the right compared with that of the control (23).

**PAFE of AMB.** For the strains preincubated with AMB, AMB induced PAFE at all concentrations tested. The majority of PAFEs were longer compared with the non-exposed wild-type strains.

However, when the same strains were preincubated with ITZ for 1 or 8 weeks, at a concentration of 4 times the MIC, the PAFE induced by AMB was longer compared with the non-exposed strains. For strains exposed to ITZ at concentrations of 1 and 0.5 times the MIC during 1 week, PAFE was not induced by AMB. When the exposure to these conditions was continued for 8 weeks AMB was again able to induce PAFE. These PAFE values were similar than those observed for the non-exposed strains.

For the strains preincubated with FCZ, PAFE was induced by AMB at all the concentrations tested. (Table 3).

For ISS and IRM, statistical significant difference ($p<0.05$) was found between the wild-type and the pre-incubated strains at 4 times the MIC for all the drugs after 1 and 8 weeks of incubation. However, no difference ($p>0.05$) was observed for IRS at the same conditions. Comparing ISS with IRS only at 4 times the MIC statistical significant difference ($p<0.05$) was obtained analysing all drugs after 1 and 8 weeks of incubation.

**PAFE of ITZ and FCZ.** Both azole drugs failed to induce PAFE at any of the condition tested. Even concentrations of ITZ as high as 10 and 20 times the MIC failed to induce a PAFE (data not shown).

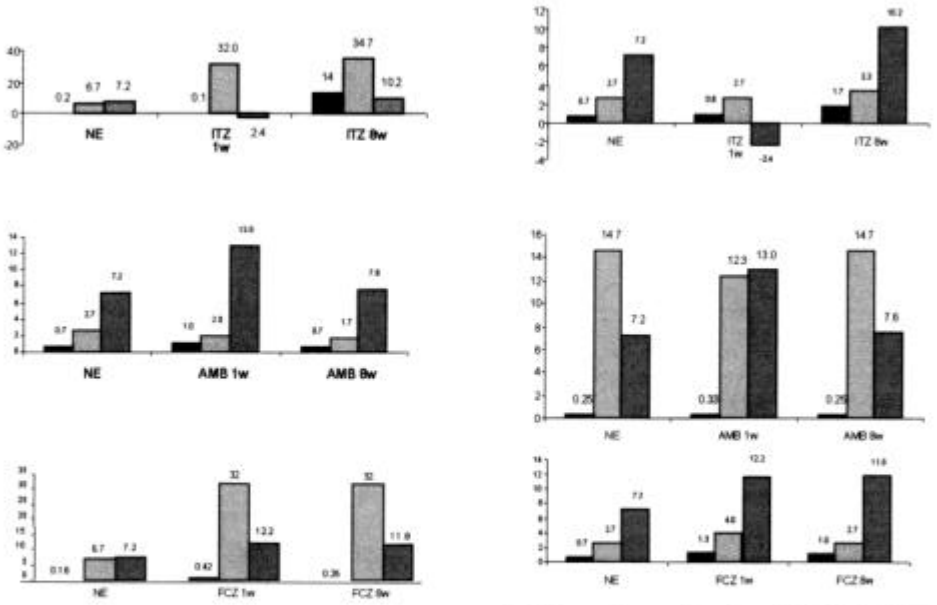
The comparison of the mean MICs, MFCs and PAFEs for the ISS are given in Figure 1.

Table 3. PAFE (h) after 4h exposure to amphotericin B (AMB) at 4,1 and 0.5 times the corresponding MIC for IS *A. fumigatus* strains and the corresponding IRM strains after incubation for1 or 8 weeks with amphotericin B (AMB), itraconazole (ITZ) or fluconazole (FCZ).

| Strain number-profile | 9339 ISS | | | 9339 IRM | | | 5161 ISS | | | 5161 IRM | | | 7820 ISS | | | 7820 IRM | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pre-incubation condition | 4x | 1x | 0.5x | 4x | 1x | 0.5x | 4x | 1x | 0.5x | 4x | 1x | 0.5x | 4x | 1x | 0.5x | 4x | 1x | 0.5x |
| Wild-type | 11.3 | 9.0 | 6.4 | 9.2 | 6.8 | 5.2 | 6.9 | 5.0 | 2.5 | 10.3 | 7.5 | 5 | 10.2 | 7.7 | 4.5 | 7.8 | 7.1 | 6.7 |
| 1 week ITZ | 20.6 | -4.3^ | -1.9^ | 22.5 | -0.5^ | -1.3^ | 18.3 | -0.5^ | -1.1^ | 17.4 | -0.4^ | -2.1^ | 18.5 | -2.3^ | -2.7^ | 20.5 | -1.2^ | -1.1^ |
| 8 weeks ITZ | 20.3 | 10.5 | 8.5 | 20.5 | 9.8 | 7.1 | 16.0 | 7.7 | 6.6 | 12.2 | 5.1 | 2.8 | 19.0 | 12.5 | 9.8 | 20.1 | 10.3 | 7.0 |
| 1 week AMB | 20 | 12.0 | 8.3 | 20 | 12.7 | 10.3 | 16.2 | 14.1 | 10.5 | 15 | 8.92 | 6.20 | 18.2 | 12.8 | 7.6 | 20.4 | 12.8 | 11.8 |
| 8 weeks AMB | 20.4 | 10 | 6 | 20.3 | 14.2 | 9.7 | 16.8 | 10.1 | 5.5 | 15.6 | 10 | 8 | 19.3 | 2.6 | -2.0 | 16.2 | 7.3 | 3.2 |
| 1 week FCZ | 19 | 11 | 6 | 21 | 15 | 11 | 15.8 | 14 | 11 | 16 | 12 | 8 | 17 | 12 | 8 | 19 | 19.8 | 12.4 |
| 8 weeks FCZ | 18 | 10.0 | 5.5 | 19 | 13.9 | 10 | 14.9 | 13 | 9.6 | 15.2 | 11.6 | 8.2 | 16.8 | 12.3 | 7.9 | 18.4 | 19.1 | 11.9 |

ISS: itraconazole susceptible strains
IRS: itraconazole resistant strains
IMS: itraconazole resistant mutants strains
^: No PAFE present after 1 week of pre-incubation with ITZ at concentrations of 1x and 0.5x times the MIC, considering the 95% CI.

Figure 1. Mean comparison of MIC, MFC in (µg/ml) and PAFE in (h) for the ISS for all the conditions.




a: *Aspergillus* pre-incubated with ITZ. MIC and MFC to ITZ and PAFE to AMB.

b: *Aspergillus* pre-incubated with ITZ. MIC and MF PAFE to AMB.

c: *Aspergillus* pre-incubated with AMB. MIC and MFC PAFE to AMB.

d: *Aspergillus* pre-incubated with AMB. MIC and M to ITZ and PAFE to AMB.

e: *Aspergillus* pre-incubated with FCZ. MIC and MFC to ITZ and PAFE to AMB.

e: *Aspergillus* pre-incubated with FCZ. MIC, MFC PAFE to AMB.

## Discussion

In this study we simulated *in vitro*, the *in vivo* situation where patients are exposed to antifungal agents in a prophylactic setting, followed by treatment with another drug when a break-through fungal infection is suspected or documented. In contrast with other reports we pre-incubated the strains with ITZ, FCZ or AMB during a long period of time

up to eight weeks, and several parameters were study that represent drug activity. Since there is evidence that a proportion of patients are colonized with *Aspergillus* on admission and go on to develop invasive aspergillosis, the fungus would be exposed to both antifungal agents.

In contrast to other reports, none of the investigated parameters indicated that the activity of AMB was attenuated by pre-exposure to azoles. Although RPMI-1640 is sub-optimal for detecting AMB resistance, MFC and PAFE also indicated no change in AMB activity (9, 22).

In a murine model of acute invasive pulmonary aspergillosis it was found that, despite the *in vitro* attenuation of AMB activity, when the isolates were recovered from infected lung tissue from animals which were pre-exposed to ITZ, the *in vitro* susceptibility of AMB did not change more than one dilution compared with the baseline MIC (10).

Pre-incubation of the strains with AMB has not shown to induce variation of the MIC and MFC of ITZ compared with the wild-type strains, indicating that the pre-exposure to AMB does not attenuate the activity of azoles in vitro. In contrast, it has been demonstrated that pre-treatment with AMB followed by addition of azoles potentiates their antifungal activity (12).

Azoles act as ergosterol synthesis inhibitors by binding to lanosterol demethylase, a specific enzyme in ergosterol biosynthesis. They also may target lipids of fungal plasma membranes and may interact with methylsterol biosynthesis (11).

Different potential mechanisms of azoles resistance have been proposed: alteration in drug influx; alteration in intracellular drug processing: alterations in the target enzyme: point mutations, overexpression, gene amplification, gene conversion; alterations in other enzymes in the ergosterol biosynthesis pathway; alteration in the efflux pumps: ATP binding cassette transporters (e.g., CDR1 and CDR2) and major facilitators (e.g., *Ca*MDR1) (25). The mechanism of resistance for all azoles in A. fumigatus is not known, although there is one study in which the mechanism of resistance of posaconazole was shown to be a mutation in *cyp51A*. In *A. fumigatus* resistance to posaconazole was found to be linked to resistance against ITZ but not against voriconazole. These observations suggest that different mechanisms are involved in the way that azoles bind the 14-a demethylase (14). Several investigators have proposed different mechanisms of resistance

in *A. fumigatus*. A possible modification of cytochrome P450 or intracellular reduction of the drug was suggested for ITZ in a murine model of invasive aspergillosis(4). In other report the ITZ resistance in *A. fumigatus* was suggested to be due to reduced penetration of the drug due to impermeability and modification of the target (13).

In our study we found that the MIC for ITZ in the IRS (resistant) and IRM (mutants resistant strains) remained high but these strains remained susceptible to AMB as has been shown before (13).

In the ISS we noted that pre-incubation of the strains with ITZ initially resulted in an increase of MFC while the MIC remained unchanged. After 8 weeks of pre-incubation the MIC increased. This might suggest that an increase of the MFC could be the first sign for the development of resistance. The MFC of azoles was found to be higher than the corresponding MIC in *A. fumigatus* and *A. terreus* (6).

Interesting to observe is that, despite no activity of FCZ against *Aspergillus*, the activity of ITZ varies in terms of MFC but not MIC in strains pre-exposed to FCZ suggesting again that the MFC could be a good predictor of strain susceptibility. No differences in MFC between the different pre-incubated strains were observed. It might be possible that the presence of FCZ induces cross resistance and activation of pumps, with a more rapid efflux of ITZ or another mechanisms resulting in the loss of activity of ITZ. High doses of FCZ were reported to have some effect to inhibit the growth of *Aspergillus* (17).

PAFE studies with AMB showed that the effect was concentration dependent as was described previously for *Aspergillus*, *Candida* and *Cryptococcus.* (5, 23). In the present study AMB displayed PAFE against the wid-types of the ISS, IRM and IRS as well as following pre-incubation with ITZ, FCZ or AMB. Exposure of all strains to azoles resulted in an increased PAFE compared with the unexposed controls when the strains were exposed to a high concentration of AMB. However, in strains incubated with ITZ for one week at 1 and 0.5 times the MIC, AMB induced no PAFE at low concentrations.

For ITZ and FCZ the effect was not observed even at higher concentrations being in agreement with previous studies in which PAFE was absent or short when investigated with azoles (15, 23).

The reason for this striking difference in PAFE is unknown and warrants further studies. Although multiple mechanisms of resistance to have been described and are being

reported that result in phenotypic resistance to azoles in *A. fumigatus* the effect of these mechanisms on the susceptibility of other antifungal drugs remains to be studied. Given the similar target an interaction between azoles and AMB can be anticipated. In addition, the interaction can evolve over time as was the case in our experiments.

Furthermore, our results indicate that depending on the concentrations of AMB used different effects can be observed, which might explain the differences that have been reported in observations concerning interaction between azoles and AMB in fungal pathogens.

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Chapter

4

# *General conclusions*

- Summary.
- Samenvatting (summary in Dutch).
- Resumen (summary in Spanish).
- Future perspectives.

## 4.1. Summary

The incidence of fungal infections, as well as the diversity of species involved, are increasing. *In vitro* susceptibility tests are tools to evaluate the activity of antifungal agents against fungi. However, despite large improvement of methodologies there may still be a lack of correlation between *in vitro* and *in vivo* results.

The results of testing drugs at a constant concentration, which is the case when determining the minimal inhibitory concentration (MIC), minimal fungicidal concentration (MFC) or drug combinations might correspond better with clinical outcome with the addition of tests in which the action of the drug that persists despite washing is evaluated simulating the *in vivo* situation when the drug concentration falls below the MIC. These *in vitro* assays together could give more information about the activity of antifungal agents and correspond better with *in vivo* outcome.

Therefore, **Chapter 2-1** focuses on the identification and susceptibility testing of two new species belonging to the genus *Exophiala*. Black yeasts morphologically identified as "*Exophiala spinifera*" were re-analyzed on the basis of morphology, nutritional physiology and ITS rDNA sequencing data. SSU rDNA phylogeny data revealed that the strains analyzed were located at considerable distance from the *E. spinifera* clade containing the type strain. Using ITS rDNA sequencing, the strains could not be attributed to any existing species, and therefore two new species, *E. attenuata* and *E. nishimurae* were proposed. Antimycotic susceptibility data of these new species were generated and compared with those obtained in tests of members of the *E. spinifera* clade. *E. attenuata* had lower MIC values for fluconazole compared with *E. nishimurae* and *E. spinifera.* In contrast, amphotericin B and flucytosine were less active for *E. attenuata* than for *E. nishimurae* or *E. spinifera.* The most active drugs for the new species were itraconazole, voriconazole and terbinafine showing values similar to those of *E. spinifera.*

In **Chapter 2-2** the activity of six antifungal agents against clinical and environmental *E. spinifera* strains was evaluated applying different temperature regimens: 28 ºC, 35 ºC and 37 ºC. We observed that the most active drug against *E. spinifera* was itraconazole against environmental as well as clinical strains regardless of the temperature of

incubation, with a geometric mean MIC of 0.04 and 0.06 µg/ml, respectively. Voriconazole and terbinafine were also active. Amphotericin B susceptibility values varied among the isolate classes, environmental *vs* clinical, with a geometric mean MIC at 35 ºC of 0.3 µg/ml for environmental isolates and of 0.9 µg/ml for clinical isolates. Differences were also seen in 5-fluocytosine MIC values which were 1.8 µg/ml for environmental and 5.7 µg/ml for clinical isolates respectively. In general, the activities of antifungal agents against environmental and clinical strains were closely comparable.

**In Chapter 2-3**, drug combinations were evaluated. Amphotericin B was combined with flucytosine and ciprofloxacin, while itraconazole was combined with ciprofloxacin, levofloxacin, lomefloxacin and sulfadiazine against *E. spinifera* isolates. Synergistic effects were observed for the combinations of itraconazole plus ciprofloxacin or levofloxacin, and amphotericin B plus ciprofloxacin and flucytosine.

**In Chapter 3-1** a novel method to evaluate post-antifungal effect (PAFE) in filamentous fungi was described. Due to technical problems such as the non-homogeneous growth of moulds in liquid media, colony count determination was not applicable for determining PAFE, which, in contrast, can be used for bacteria or yeasts. Therefore, for moulds, a spectrophotometric procedure was developed. The standard medium RPMI-1640 was used. Conidia were exposed to the drugs at concentrations of 4, 1, and 0.25 times the MIC, each for 4, 2, and 1 h at 37°C. The same procedure was followed for controls with drug-free medium. Following exposure, the conidia were washed, resuspended in RPMI-1640 and then incubated in a spectrophotometric reader. The optical density (OD) was measured automatically at 10-min intervals, resulting in growth curves to quantify PAFE. $OD_0$ was chosen as an arbitrary criterion due to several advantages that we observed. PAFE was defined as a significant delay in growth of exposed conidia compared with the non-exposed controls. Amphotericin B was found to induce PAFE against *Aspergillus* in a concentration dependent manner. No post drug exposure effects were observed following exposure to itraconazole. **In Chapter 2-2**, using the method described above as a basis, PAFE of anfotericin B, itraconazol and terbinafine against *E. spinifera* was evaluated. PAFE was induced by amphotericin B only, with extended inhibition times seen at high drug concentrations. After 4 h of incubation at 4 times the MIC, amphotericin B induced significant PAFE against all strains with a mean of 16.2 h and a

range of 9.7 to 19 h. For 1 time the MIC, PAFE was induced against 7 of 11 strains tested with a mean of 9.8 h and a range of 1.6 to 16 h. For the other drugs PAFE was not observed.

**In Chapter 3-1**, the post drug exposure effects of amphotericin B and itraconazole were studied against fifteen clinical isolates of *Aspergillus* species, three isolates of *A. fumigatus*, *A. flavus*, *A. terreus*, *A. nidulans*, and *A. ustus*. PAFE was found to be concentration and time dependent. PAFE induced by amphotericin B was longer for *A. fumigatus* than for the other *Aspergillus* species, with mean PAFEs of 9.9 and 4.0 h after 4 h and 2 h of exposure, respectively, to four times the MIC. Itraconazole failed to induce a PAFE at any concentration tested and for any exposure time, even after exposure to concentrations as high as 10, 20, and 50 times the MIC.

**In Chapter 3-2**, PAFE of amphotericin B and nystatin was evaluated against 30 zygomycetes including *Absidia corymbifera*, *Rhizopus oryzae*, *Rhizopus microsporus*, *Mucor* spp and *Rhizomucor* spp., in two media: RPMI-1640 and antibiotic medium 3 (AM3). Both drugs induced PAFE against all *A. corymbifera* strains at 4 and 1 times the MIC after 4 h exposure. However, for nystatin longer PAFEs were seen in AM3 for both concentrations than in RPMI-1640. PAFE was not induced by amphotericin B or nystatin after 1 h exposure. For *Rhizopus* spp. no differences in PAFE values were seen when both drugs were compared. Both drugs tended to induce longer PAFE in *R. oryzae* than in *R. microsporus*. For *Mucor* spp. nystatin tended to induce longer PAFE values than amphotericin B in the same medium. Both, amphotericin B and nystatin failed to induce PAFE in *Rhizomucor* strains. In RPMI-1640, for amphotericin B the rank order for PAFE was *A. corymbifera* > *R. oryzae* > *Mucor* sp. > *R. microsporus* and for nystatin the rank order was *Mucor* sp > *R. oryzae* > *A. corymbifera* > *R. microsporus*.

**In Chapter 3-3** the activity and PAFE of non-antifungal drugs is investigated. Two phenotiazines were evaluated based on their presumed antifungal acitivity against yeasts. The in vitro activity of chlorpromazine and trifluopherazine was evaluated against *Aspergillus* species, zygomycetes and *Scedosporium* species, 80 isolates in total. Both drugs inhibited the growth of all species tested at concentrations of 16 to 64 mg/L. Growth inhibition was also observed after exposure and removal of the drug (PAFE). For *Aspergillus* species the mean PAFE was 3.7 h and 4.7 h; for zygomycetes, 3.1 h and 3.4

h; for *Scedosporium*, 4.3 h and 5.3 h for chlorpromazine and trifluoroperazine, respectively. This observation indicates that these drugs inhibit the growth of moulds and might be of interest for antifungal drug discovery.

**In Chapter 3-4**, MIC, MFC and PAFE of amphotericin B , itraconazole and fluconazole were studied in nine clinical strains of *A. fumigatus* of which three were itraconazole susceptible (ISS), three itraconazole resistant (IRS) and three itraconazole mutants (IRM). The evaluation was carried out by pre-incubating all the strains at sub-inhibitory concentrations of itraconazole, amphotericin B and fluconazole and by comparing the results with those from the wild-type strains.

The MICs and MFCs of amphotericin B were similar for the pre-incubated and wild-type strains. The MICs for itraconazole were similar after 1 week of pre-incubation although the MFC values for the ISS increased. However, after 8 weeks of pre-incubation the MICs of the ISS had also increased. When pre-incubated with fluconazole, the MICs of itraconazole for the non and pre-incubated strains were similar whereas the MFCs values were higher for the pre-incubated strains. Similar values in MICs and MFCs for itraconazole between non and pre-incubated strains was observed when the strains had been pre-incubated with amphotericin B.

For the wild-type strains, PAFE was induced by amphotericin B at 4, 1 and 0.5 times the corresponding MIC but not by itraconazole or fluconazole. PAFE induced by amphotericin B against pre-incubated strains, was observed for the ISS, IRM, IRS with longer values compared with the original strains, the exception was those strains pre-incubated with itraconazole during 1 week at 1 and 0.5 times the MIC. In all cases, the effect was concentration dependent. In summary, the MFCs increased earlier than the MICs for itraconazole. For amphotericin B no differences were observed and PAFE was induced against both wild-type and the pre-incubated strains.

## 4.2. Samenvatting (Summary in Dutch)

De problematiek van levensbedreigende schimmelinfecties neemt steeds verder toe, zowel in incidentie als met betrekking tot het aantal veroorzakende soorten. Een van de methoden om de activiteit van antifungale middelen te toetsen is de *in vitro* gevoeligheidsbepaling. Ondanks grote verbeteringen in de methodiek is er echter lang niet altijd een direkte correlatie tussen uitkomsten *in vitro* en *in vivo.*

De resultaten van het testen van middelen bij een constante concentratie, zoals het geval is bij het bepalen van de minimale remmende concentratie (MIC), minimale fungicide concentratie (MFC) of combinaties van antifungale middelen, zouden beter met de klinische uitkomst kunnen corresponderen door het toevvegen van testen waarin de activiteit van het middel dat aanwezig blijft ondanks het wassen wordt geevalueerd. Dit bootst de in vivo situatie waarin de concentratie van het middel daalt beneden de MIC na. In combinatie leveren de verschillende *in vitro* tests meer informatie op over hun werkelijke activiteit, en weerspiegelen mogelijk de *in vivo* situatie beter.

**Hoofdstuk 2-1** behandelt identificatie en gevoeligheidsbepaling van twee nieuwe soorten van het geslacht *Exophiala*. Zwarte gisten die morfologisch werden geïdentificeerd als "*Exophiala spinifera*" werden opnieuw onderzocht op basis van morphologische gegevens, nutritieve fysiologie en ITS rDNA sequencing. Met fylogenie op basis van SSU rDNA bleek dat de onderzochte stammen op een aanmerkelijke afstand stonden van de *E. spinifera* clade, waarin zich de type-stam van de soort bevindt. Met ITS rDNA sequentiëring konden de stammen in geen enkele bestaande soort worden ingedeeld, en om die reden werden twee nieuwe soorten, *E. attenuata* en *E. nishimurae,* beschreven. Antimycotische gevoeligheidsbepalingen van deze nieuwe soorten werden uitgevoerd, en vergeleken met die van vertegenwoordigers van de *E. spinifera* clade. *E. attenuata* had lagere MIC waarden voor fluconazole dan *E. nishimurae* en *E. spinifera.* Amphotericine B en flucytosine waren daarentegen minder actief tegen *E. attenuata* dan tegen *E. nishimurae* of *E. spinifera*. De meest actieve stoffen tegen de nieuwe soorten waren itraconazole, voriconazole en terbinafine, met waarden de vergelijkbaar waren met die gevonden in *E. spinifera*.

In **Hoofdstuk 2-2** werd de activiteit van zes antifungale middelen tegen klinische stammen van *E. spinifera* en stammen uit de omgeving getest bij verschillende temperaturen: 28 ºC, 35 ºC en 37 ºC. We stelden vast dat de stof met de hoogste activiteit tegen zowel omgevings- als klinische stammen van *E. spinifera* itraconazole was, onafhankelijk van incubatie-temperatuur, met geometrisch gemiddelde MICs van respectievelijk 0.04 en 0.06 µg/ml. Voriconazole en terbinafine waren eveneens werkzaam. Gevoeligheid voor amphotericin B verschilde tussen de groepen van isolaten, hetzij uit de omgeving of uit de kliniek, met een geometrisch gemiddelde MIC bij 35 ºC van 0.3 µg/ml voor omgevings-stammen en 0.9 µg/ml klinische isolaten. Verschillen werden ook waargenomen bij MIC waarden voor 5-flucytosine, welke 1.8 µg/ml waren voor stammen uit de omgeving en 5.7 µg/ml voor klinische isolaten. In het algemeen waren de resultaten met stammen uit de omgeving of uit de kliniek in hoge mate vergelijkbaar.

In **Hoofdstuk 2-3** werd amphotericine B gecombineerd toegediend met flucytosine en ciprofloxacin, en itraconazole met ciprofloxacin, levofloxacin, lomefloxacin en sulfadiazine; de combinaties werden gebruikt tegen isolaten van *E. spinifera*. Synergistisch effect werd waargenomen voor de combinaties van itraconazole met ciprofloxacin of levofloxacin, en amphotericin B met ciprofloxacin en flucytosine.

In **Hoofdstuk 3-1** werd een nieuwe methode geïntroduceerd om het post-antifungale effect (PAFE) in filamenteuze fungi te beschrijven. Door technische problemen, zoals de inhomogene groei van filamenteuze schimmels in vloeibaar medium, was het tellen van kolonies niet te gebruiken voor het vaststellen van PAFE, zoals dat wel goed te gebruiken is in bacteriën en gisten. Daarom werd een spectrofotometrische procedure ontwikkeld. Het standaard medium RPMI-1640 werd gebruikt. Suspensies van conidiën werden blootgesteld aan concentraties van 4, 1, en 0.25 maal de MIC, gedurende 4, 2, en 1 h bij 37°C. Dit werd herhaald voor controles met medium zonder antifungale middelen. Na blootstelling werden de conidiën gewassen en werden de aantallen CFU per milliliter geteld. De conidiën werden vervolgens geïncubeerd in een spectrofotometer en de optische dichtheid (OD) werd automatisch elke 10 minuten gemeten; de resulterende groeicurven waren een maat voor de geconstateerde PAFE. $OD_0$ was gekozen als een arbitrair citerium om verschillende redenen. PAFE werd gedefinieerd als een significante

remming van de groei in de blootgestelde conidiën-suspensie in vergelijking tot de niet-blootgestelde controle-suspensies. Amphotericine B resulteerde in PAFE tegen *Aspergillus* species; de mate waarin PAFE werd waargenomen was afhankelijk van de concentratie. Er werd geen effect geconstateerd na incubatie met itraconazole.

In **Hoofdstuk 2-2** werd PAFE in *E. spinifera* gemeten, aan de hand van de hierboven beschreven methode. PAFE werd alleen geconstateerd met amphotericine B, vooral als hogere concentraties van het middel werden gebruikt. Na 4 uur incubatie met 4 maal de MIC concentratie amphotericine B werd inderdaad significante PAFE waargenomen in alle stammen, met een gemiddelde van 16.2 uur en een spreiding van 9.7 tot 19 uur. Bij een concentratie gelijk aan de MIC was PAFE waarneembaar in 7 van de 11 geteste stammen, met een gemiddelde van 9.8 uur en een spreiding van 1.6 tot 16 uur. Bij de overige gesteste antifungale middelen werd geen PAFE geconstateerd.

In **Hoofdtuk 3-1** werden de post-antifungale effecten van amphotericine B and itraconazole bestudeerd tegen vijftien klinische isolaten van diverse *Aspergillus* soorten, namelijk drie isolaten per species van *A. fumigatus*, *A. flavus*, *A. terreus*, *A. nidulans*, en *A. ustus*. PAFE bleek afhankelijk te zijn van concentratie en incubatieduur. De door amphotericine B geïnduceerde PAFE was langer bij *A. fumigatus* dan bij andere *Aspergillus* species, met gemiddelde PAFEs van respectievelijk 9.9 en 4.0 uur na 4 en 2 uur incubatie, tot vier maal de MIC. Met itraconazole was bij geen enkele concentratie of incubatietijd enige PAFE waarneembaar, zelfs niet bij blootstelling aan concentraties van 10, 20 en 50 maal de MIC.

In **Hoofdstuk 3-2** werd PAFE van amphotericine B en nystatine geëvalueerd tegen 30 zygomyceten, waaronder *Absidia corymbifera*, *Rhizopus oryzae*, *Rhizopus microsporus*, *Mucor* spp. en *Rhizomucor* spp. Bij beide middelen werd PAFE geconstateerd in alle stammen van *A. corymbifera* bij 4 maal resp. eenmaal de MIC na 4 uur incubatie in hetzelfde medium. Bij nystatine werden echter bij beide concentraties langere PAFEs waargenomen in antibiotisch medium 3 (AM3) dan in RPMI-1640. PAFE werd niet waargenomen in amphotericine B of nystatine na 1 uur blootstelling. Voor *Rhizopus* spp. werden tussen beide componenten geen verschil in PAFE waarden geconstateerd. Beide stoffen induceerden langere PAFE in *R. oryzae* dan in *R. microsporus*. In *Mucor* spp. was PAFE in het algemeen groter met nystatine dan met amphotericine B in hetzelfde

medium. Zowel amphotericine B als nystatine induceerden geen PAFE in stammen van *Rhizomucor*. In RPMI-1640 was voor amphotericine de volgorde in afnemend PAFE B alsvolgt: *A. corymbifera* > *R. oryzae* > *Mucor* sp. > *R. microsporus.* Voor nystatine was deze *Mucor* sp > *R. oryzae* > *A. corymbifera* > *R. microsporus.*

In **Hoofdstuk 3-3** werd de werkzaamheid en PAFE van minder gebruikelijke antifungale middelen onderzocht. Twee phenotiazines werden geëvalueerd op grond van hun veronderstelde activiteit tegen gisten. De *in vitro* activiteit van chlorpromazine en trifluopherazine tegen *Aspergillus* species, zygomyceten en *Scedosporium* species werd onderzocht aan de hand van in totaal 80 isolaten. Beide stoffen remden de groei van alle geteste soorten bij concentraties van 16 tot 64 mg/L. Groeiremming werd eveneens waargenomen na incubatie met, en na verwijdering van de componenten (PAFE). In *Aspergillus* species was de gemiddelde PAFE voor chlorpromazine en trifluoroperazine respectievelijk 3.7 en 4.7 uur, in zygomyceten 3.1 en 3.4 uur, en in *Scedosporium* 4.3 en 5.3 uur. Deze resultaten laten zien dat de componenten inderdaad de groei van schimmels kunnen remmen, en dat ze dus van belang kunnen zijn bij het zoeken naar nieuwe antifungale middelen.

In **Hoofdstuk 3-4** werden MIC, MFC en PAFE bestudeerd voor amphotericine B, itraconazole and fluconazole in negen klinische isolaten van *A. fumigatus,* waarvan drie gevoelig waren voor itraconazole (ISS), drie resistent tegen dit middel (IRS), en drie itraconazole mutanten (IRM). Het onderzoek werd gestart met voorincubatie van alle stammen bij concentraties beneden MIC voor itraconazole, amphotericine B en fluconazole, en de resultaten werden vergeleken met die van de wild-type stammen. De MICs en MFCs voor amphotericine B waren vrijwel gelijk in de voorgeïncubeerde en de wild-type stammen. De MICs voor ITZ waren gelijk na 1 week voorincubatie, hoewel de MFC waarden bij de ISS stammen stegen. Na 8 weken voorincubatie stegen ook de MICs van de ISS stammen. Na voorincubatie met fluconazole waren de MICs voor itraconazole in de niet resp. wel voor-geïncubeerde stammen aan elkaar gelijk, terwijl de MFC waarden in de voorgeïncubeerde stammen hoger waren. Soortgelijke resultaten met MIC en MFC voor itraconazole tussen al of niet voorgeïncubeerde stammen wanneer de voor-incubatie had plaatsgevonden met amphotericine B. In de wild-type stammen werd PAFE gestimuleerd door amphotericine B in concentraties van 4, 1 en 0,5 maal de

oorspronkelijke MIC, maar dat gold niet voor itraconazole en fluconazole. Het feit dat PAFE wordt gegenereerd door amphotericin B in voorgeïncubeerde stammen werd waargenomen in de ISS, IRM en IRS, waar de waarden langer waren dan in de oorspronkelijke stammen, met als uitzondering de stammen voor-geïncubeerd met itraconazole gedurende 1 week bij concentraties van 1 en 0.5 maal de MIC. In alle gevallen was het effect afhankelijk van de concentratie. Samengevat stijgen de MFCs voor itraconazole eerder dan de MICs voor dit middel. In amphotericine B werden geen verschillen waargenomen en werd PAFE geïnduceerd in zowel het wild-type als in de voorgeïncubeerde stammen.

## 4.3. Resumen (Summary in Spanish)

La incidencia de infecciones fúngicas se han incrementado, como así también las diversidad de especies involucradas. Los tests de susceptibilidad *in vitro* son herramientas para evaluar la actividad antifúngica frente a hongos. Sin embargo y a pesar de los adelantos y mejoras en las metodologías empleadas, sigue existiendo un problema de la falta de correlación de los resultados *in vitro* e *in vivo*.

Los resultados obtenidos al evaluar concentraciones constantes de una droga, como serían la determinación de la concentración inhibitoria mínima (CIM), la concentración fungicida mínima (CFM) o las combinaciones de drogas, tal vez correlacionarían mejor con el resultado del tratamiento clínico, si se adicionaran tests en donde la acción de la droga persista después de ser eliminada, simulando la situación *in vivo*, en la cual la concentración de la droga disminuye por debajo de la CIM. Todos éstos tests juntos podrían dar más información acerca de la actividad antifúngica y correlacionar mejor con la clínica.

Por lo tanto, el **Capítulo 2-1**, se basa en la identificación y el estudio de susceptibilidad antifúngica de dos nuevas especies pertenecientes al género *Exophiala*. Las levaduras negras pre-identificadas como *Exophiala spinifera*, fueron re-analizadas en base a estudios morfológicos, fisiológicos y de secuenciación (ITS rDNA). Los datos filogenéticos obtenidos de SSU rDNA, revelaron que las cepas analizadas se encontraban distanciados considerablemente respecto del grupo *Exophiala spinifera*. Los datos

obtenidos de los estudios de secuenciación (ITS rDNA), demostraron que las cepas estudiadas no se podían relacionar con ninguna cepa existente, por lo que, *E. attenuata* y *E. nishimurae* han sido propuestas como dos nuevas especies. Paralelamente se han realizado estudios de susceptibilidad antifúngica de las nuevas especies mencionadas y se compararon los resultados con las cepas de *E. spinifera.* Se observó que *E. attenuata* presentaba valores de CIM más bajos para fluconazol comparándola con los obtenidos para *E. nishimurae* y *E. spinifera*. Por el contrario, anfotericina B y flucitocina fueron menos activas para *E. attenuata*, al compararla con los resultados observados para *E. nishimurae* o *E. spinifera.*

Las drogas más activas para las nuevas especies han sido itraconazol, voriconazol y terbinafina con valores similares a los obtenidos para *E. spinifera*.

En el **Capítulo 2-2** se estudió la actividad de seis agentes antifúngicos en cepas ambientales y clínicas de *E. spinifera*, utilizándose distintos regímenes de temperaturas: 28 ºC, 35 ºC y 37 ºC. Se observó que la droga más activa tanto para las cepas ambientales como para las clínicas fue itraconazol para todas las temperaturas, con una media geométrica de CIM de 0.04 y 0.06 µg/ml, respectivamente.

También se observó que voriconazol y terbinafina fueron activas. En el caso de anfotericina B se obtuvieron algunas diferencias entre las cepas ambientales y clínicas, con una media geométrica de CIM a 35 ºC de 0.3 µg/ml para las cepas ambientales y de 0.9 µg/ml para las cepas clínicas. También se observaron diferencias en el caso de flucitocina con valores de CIM de 1.8 µg/ml y 5.7 µg/ml para las cepas ambientales y clínicas, respectivamente. En general, la actividad de los agentes antifúngicos fue comparable entre las cepas ambientales y clínicas.

En el **Capítulo 2-3**, se estudiaron drogas en combinación. Para ello, anfotericina B se combinó con flucitocina y ciprofloxacina, mientras que itraconazole se combinó con ciprofloxacina, levofloxacina, lomefloxacina y sulfadiazina contra *E. spinifera*. Se observó efecto sinérgico para las combinaciones entre itraconazol y ciprofloxacina o levofloxacina y para la combinación entre anfotericina B y ciprofloxacina o flucitocina.

En el **Captítulo 3-1**, se describió un nuevo método para la evaluación del efecto post-antifúngico (PAFE), en hongos filamentosos. Este efecto puede evaluarse tanto para levaduras como bacterias utilizando la técnica de recuento de unidades formadoras de

colonias. En el caso de los hongos filamentosos, debido a problemas técnicos como ser su crecimiento no homogéneo en medios líquidos, la técnica de recuento mencionada anteriormente no puede aplicarse. Por lo tanto, para hongos filamentosos, se desarrolló un método espectrofotométrico. Se utilizó el medio de cultivo estándar, RPMI-1640. Las conidias fueron expuestas a los agentes antifúngicos a concentraciones correspondientes a 4, 1 y 0.25 veces la CIM. El tiempo de incubación para cada caso ha sido 4, 2 y 1 hora a 37 ºC. El mismo procedimiento se realizó con los controles, para los cuales no se adicionó droga al medio de cultivo. Luego de la incubación, las conidias fueron lavadas, resuspendidas en el medio RPMI-1640 e incubadas en un lector espectrofotométrico, el cual realiza mediciones de la densidad óptica (OD) automáticamente en intervalos de 10 minutos. La resultante de estas mediciones es una curva de crecimiento del hongo que fue utilizada para calcular el valor del PAFE. La $OD_0$, fue elegida arbitrariamente como criterio de cálculo. El PAFE se ha definido como la diferencia en tiempo de crecimiento entre la $OD_0$ de las conidias tratadas con el agente antifúngico y la $OD_0$ del control correspondiente. Se observó que anfotericna B ha sido capaz de inducir PAFE en *Aspergillus* y fue concentración dependiente. Por otro lado, con itraconazol el effecto no fue observado.

En el **Capítulo 2-2**, se evaluó el PAFE de anfotericina B en *E. spinifera*, utilizando como base el método descripto anteriormente. El PAFE inducido por anfotericina B fue concentración dependiente luego de una exposición de 4 horas a 4 veces la concentración de la CIM. Anfotericina B indujo PAFE para todas las cepas evaluadas con una media de 16.2 h y un rango de 9.7 a 19 h. Considerando la exposición de 1 vez la CIM, el PAFE fue inducido en 7 cepas de las 11 estudiadas con una media de 9.8 h y un rango de 1.6 a 16 h. Para las otras drogas analizadas como itraconazol y terbinafina, el efecto no se observó.

En el **Capítulo 3-1**, se estudió el PAFE de anfotericina B e itraconazol en quince cepas clínicas de especies de *Aspergillus*, de las cuales 3 han sido cepas de *A. fumigatus*, 3 de *A. flavus*, 3 de *A. terreus* y 3 de *A. ustus*. Se observó también en esta ocasión que el PAFE fue concentración y tiempo dependiente. Anfotericna B ha inducido PAFE, más prolongado para *A. fumigatus*, al comparar esta especie con las demás, con una media de 9.9 h y 4 h luego de 4 h y 2 h de exposición, respectivamente, a 4 veces la CIM. Para

itraconazol, no se ha observado PAFE aún cuando la exposición se realizó a concentraciones tan altas como 10, 20 y 50 veces la CIM.

En el **Capítulo 3-2**, se estudió el PAFE de anfotericina B y nistatina a 30 zygomycetes, utilizando dos medios de cultivos, RPMI-1640 y AM3. Se han incluído: *Absidia corymbifera*, *Rhizopus oryzae*, *Rhizopus microsporus*, *Mucor* spp y *Rhizomucor* spp. Analizando cada medio de cultivo, se ha observado PAFE para ambas drogas en *Absidia corymbifera* luego de 4 h de exposición a concentraciones de 4 y 1 vez la CIM. Sin embargo, al comparar los dos medios, se ha observado que nistatina ha inducido un PAFE más prolongado en AM3 que en RPMI-1640 para ambas concentraciones. Luego de 1 h de incubación, no se ha observado PAFE para ninguna de las dos drogas estudiadas. En el caso de *Rhizopus* spp., ambas drogas han inducido valores de PAFE similares. En general, el PAFE fue más prolongado para *R. oryzae* que para *R. microsporus*. Comparando ambas drogas en el mismo medio de cultivo, se han observado valores más prolongados de PAFE para nistatina en *Mucor* spp. Por otro lado, en *Rhizomucor* spp., no se ha observado el efecto.

En RPMI-1640 los valores de PAFE para anfotericina B fueron en orden decreciente: *A. corymbifera* > *R. oryzae* > *Mucor* sp. > *R. microsporus* y para nistatina: *Mucor* sp > *R. oryzae* > *A. corymbifera* > *R. microsporus.*

En el **Capítulo 3-3**, se han investigado la actividad antifúngica y el PAFE de drogas no antifúngicas. Para ello, se han elegido dos fenotiazinas dada su presunta actividad antifúngica en levaduras. Se evaluó *in vitro* la susceptibilidad de clorpromazina y trifluperazina en 80 aislamientos incluyendo los siguientes microorganismos: *Aspergillus* species, zygomycetes y *Scedosporium* species. Ambas drogas inhibieron el crecimiento de los hongos a concentraciones de 16 hasta 64 mg/L. Se observó la presencia de PAFE para clorpromazina y trifluperazina en *Aspergillus* spp. (media de 3.7 h y 4.7 h); en zygomycetes (media 3.1 h y 3.4 h) y en *Scedosporium* spp. (media 4.3 h y 5.3 h), respectivamente. Estas observaciones indicarían que estas drogas son capaces de inhibir el crecimiento e inducir PAFE en estos hongos filamentosos, pudiendo representar drogas potenciales en el desarrollo de nuevos compuestos antifúngicos.

En el **Capítulo 3-4**, se evaluó la CIM, CFM y el PAFE de anfotericina B, itraconazol y fluconazol en nueve cepas clínicas de *A. fumigatus*. Entre éstas cepas, 3 fueron sensibles

a itraconazol (ISS), 3 fueron resistentes a itraconazol (IRS) y 3 fueron cepas mutadas en el laboratorio, resistentes a itraconazol (IRM). La evaluación se ha realizado pre-incubando todas las cepas a concentraciones sub-inhibitorias de itraconazol, anfotericina B y fluconazol. Los resulatdos obtenidos han sido comparados con las cepas sin pre-incubar.

La CIM y la CFM de anfotericina B fue similar entre las cepas pre-incubadas y aquellas no pre-incubadas. La CIM de itraconazol para las cepas pre-incubadas luego de 1 semana fue similar aunque la CFM se ha elevado en las cepas ISS. Sin mebargo, la CIM en éstas cepas también se elevó luego de 8 semanas de incubación. En el caso de la pre-incubación con fluconazol, los valores de CIM a itraconazol han sido similares tanto para las cepas pre-incubadas como para las cepas no pre-incubadas, mientras que los valores de la CFM se ha elevado para las cepas pre-incubadas. Por otro lado, se han observado valores similares tanto en la CIM como en la CFM para ambos grupos (cepas sin pre-incubar y cepas pre-incubadas), cuando han sido incubadas con anfotericina B.

Para las cepas no pre-incubadas, se ha observado que anfotericina B indujo PAFE a concentraciones de 4, 1 y 0.5 veces la CIM, mientras que, tanto itraconazol como fluconazol no han inducido PAFE. Anfotericina B indujo PAFE en las cepas ISS, IRM y IRS, se obtuvieron valores más prolongados que aquellos observados en las cepas sin pre-incubar, a excepción de las cepas pre-incubadas con itraconazol durante 1 semana a concentraciones de 1 y 0.5 veces la CIM. En todos los casos, el PAFE ha sido concentración dependiente. En conclusión, la CFM para itraconazol, se elevó primero que la correspondiente CIM; en el caso de anfotericina B no se han observado diferencias y el PAFE fue inducido para ambos grupos de cepas: aquellas pre-incubadas y aquellas sin incubar.

## 4.4. Future perspectives

The increment of fungal infections, the deterioration in host defenses due to several factors, the limited number of antifungal drugs, the adverse effects particularly associated with amphotericin B, the time of diagnosis, non-specific clinical manifestations, and site of infection makes that treatment failure is frequently observed highlighting the need to better understand the pathogenesis of invasive fungal infections. In this thesis different studies are described that investigate the in vitro activity of drugs with antifungal activity against fungi and methods which can be used to investigate antifungal activity in vitro.

Basic to successful treatment is the correct identification of pathogenic fungi. For example, empiric therapy with amphotericin B might be necessary in an immunocompromised patient with the typical fever that persists despite antibiotic treatment, because of the suspicion of *Aspergillus* infection. Correct identification of an isolate from this patient is vital since other fungi resistant to amphotericin B such as *Scedosporium* could be the cause. The sole recognition of hyphae is not enough and culture eventually supplemented by molecular analysis is necessary for the correct identification.

Taken into account the limited efficacy of antifungals available, investigations of conventional and new antifungal drugs are important as well as alternative agents as antibiotics or other group of drugs, alone or in combination might give novel approaches to therapy.

The study of post drug-exposure effects could have an impact for dosing regimens. Drugs that demonstrate post drug effect might require less frequent administration, with the consequent reduction of adverse effects. The in vitro experiments described in this thesis are a first step in the study of post drug exposure effects in moulds which needs validation in *in vivo* models.

In this thesis, not only the activity of current antifungal drugs was evaluated but also the activity of antibiotics such as quinolones combined with conventional antifungal drugs was studied in black yeasts. It has been demonstrated that quinolones augment the

activity of amphotericin B and azoles (6, 7). In addition, the combination of fluconazole with ciprofloxacin was observed to be more effective in a mouse model of invasive candidiasis (7). Our studies demonstrate that this combination could interact synergistically in *Exophiala*. Animal models need to be performed in order to confirm the *in vitro* findings.

Other non-antifungal drugs that exhibit antimicrobial activity are antiphychotic drugs which have been shown to be active against bacteria and yeasts including *Candida* or *Cryptococcus.* Activity was demonstrated *in vitro* as well as in animal models (1, 2). In this study, chlorpromazine and trifluoroperazine showed activity against filamentous fungi and also induced post antifungal effect These drugs are not suitable for treatment of invasive fungal infection due to the neuroleptic side effects and non-specific activity against eukaryotic cells. However, the mechanism of action should be explored further since the drug might interact with the calcium metabolism of the fungus which is new target. Furthermore, the existence of the *cis* and *trans* stereoisomerism in phenotiazine compounds is described, for which only the *cis* form possesses neuroleptic activity, but both the *cis* and *trans* forms have equal antimicrobial potency (4).

Patients are exposed to antifungal agents in a prophylactic setting which is followed by treatment with another drug when a breakthrough fungal infection is suspected. The activity of the second antifungal agent could be augmented by exposure of the fungus to the first. The studies performed in this thesis indicate that exposure of *Aspergillus* to itraconazole changes the ability of amphotericin B to induce PAFE *in vitro*. Although the mechanism by which this occurs was not studied one could hypothesize that the ergosterol contents in the cell membrane is altered in quantity or quality following the exposure to itraconazole after long exposure period thereby altering the target for amphotericin B. Our studies further show that resistance to itraconazole was first detected by increase of MFC followed by an increase of MIC. The appropriateness of MFC testing for detecting resistance in moulds needs further study as well as the correlation with clinical response. The strains with increased MIC and MFC need further analysis in order to establish which mechanism is related to the observed changes.

Finally, further steps are needed to confirm the post drug exposure effects in different filamentous fungi, as described in this thesis . Some *in vitro* factors could be involved in the determination of PAFE such variation of pH, temperature of incubation, presence of serum, mechanism of action of the drug tested and others. Further studies are required to study the impact of these factors and other parameters on the PAFE. For example for *Candida* it was observed that the presence of serum or leucocytes enhanced the PAFE induced by amphotericin B (3, 5).

The spectrophotometric system can be used to study the impact of sequential exposure of moulds to different antifungals or the effect of drug exposure at different stages of growth

Finally, animal experiments should also be performed to confirm these *in vitro* findings to evaluate the impact of PAFE in filamentous fungi on dosing regimens of antifungal agents. The technical problems in establishing fungal burden in tissue can be overcome by quantitative PCR techniques.

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# Curriculum vitae

**Roxana G. Vitale** was born on the 10th of April 1966, in Buenos Aires, Argentina.

In 1979 she started secondary school.

In 1984 she has started her University studies. After 6 years, in 1990 she got the degree of Biochemistry at the University of Morón, Faculty of Natural and Exact Sciences.

From 1990 until 1994, she has been trained in a General Hospital at the Departments of Internal Medicine, Neonatology, Biochemistry and Haematology.

In 1994 she has started clinical training in: Bacteriology at the Infectious Diseases Department, Hospital Francisco Muñiz and as Researcher at the Mycology Unit, National Institute of Microbiology, INEI, ANLIS, Dr. Carlos Malbrán.

In 1996 she has started working at the Mycology Unit, Infectious Diseases Department, Hospital Francisco Muñiz and continued as Researcher at the Mycology Unit at the Institute of Microbiology, INEI, ANLIS, Dr. Carlos Malbrán.

In 1999 she has got the speciality as Human Mycologist given by the Argentina Association of Mycology.

From 1996 up to date she was involved in teaching activity.

In 2000 she has started her PhD studies at the University Medical Center, Nijmegen, The Netherlands.

## Professional training in mycology

Clinical and diagnosis training in endemic, superficial, sub-cutaneous and opportunistic mycoses. Mycology Unit at the Infectious diseases Hospital Francisco Muñiz. Unit Chief: Dr. Prof Ricardo Negroni. April 1996- Dec.1999.

Identification of yeasts and moulds, susceptibility testing and prove of synergistic action of different drugs . Mycology Unit of the National Institute of Microbiology Dr. C.Malbrán. April 1994-1998. Unit Chief: Dr. Laura Rodero.

Taxonomy and molecular biology in black yeasts. Centraalbureau voor Schimmelcultures. Utrecht. The Netherlands. Year 2000-2001. Unit Chief: Dr. G.S. De Hoog.

Susceptibility tests and post antifungal effect studies in different moulds, yeasts and black yeasts. University Medical Center, Nijmegen, The Netherlands. Year 2000-2003. Unit Chief: Dr. PE Verweij.

# Scientific Work in Medical Mycology

## Congress presentations

**1**. Rodero L., **Vitale R**, Vivot W., Canteros C., Davel G. Evaluation of Chromagar as differential medium for yeast species. VI Mycology Congress. Rosario, Argentina. April 1996.

**2**. Rodero L., Demkura H., Burkett A., Boutureira M., Hochenfellner F., Vivot W., **Vitale R**., Cordoba S., Canteros C., Perrotta D., Davel G. Nosocomial candidiasis: agentes causales y su sensibilidad antifúngica. II Congress of Infectology. Buenos Aires, Argentina. May 1996.

**3**. Rodero L., **Vitale R**, Canteros C., Davel G. In vitro activity of terbinafine in combination with triazole drugs against fluconazole resistant *Candida* species. 36th ICAAC Meeting. American Society for Microbiology. New Orleans, USA. September 1996.

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**16**. C. Gil-Lamaignere, **R.G.Vitale**, J. Curfs, P. Verweij, E. Roillides. In vivo treatment of mice with interferon-gamma and granulocyte-monocyte colony-stimulating factor (GM-CSF) enhances neutrophil-induced damage of *Scedosporium prolificans* and *apiospermum* hyphae. Clinical Microbiology and Infection. Vol.7, Suppl.1; pag.125. 11th European Congress of the Clinical Microbiology and Infectious Diseases. Istambul. Turkey. April; 2001.

**17**. **Vitale R.**, Afeltra J., J.F.G.M. Meis, Rodriguez Tudela J.L., L. Rodero, P.E.Verweij. Reversion of resistance of *Candida* spp to fluconazole by efflux pump blockers. Clinical Microbiology and Infection. Vol.7, Suppl.1; pag.343. 11th European Congress of the Clinical Microbiology and Infectious Diseases. Istanbul. Turkey. April; 2001.

**18**. J. Afeltra, **R.Vitale**, J.F.G.M. Meis, P.E.Verweij. In vitro interaction of itraconazole and nifedipine against clinical isolates of *Aspergillus fumigatus* susceptible and resistant to itraconazole. Clinical Microbiology and Infection. Vol.7, Suppl.1; pag.344. 11th European Congress of the Clinical Microbiology and Infectious Diseases. Istanbul. Turkey. April; 2001.

**19**. **Vitale R.,** Afeltra, J., Meis J.F.G.M., Mouton J.W., Verweij P.E. In vitro synergistic activity of fluconazole and nifedipine against clinical isolates of *Candida albicans* resistant or susceptible dose dependent to fluconazole. International Journal of Antimicrobial Agents. Supplement. P27.157. International Congress of Chemotherapy. Amsterdam, The Netherlands, June 30th-July 3rd 2001.

**20**. Afeltra, J., **Vitale R.**, Meis J.F.G.M., Mouton J.W., Verweij P.E. In vitro synergistic activity of itraconazole and six different pumps blockers against 16 clinical isolates of *Aspergillus fumigatus* resistant and susceptible to itraconazole. International Journal of Antimicrobial Agents. Supplement. P27.158. International Congress of Chemotherapy. Amsterdam, The Netherlands, June 30th-July 3rd 2001.

**21**. **R.Vitale,** J. Mouton, J. Afeltra, J.F.G.M. Meis, P.E. Verweij. Development of a method to determine post antifungal effect (PAFE) in *Aspergillus* species. 41st ICAAC Meeting. American Society for Microbiology. Chicago, USA, December 16-19, 2001.

**22**. **R.G. Vitale.** Evaluation of post antifungal effect (PAFE) of amphotericin B and itraconazole in *Aspergillus* species.. 41st ICAAC Meeting. American Society for Microbiology. Chicago, USA, December 16-19, 2001. Oral presentation.

**23**. **R.G. Vitale**. Post-antifungal effect in *Exophiala spinifera*. Nederland Vereniging voor Medische Microbiologie. Arnhem, The Netherlands. April 9-10 2002. Oral presentation.

**24**. **R.G.Vitale**, J.F.G.M. Meis, G.S. De Hoog, and P.E. Verweij. Evaluation of the post antifungal effect of 11 clinical *Exophiala spinifera* strains against Amphotericin B, Itraconazole, Terbinafine and 5-fluocytosine. Mycoses Vol 45, Supp. 2. 8$^{th}$ Congress of the European Confederation of Medical Mycology. Budapest, Hungary. August, 2002.

**25**. **R.Vitale,** J. Afeltra, A. Rijs, J.F.G.M. Meis, P. Verweij. In vitro activity of amphotericin B and itraconazole in combination with flucytosine, sulfadiazine and quinolones against *Exophiala spinifera* strains. Mycoses, Vol 45, Supp. 2. 8$^{th}$ Congress of the European Confederation of Medical Mycology. Budapest, Hungary. August, 2002.

**26**. **R.G. Vitale**, L. Rodero, J. Afeltra. In vitro synergistic activity of amlodipine in combination with fluconazole, itraconazole and terbinafine against clinical isolates of *Candida albicans.* 42nd ICAAC Meeting. American Society for Microbiology. San Diego, USA, September 26-30, 2002.

**27**. J. Afeltra, **R.G.Vitale**, J.F.G.M. Meis, P.E. Verweij. In vitro activity of sulphonamides and antiparasitic agents against *Cryptococcus neoformans* (Cn). 42nd ICAAC Meeting. American Society for Microbiology. San Diego, USA, September 26-30, 2002.

## List of publications

**1**. Rodero L, Boutureira M, Demkura H, Burkett A, Fernandez C, Losso M, Jauregui Rueda H, Monticelli A, **Vitale R**, Canteros C, Hochenfellner F, Vivot W, Davel G. Yeasts infections: Identification and antifungals resistant in pediatric and HIV patients. Revista Argentina de Microbiología. 1997;29:7-15.

**2**. Bianchi M, Robles AM, **Vitale R**, Helou S, Arechavala A, Negroni R. The usefulness of blood culture in diagnosing HIV-related systemic mycoses: evaluation of a manual lysis centrifugation method. Medical Mycology. 2000;38:77-80.

**3**. **Vitale RG,** Mouton JW, Afeltra J, Meis JF, Verweij PE. Method to measuring postantifungal effect in *Aspergillus* species. Antimicrobial Agents and Chemotherapy. 2002;46:1960-5.

**4. Vitale RG**., De Hoog GS. Molecular diversity, new species and antifungal susceptibilities in the *Exophiala spinifera* clade. Medical Mycology 2002;40:545-56.

**5.** Afeltra J, Meis JF, **Vitale RG**, Mouton JW, Verweij PE. In vitro activities of pentamidine, pyrimethamine, trimethoprim and sulfonamides against *Aspergillus* species. Antimicrobial Agents and Chemotherapy. 2002;46:2029-31.

**6. Vitale RG**, Afeltra J, De Hoog GS, Rijs AJ, Verweij PE. In vitro activity of amphotericin B and itraconazole in combination with flucytosine, sulfadiazine and quinolones against *Exophiala spinifera*. Journal of Antimicrobial Chemotherapy 2003 51: 1297-1300.

**7. Vitale RG**, De Hoog GS, Verweij PE. In vitro activity of amphotericin B, itraconazole, terbinafine and 5-fluocytosine against *Exophiala spinifera* and evaluation of post antifungal effects. Medical Mycology 2003. 41:301-7.

**8. Vitale RG**, Meis JF, Mouton JW, and Verweij PE. Evaluation of post antifungal effect (PAFE) of amphotericin B and nystatin using two different media in 30 Zygomycetes. Journal of Antimicrobial Chemotherapy 2003. 52:65-70.

**9. Vitale RG**, Afeltra J, Meis JF, Verweij PE. Evaluation of post antifungal effect (PAFE) of chlorpromazine and trifluopherazine against *Aspergillus*, *Scedosporium* and Zygomycetes. (Submitted for publication).

**10. Vitale RG**, Afeltra J, Meis JF, Verweij PE. Evaluation of minimal inhibitory concentration, minimal fungicidal concentration and post antifungal effect (PAFE) of *Aspergillus fumigatus* after exposure to subinhibitory concentrations of itraconazole, fluconazole and amphotericin B. (Submitted for publication)

## Awards

**ECMM Young Investigators Travel Award.** Given by the European Confederation of Medical Mycology. 6th Congress of ECMM. Barcelona. Spain; November 2000.

**American Society Microbiology (ASM) Student Travel Grant**. 41st ICAAC, Chicago, Illinois. December 16-19, 2001.

**BSAC Project Grant**. Given by the British Society for Antimicrobial Chemotherapy. September 2002.

## Teaching activities

**Instructor and lecturer** in the International Course of Veterinary and Medical Mycology for graduates. Held at Muñiz Hospital and in the Mycology Center, Faculty of Medicine of the Universidad de Buenos Aires 1997 - 1998. Director: Prof. Dr. Ricardo Negroni.

**Instructor** in the Course of Superficial Mycoses. National Institute of Microbiology, Dr. Carlos Malbrán. Buenos Aires 1996.

**Instructor** in the Course of Identification and Antifungal susceptibility tests for yeasts. National Institute of Microbiology, Dr. Carlos Malbrán. Buenos Aires 1996.

**Associate professor** in Microbiology at the Faculty of Medicine. Private University "Del Salvador"; Buenos Aires. 1998-1999.

**Associate professor** in Microbiology at the Faculty of Medicine. UBA, University of Buenos Aires; Buenos Aires. 1998-2000.

**Instructor and lecturer** in the Intensive Course in Medical Mycology. April, 2003. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. Director: Prof. Dr. G.S. de Hoog.

In vitro activity and post-drug-exposure effect of antifungal agents and other drugs in *Exophiala spinifera* and filamentous fungi – R. G..*Vitale*