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Mechanisms involved in Aspergillus fumigatus antifungal drug adaptation

Aruanno Marion

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UNIL | Université de Lausanne
Faculté de biologie et de médecine

INSTITUT DE MICROBIOLOGIE DE LAUSANNE (IMUL)
Département de médecine de Laboratoire et Pathologie - CHUV

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Thèse de Doctorat ès sciences de la vie (PhD)

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par

Marion ARUANNO

Jury

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Mechanisms involved in *Aspergillus fumigatus* antifungal drug adaptation

Lausanne, le 1er décembre 2020

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Niko GELDNER
Directeur de l'Ecole Doctorale

Let me tell you the secret that has led me to my goal.
My strength lies solely in my tenacity.

- Louis Pasteur

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You are the best thing that happened to me.

English Abstract

Aspergillus fumigatus is an opportunistic pathogenic mold responsible of invasive aspergillosis (IA) in immunocompromised patients. Antifungal therapies for the treatment of IA are limited to three drug classes. The azoles (e.g. voriconazole) represent the first-line treatment, while polyenes (amphotericin B) and echinocandins (e.g. caspofungin) are alternative therapies. Resistance to antifungal drugs is a concern. *A. fumigatus* exhibits some degree of natural tolerance to caspofungin with loss of efficacy of the drug at higher concentrations (a phenomenon known as the "paradoxical effect"). Moreover, acquired resistance to azoles consisting of mutations in the target gene (*cyp51A*) is emerging as a consequence of the widespread use of fungicides in agriculture.

This work aims at deciphering the different mechanisms of the adaptive response of *A. fumigatus* leading to caspofungin tolerance and azole resistance.

In the first part of the project, we investigated the network of the heat shock protein 90 (Hsp90) in caspofungin tolerance. Hsp90 is an essential molecular chaperone known to be essential for this process, but its pathway remains to be elucidated. We identified a yet unrevealed role of the mitochondrial respiratory chain (MRC) in caspofungin tolerance and paradoxical effect, which is dependent from Hsp90.

In the second part of the project, we investigated the mechanisms of azole resistance of a laboratory generated *A. fumigatus* strain exhibiting pan-azole resistance in the absence of *cyp51A* mutations. Overexpression of efflux pumps genes (ABC and MFS transporters) were found to play a role in azole resistance of this strain. Transporter inhibitors such as milbemycin and Phe-Arg β-naphthylamide could potentiate the activity of azoles against *A. fumigatus*.

This work provides further insights in these mechanisms of antifungal adaptation and resistance in *A. fumigatus* and could possibly identify novel therapeutic targets.

Résumé français

Aspergillus fumigatus est une moisissure pathogène opportuniste responsable de l'aspergillose invasive (AI) chez les patients immunocompromis. Les thérapies antifongiques pour le traitement des AI sont limitées à trois classes d'antifongiques. Les azoles (ex.: voriconazole) représentent le traitement de première ligne alors que les polyènes (amphotéricine B) et les échinocandines (ex.: caspofungine) sont des traitements alternatifs. La résistance aux antifongiques représente un véritable problème. *A. fumigatus* présente un certain degré de tolérance naturelle à la caspofungine avec une perte d'efficacité à haute concentration (phénomène appelé « effet paradoxal »). De plus, la résistance acquise aux azoles, consistant principalement en des mutations dans le gène cible (*cyp51A*), est un problème émergeant dû à l'utilisation importante de fongicides dans l'agriculture.

Les objectifs de ce travail sont d'élucider les différents mécanismes de la réponse adaptative d'*A. fumigatus* impliqués dans la tolérance à la caspofungine et dans la résistance aux azoles.

Dans la première partie du projet, nous avons étudié le réseau de la « heat shock protein 90 » (Hsp90) dans la tolérance à la caspofungine. Hsp90 est une protéine « chaperon » essentielle connue pour jouer un rôle dans l'effet paradoxal, mais le mécanisme exact est inconnu. Nous avons identifié un rôle de la chaîne respiratoire mitochondriale dans la tolérance à la caspofungine et l'effet paradoxal, qui est dépendant de Hsp90.

Dans la deuxième partie du projet, nous avons étudié les mécanismes de résistance aux azoles d'une souche d'*A. fumigatus* générée en laboratoire et présentant une résistance aux azoles en l'absence de mutations du *cyp51A*. La surexpression des gènes des transporteurs ABC et MFS semblait impliquée dans la résistance aux azoles de cette souche. Nous avons montré que les inhibiteurs de transport tels que la milbémycine et le Phe-Arg β-naphtylamide potentialisent l'activité des azoles contre *A. fumigatus*.

Ces travaux ont permis de mieux comprendre ces mécanismes d'adaptation et de résistance aux antifongiques chez *A. fumigatus*, et possiblement d'identifier de nouvelles cibles thérapeutiques.

Table of content

List of abbreviations

ABC: ATP-Binding Cassette

ABPA: Allergic Bronchopulmonary Aspergillosis

AIDS: Acquired immunodeficiency syndrome

ATP: Adenosine triphosphate

BAL: Bronchoalveolar lavage

CD: Cluster of Differentiation

Cna: Calcineurin

CPA: Chronic Pulmonary Aspergillosis

CPE: caspofungin paradoxical effect

CT-scan: Computerized tomography scanner

EBUS: Endobronchial ultrasound

ER: Endoplasmic reticulum

HIV: Human immunodeficiency virus

Hsp90: Heat shock protein 90

IA: Invasive Aspergillosis

ICU: Intensive care unit

IP3: Inositol trisphosphate

ISA: Isavuconazole

KOH: Potassium hydroxide

MFS: Major Facilitator Superfamily

MIC: Minimal Inhibitory Concentration

MLB: Milbemycin

MRC: Mitochondrial respiratory chain

NADPH: Nicotinamide adenine dinucleotide phosphate

NETs: Neutrophil extracellular traps

PAMPs: Pathogen associated molecular patterns

PAβN: Phe-Arg β-naphthylamide

PCR: Polymerase chain reaction

POSA: Posaconazole

PRRs: Pattern recognition receptors

ROS: Reactive oxygen species

RT-qPCR: reverse transcription quantitative PCR

SNPs: Single nucleotide polymorphisms

TLR: Toll-like receptor

TR: Tandem repeat

VRC: Voriconazole

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INTRODUCTION

I. Fungi

The kingdom of fungi consists of diverse eukaryotic organisms, which are ubiquitous in the environment. They share some similarities with both animals and plants, and also exhibit some important distinct characteristics. Like animals, fungi are heterotroph and are not capable of photosynthesis. They also have a cell membrane, which contains ergosterol (instead of cholesterol in animals). Like plants, they possess a cell wall, which mainly consist of chitin and glucans (instead of cellulose in plants) (Figure 1).

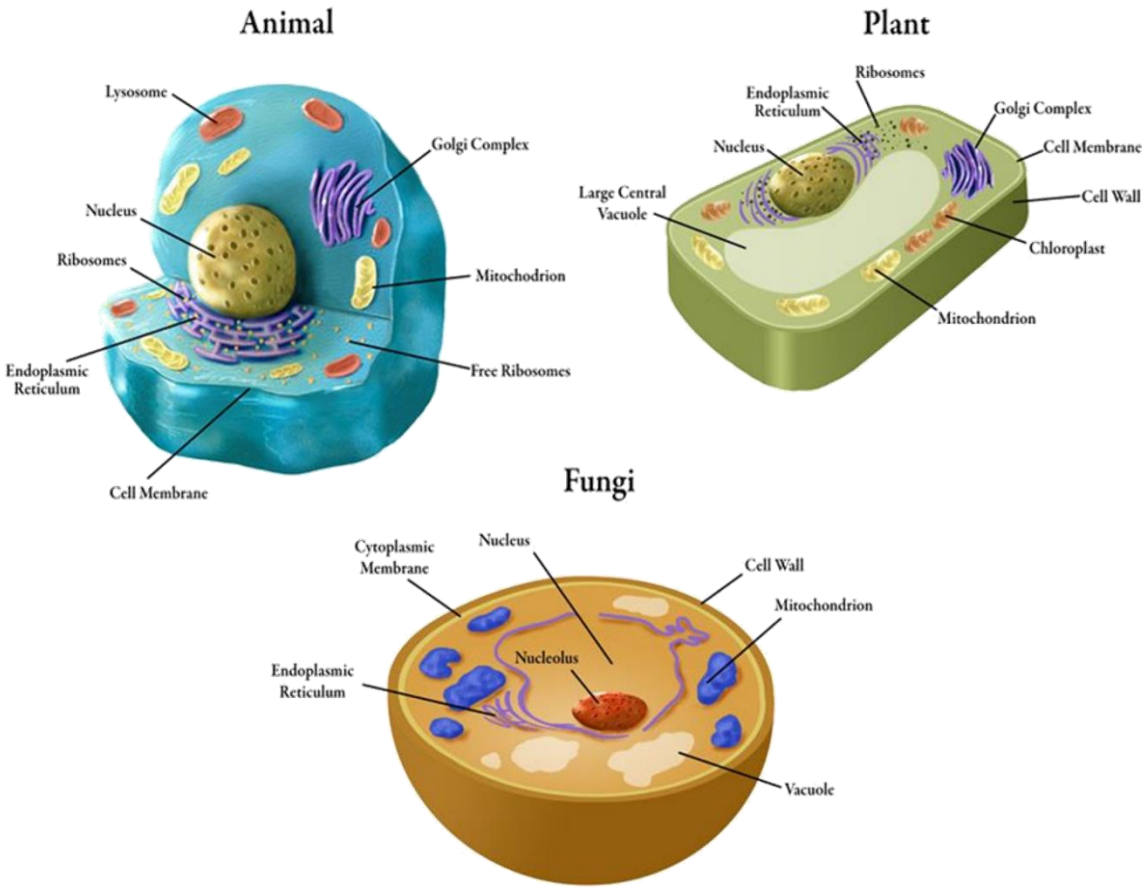


Figure 1: Composition and structure of animal, plant and fungal cell[1]

As saprophytes or parasites, fungi play an important role in decomposition of organic matter or sequestration of nutrients from living hosts such as plants or animals.

1) Classification and mode of reproduction

Fungi can be unicellular or multicellular and exhibit different modes of reproduction and growth.

Fungi have been historically classified according to their sexual mode of reproduction in three phyla (Ascomycota, Basidiomycota and Zygomycota). Sexual structures (cleistothecia, zygosporangia, basidia or perithecia) produce propagules after fusion of two haploid nuclei and based on the phylum, these spores are either contained within asci (e.g Ascomycota) or can be external (e.g. Basidiomycota or Zygomycota).

The asexual mode of reproduction of fungi is efficient for their rapid propagation and is essential for their human pathogenicity. Fungi can be classified according to their asexual mode of reproduction, as yeasts and molds. Yeasts are unicellular fungi that divide by budding or fission (e.g. *Candida* spp., *Cryptococcus* spp.). Molds, also called filamentous fungi (e.g. *Aspergillus* spp., *Mucorales*), grow by apical extension and branching to form filaments (hyphae) in complex networks called mycelia. Under appropriate conditions, filaments can form fruiting bodies (conidiophores or sporangiophores) that produce asexual propagules called spores or conidia. These spores can disseminate by air and germinate to develop other mycelia.

(Figure 2)

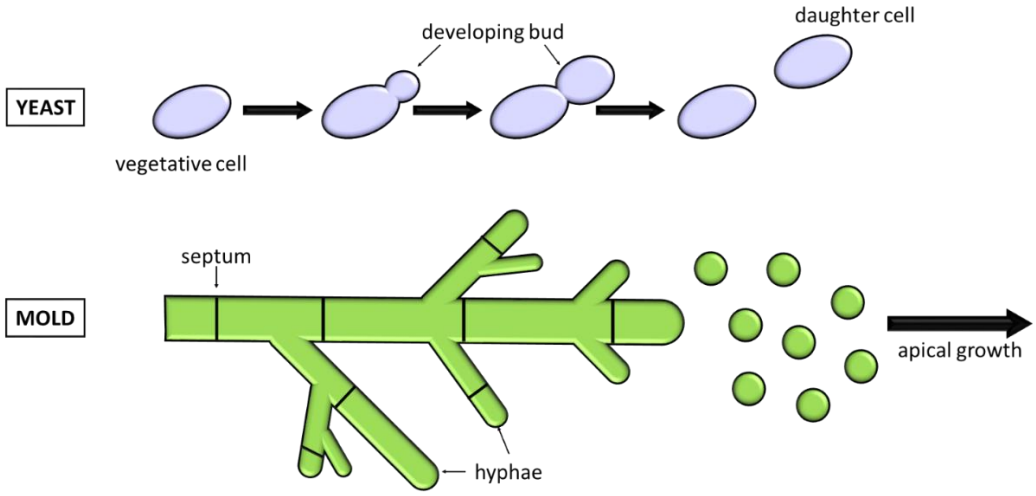


Figure 2: Schematic representation of yeast and mold division and growth.

2) Pathogenic fungi

Around 1.5 to 5 million of fungal species exist [4] but less than one hundred have been associated with human diseases. The human pathogenicity of fungi requires several virulence traits:

- The ability to grow at human body temperature (37°c or above)
- The ability to reach internal tissues by penetration and bypassing host barrier
- The ability to lyse and absorb components of human tissue
- The ability to resist to human immune system

3) Human fungal diseases

Human fungal diseases can be divided in superficial mycoses (affecting the skin or mucosa) and invasive mycoses (with invasion of deep tissues and potential for dissemination to other internal organs). Superficial fungal diseases, such as dermatophytoses (skin infection) or *Candida* vulvovaginitis are among the most

frequent human diseases. Invasive mycoses are rare but life-threatening diseases. These latter include endemic mycoses and opportunistic mycoses. Endemic mycoses (e.g. histoplasmosis, talaromycosis, blastomycosis, coccidioidomycosis and paracoccidioidomycosis), caused by dimorphic fungi, are limited to specific geographical areas and can affect both immunocompetent and immunocompromised hosts. In contrast, other invasive mycoses are opportunistic, as they mainly affect individuals with depressed immune system. These later ones have emerged as a major health concern over the last decades of the 20th century with the emergence of the human immunodeficiency virus (HIV), cause of the acquired immunodeficiency syndrome (AIDS), the progresses in transplantation medicine and anti-rejection immunosuppressive therapies, and the advent of anti-cancer chemotherapies.

The most important invasive opportunistic mycoses in humans and their respective host population at risk are shown in Table 1.

Disease	Fungus	Population
Pneumocystosis	*Pneumocystis jirovecii*	HIV Hematologic cancer
Cryptococcosis	*Cryptococcus neoformans*	HIV
Invasive candidiasis	*Candida albicans* (other *Candida* spp.)	ICU patients Hematologic cancer Transplant patients
Invasive aspergillosis	*Aspergillus fumigatus* (other *Aspergillus* spp.)	Hematologic cancer Transplant patients ICU patients (Influenza)
Invasive mucormycosis	*Mucorales*	Hematologic cancer Transplant patients

Table 1: Most important invasive opportunistic mycoses in humans and their respective host population at risk

In this work, we will focus on the filamentous fungi of the genus *Aspergillus* and particularly on *Aspergillus fumigatus*, the main cause of invasive aspergillosis

II. *Aspergillus*

Molds of the genus *Aspergillus* are characterized by the presence of long conidiophores terminated by vesicles covered with phialides and supporting conidia (Figure 3). These conidia with ovoid or elliptic shape present different colors

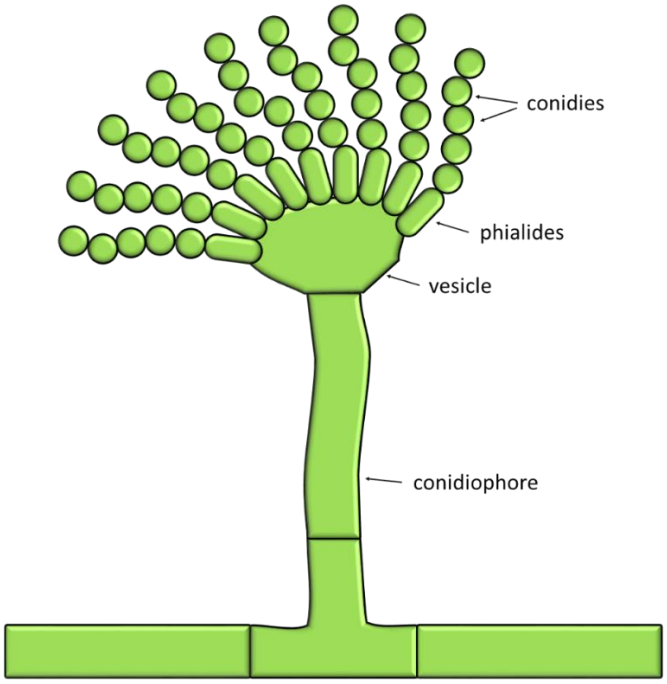


Figure 3: Schematic representation of an aspergillus head

The genus *Aspergillus* includes 339 species divided in four subgenera (*Fumigati*, *Circumdati*, *Nidulantes* and *Aspergillus*) and 18 sections [5]. These species are found in different environments and substrates like soil, compost, organic debris or indoor dust[6]. Only a limited number of them have been associated with human diseases.

The most common cause of aspergillosis in humans is *A. fumigatus* (50-90%), followed by *Aspergillus flavus* (10-20%), *Aspergillus niger* (<5%), and *Aspergillus terreus* (<5%). Other species, such as *Aspergillus calidoustus*, *Aspergillus nidulans, Aspergillus glaucus* and *Aspergillus versicolor*, can cause human infections in rare occasions. Identification of these species can be done in the laboratory based on the macroscopic aspects of the colonies and the microscopic aspect of mycelia, as shown in Figure 4.

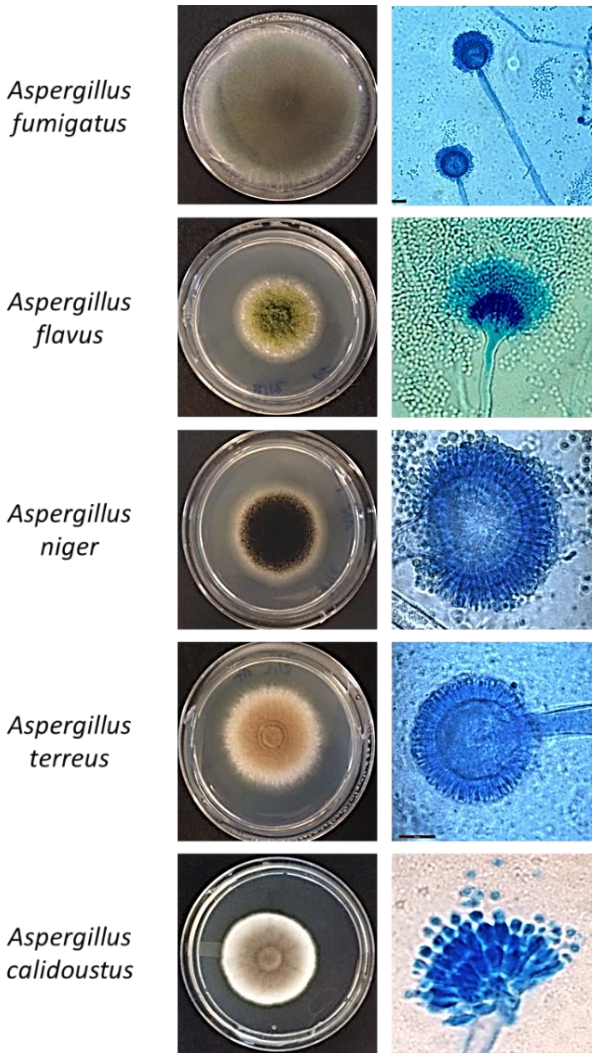
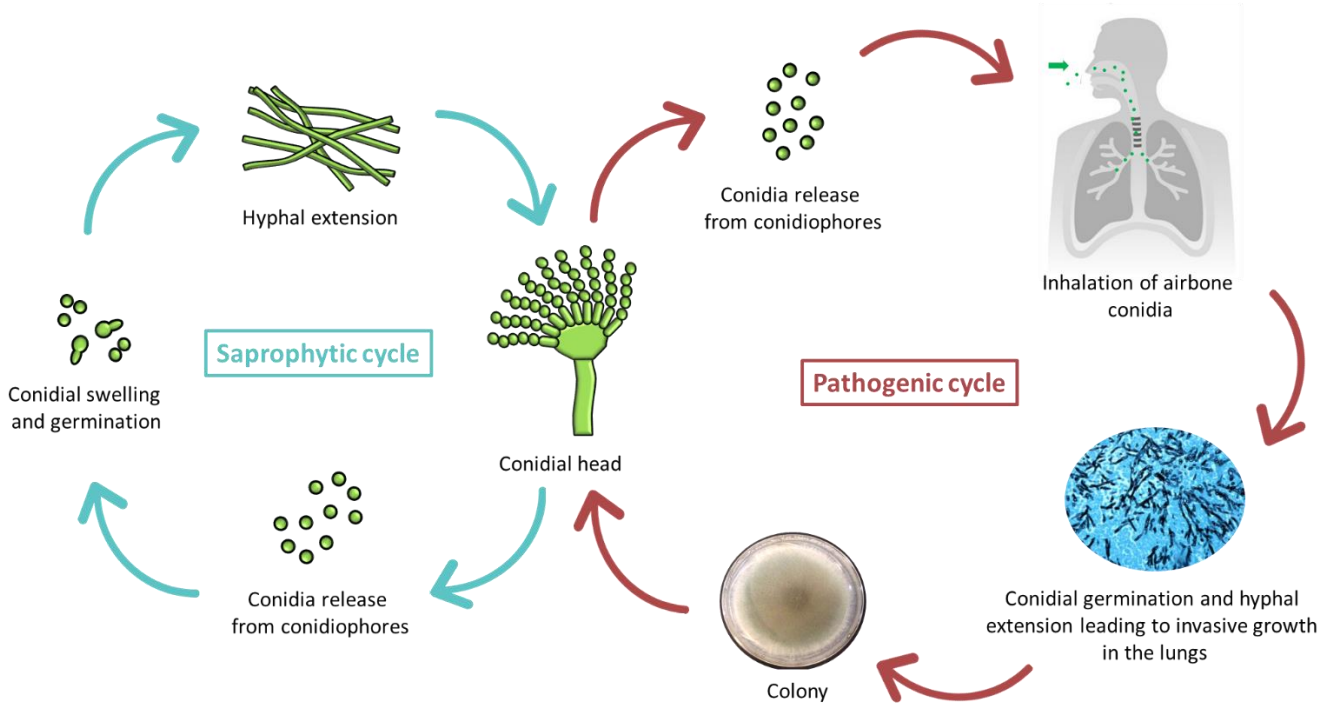


Figure 4: Most common pathogenic *Aspergillus* spp. macroscopic and microscopic aspects.

III. *Aspergillus fumigatus*

Aspergillus fumigatus is a saprophytic fungus involved in recycling environmental carbon and nitrogen. It can adapt easily to elevated temperatures, oxidative stress, limitations in nutrient and hypoxic conditions, which makes of it the most virulent *Aspergillus* species for humans.

A. fumigatus produces airborne spores that are inhaled by humans and can reach lung alveoli (Figure 5). Humans can inhale hundreds of these conidia per day.



<u>Figure 5</u>: Saprophytic and pathogenic cycles of *A. fumigatus* (adapted from Sugui et al., 2014) [2]

In immunocompetent hosts, *A. fumigatus* conidia are cleared by the immune system but in some cases, they can lead to allergic diseases (bronchopulmonary aspergillosis) or chronic forms of the disease (aspergilloma, i.e. a fungus ball in a preexisting lung

cavity). However, in immunocompromised patients, the inhaled conidia can invade the lung tissue and provoke invasive aspergillosis (IA), a severe infection associated with high mortality rates.

1) Immune defenses against *A. fumigatus*

Both innate and adaptive immune responses are important against *A. fumigatus*.

Respiratory epithelial cells are the first cells in contact with fungal conidia. Some of these cells showed the ability to internalize conidia but with less efficacy in fungal elimination than phagocytosing cells (macrophages and neutrophils). Alveolar macrophages represent the first line of phagocytic host defences against inhaled spores.

Pathogen associated molecular patterns (PAMPs) of the fungus are recognized by pattern recognition receptors (PRRs) at the surface of macrophages or dendritic cells (e.g. TLR2, TLR4, dectin), which activate intracellular signaling pathways resulting in the production of cytokines and the differentiation of T-helper CD4+ lymphocytes [7]. Pentraxin 3, a soluble PRR, binds to *Aspergillus* conidia facilitating their phagocytosis and killing by macrophages.

After spore germination, Neutrophils belong to the first recruited cells in the response and are essential innate effectors against *A. fumigatus* hyphae. They employ several mechanisms for the spores elimination such as ROS generation using NADPH oxidase, production of lactoferrine and release of antimicrobial protease by degranulation. Neutrophils can produce Neutrophil extracellular traps (NETs, consisting of chromatin mixed with proteins from neutrophilic granules and cytoplasmic proteins) to trap the pathogen.

Other cells involved in the immune response against *A. fumigatus* include eosinophils, mast cells, dendritic cells, natural killers, and platelets.

A defect in neutrophil count or function, such as neutropenia resulting from anti-cancer chemotherapy or some hereditary disorders such as chronic granulomatous disease, represent the major risk factor for IA. Drugs crippling the T-cell function (e.g. anti-calcineurin inhibitors, corticosteroids) also predispose to IA.

Recently, some single nucleotide polymorphisms (SNPs) have been identified in different PRRs (e.g. pentraxin, dectin, TLR4) and have been associated with an increased risk of IA.

2) Invasive aspergillosis (IA)

- Epidemiology of IA

IA represents the most severe form of aspergillosis and essentially affects patients with severe alteration of the immune system [8]. Patients at particularly high risk of IA are those with hematologic cancers (e.g. acute leukemia) who undergo myeloablative chemotherapy resulting in prolonged neutropenia. Another category of high-risk patients consists of transplant recipients, such as allogeneic hematopoietic stem cell transplant recipients or solid-organ (in particular lung) transplant recipients. These patients have long-term immunosuppressive therapies (anti-calcineurin drugs, corticosteroids) that prevent graft rejection and represent a risk of opportunistic infections. IA can also affect other categories of immunocompromised patients, such as those with prolonged corticosteroid treatment or other immunosuppressive therapies for auto-immune diseases. Recently, IA was also recognized as a

complication of severe Influenza or COVID-19 among patients requiring invasive mechanical ventilation [9,10].

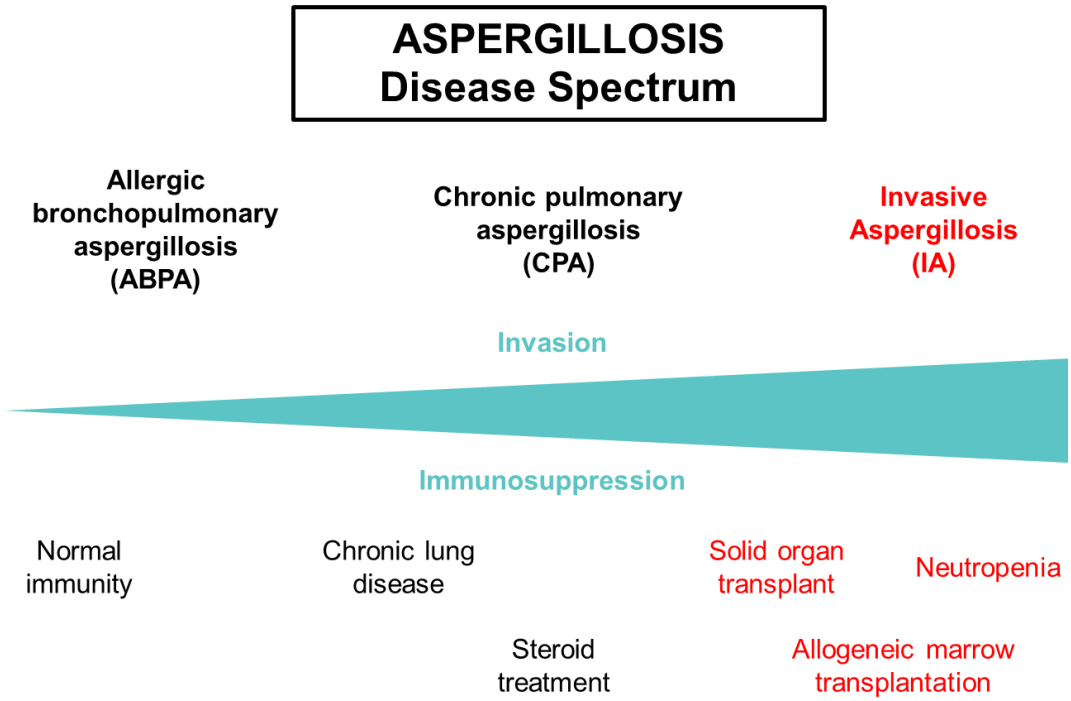


Figure 6: Risk factors and incidence of IA

- **Pathogenesis and clinical manifestations of IA**

In immunocompromised patients, The *A. fumigatus* conidia can penetrate the lung epithelial barrier and invade lung tissue and blood vessels with subsequent dissemination to other organs.

- **Clinical manifestations**

The most common affected organ in IA is the lung. Inhaled spores reach the alveoli and develop invasive pulmonary aspergillosis. Hyphae invade the lung tissue and cause an hemorrhagic and necrotic lesion. Patients usually have fever with only mild or no respiratory symptoms. Imaging of the lungs by CT-scan shows a well-

circumscribed nodule or mass with surrounding ground-glass opacity called “halo sign” and corresponding to angio-invasion and hemorrhage (Figure 7).

After angio-invasion, *A. fumigatus* can disseminate to other organs, such as the skin, kidney, liver, spleen or brain.

Sinuses can also represent a primary site of IA with secondary dissemination to brain by contiguous extension (Figure 7).

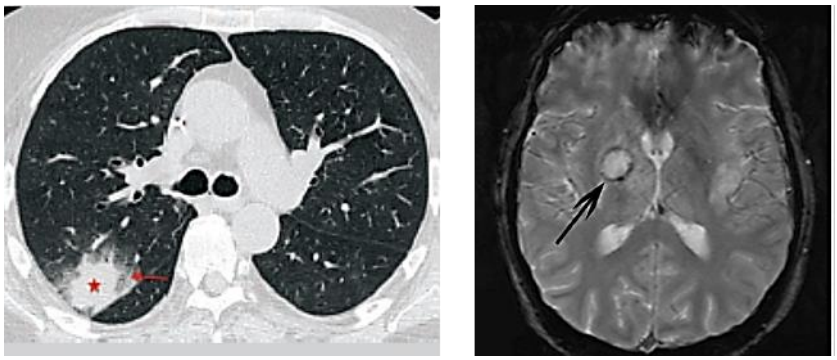


Figure 7: Radiological aspects of pulmonary IA showing a mass with halo sign (left) and cerebral IA showing a fungal abscess (right).

- **Diagnosis of IA**

Diagnosis of IA requires the acquisition of deep tissue samples for detection of the fungus. For pulmonary IA, bronchoscopy is needed to get bronchoalveolar lavage (BAL) fluid and endobronchial ultrasound (EBUS) guided lung tissue biopsy.

The standard diagnostic methods used are:

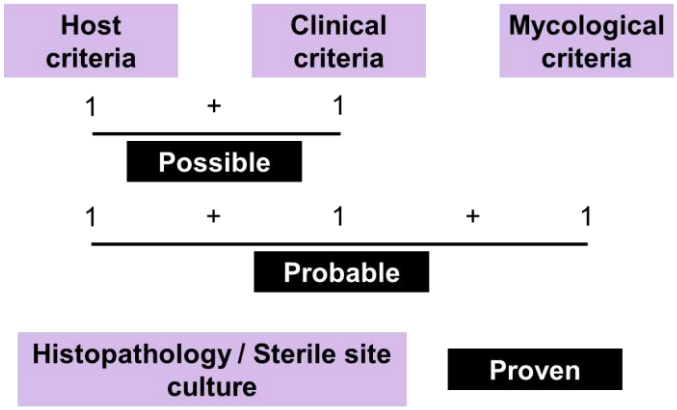
- Direct examination with silver staining or KOH-calcofluor (staining the chitin component of the cell wall).
- Culture on selective Sabouraud dextrose agar (or other specific media for the recovery of fungi).

However, these methods of direct identification of the fungus have low sensitivity and IA diagnosis usually requires addition of serological and/or molecular diagnostic tools.

- Detection of fungal polysaccharides that are released in blood or other fluids, such as the galactomannan (specific cell wall component of *Aspergillus* spp.) and the β-D-glucan (major cell wall component of most ascomycetous fungi). Galactomannan can be detected in serum, BAL or cerebrospinal fluid.
- Polymerase chain reaction (PCR) currently represents the most sensitive method for the detection of *Aspergillus* spp. in any kind of tissue or biological fluid, but its availability is limited.

Because of the relative low yield of diagnostic methods, the diagnosis of IA is classified on a scale of probability (possible, probable, proven) considering the three following criteria i) the presence of host criteria (immunosuppressive conditions at risk of IA), ii) the presence of clinical criteria (radiological findings, such as lung lesions on CT-scan, consistent with IA) and iii) the presence of mycological criteria (a positive microbiological test for the detection of *Aspergillus* spp. as above described) [3]

(Figure 8)



<u>Figure 8</u>: Classification of invasive aspergillosis according to EORTC-MSG criteria[3].

- **Treatment of IA**

Three antifungal drug classes are currently available for the treatment of IA. These antifungals target either the cell membrane or the cell wall of the mold, which are essential for its growth and survival (Figure 9). Fungicidal drugs such as triazoles (e.g. voriconazole, posaconazole or isavuconazole) and polyenes (amphotericin B formulations) represent the first-choice treatments, and disturb the cell membrane. Echinocandins (caspofungin, anidulafungin and micafungin), inhibiting the synthesis of the β-1,3-d-glucan polysaccharidic compound of the cell wall, only have fungistatic activity against *A. fumigatus* and represent an alternative therapy.

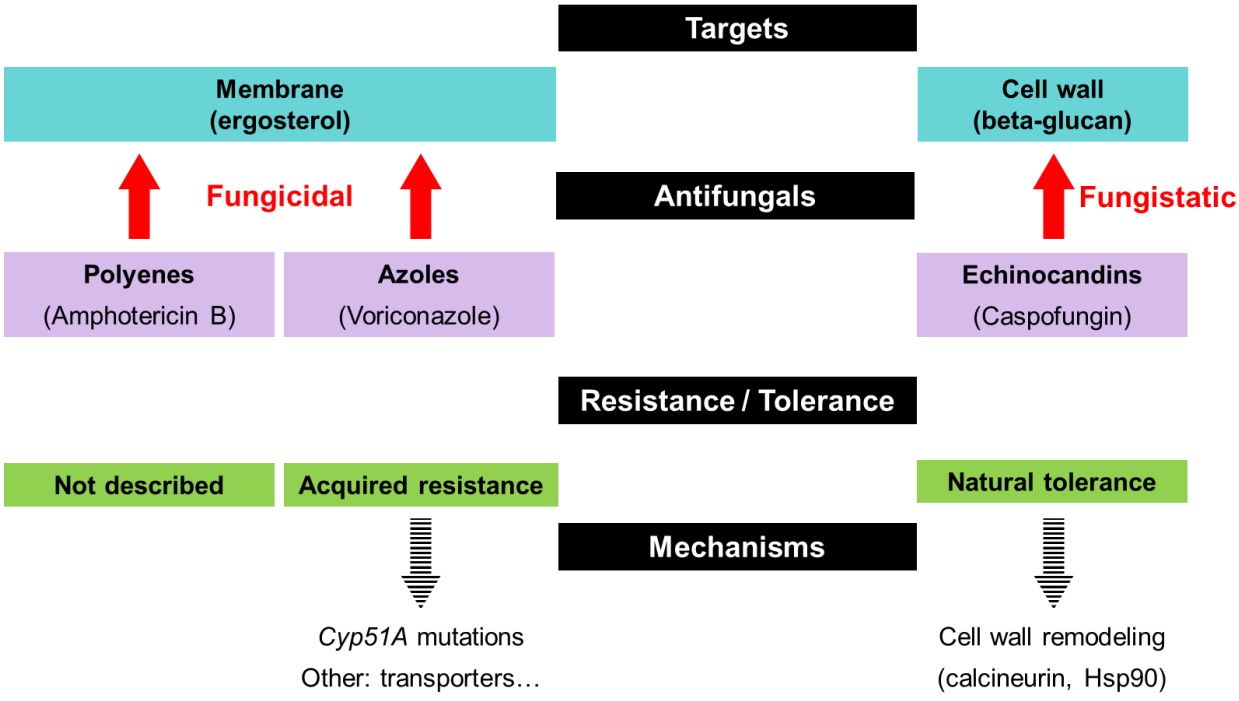


Figure 9: Mechanisms of action and resistance for the different anti-*Aspergillus* drugs (polyenes, azoles and echinocandins).

1- Azoles

Chemical properties: these antifungals contain an azole ring. They are classified into two groups: those with two nitrogens in the azole ring (the imidazoles including clotrimazole, econazole, ketoconazole, miconazole, and tioconazole) and those with three nitrogens in the azole ring (the triazoles which include fluconazole, itraconazole, posaconazole, and voriconazole) (Figure 10).

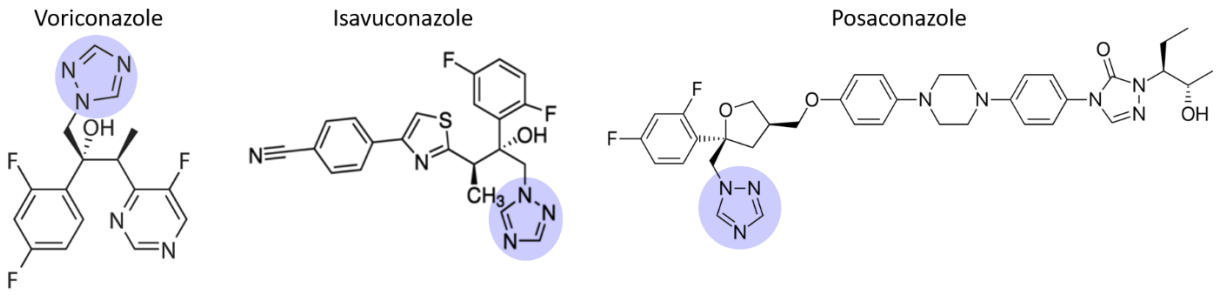


Figure 10: Main Triazoles used to treat aspergillosis represented with azole ring

Mechanism of action: azoles inhibit the lanosterol 14 α-demethylase, enzyme of the ergosterol synthesis pathway thus preventing the formation of ergosterol, an important component of the fungal cell membrane, which results in loss of ergosterol, accumulation of toxic sterol intermediates and cell death. (Figure 11).

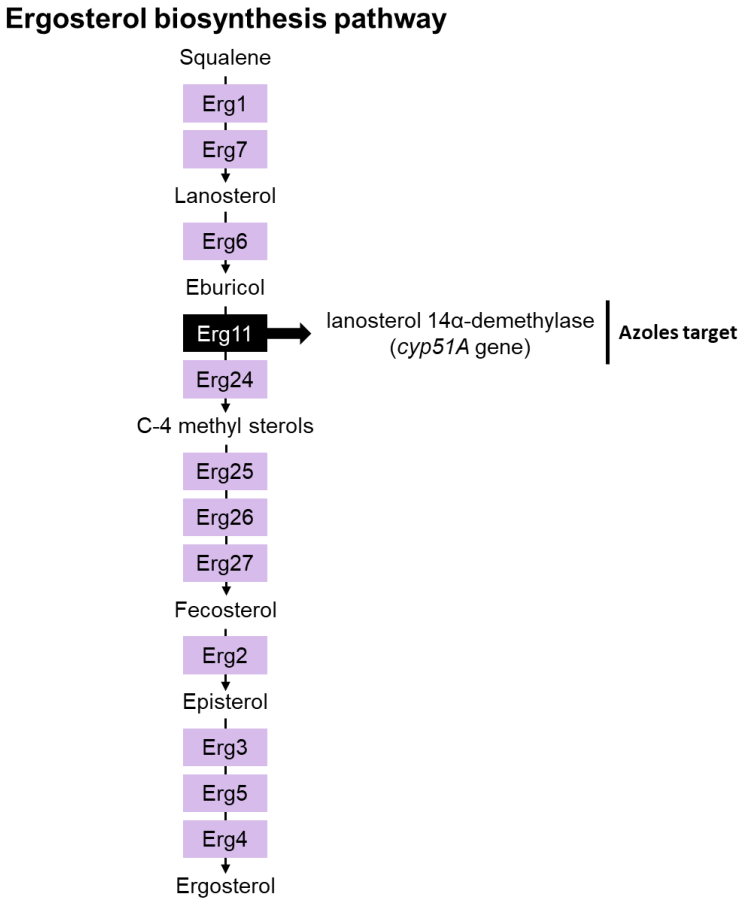


Figure 11: Ergosterol synthesis pathway and azole target

Antifungal activity and spectrum: fluconazole and itraconazole correspond to the first generation of azole drugs, while voriconazole (VRC), posaconazole (POSA) and isavuconazole (ISA) belong to the second generation and are the most effective drugs in treating IA. Azoles, with the exception of fluconazole (not active against molds), are fungicidal and very active against *Aspergillus fumigatus* and most *Aspergillus* spp. with rare exceptions (e.g. *A. calidoustus*, *A. lentulus*).

Clinical application: azoles can be administered to the patient intravenously or orally, which represents an advantage. However, azoles present several limitations, such as

interactions with other drugs (as inhibitors of the cytochrome p450 isoenzymes) and hepatotoxicity.

Itraconazole is often used in patients with chronic pulmonary aspergillosis, while voriconazole and isavuconazole are the first-line treatments of IA [11]. Posaconazole is approved for use as prophylaxis to prevent IA in high-risk hematologic cancer patients.

2- Polyenes

Chemical properties: polyenes are characterized by a conjugated double bond structure, part of their macrolide ring structure (Figure12)

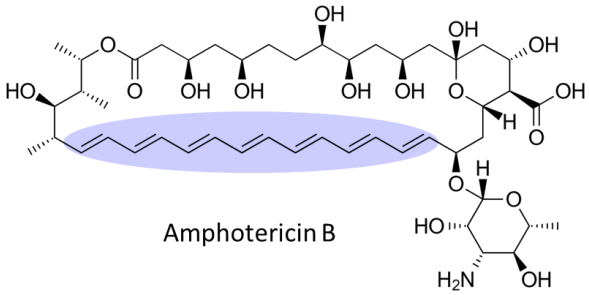


Figure 12: Amphotericin B structure with conjugated double bond structure

Amphotericin B is the only polyene approved for the treatment of IA. Several drug formulations exist. Historically, amphotericin B deoxycholate was the first formulation used in clinical practice. This formulation has been substituted by novel lipid formulations of amphotericin B, which are better tolerated, such as liposomal amphotericin B and amphotericin B lipid complex. In these formulations, the drug, after being released from the liposomes, is transferred through the cell wall to bind the ergosterol in the fungal cell membrane. [12]

Mechanism of action: polyenes bind to ergosterol forming pores and causing a depolarization of the membrane, with increase of the membrane permeability which leads to ion leakage and cell death. Another relevant mechanism of action of amphotericin B may consist of the accumulation of reactive oxygen species, which is toxic for the fungal cell.

Antifungal activity and spectrum: Amphotericin B has a fungicidal activity and a wide spectrum of action against most pathogenic molds including *Aspergillus* spp, with the exception of *A. terreus*.

Clinical application: Amphotericin B is only available for parenteral use, as its absorption from the gastrointestinal tract is very low. Amphotericin B deoxycholate, the first developed formulation of the drug, was associated with important toxicity in humans, in particular nephrotoxicity, which limited its use. Novel lipid formulations of amphotericin B are associated with a lower occurrence of renal toxicity. Amphotericin B formulations are second-line treatments of IA, as their efficacy was found to be somewhat lower compared to that of voriconazole [11,13].

3- Echinocandins

Chemical properties: Echinocandins are lipopeptides molecules, derived from fungal secondary metabolite. These molecules are characterized by a cyclic hexapeptide core linked to a variably configured lipid side chain (Figure 13)

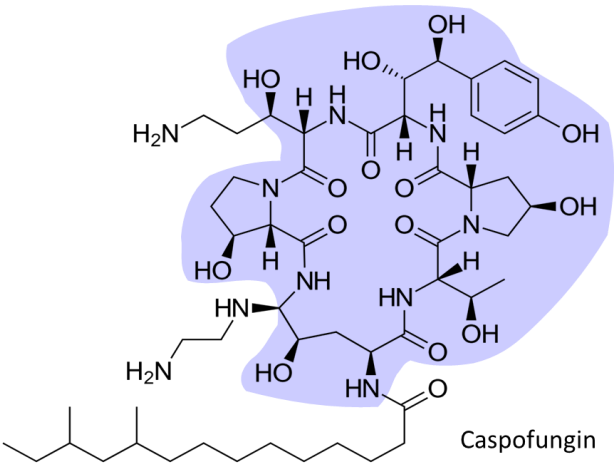


Figure 13: Caspofungin structure represented with its cyclic hexapeptide core

This antifungal class currently consists of three available drugs, i.e., caspofungin, micafungin, and anidulafungin. A novel molecule with a prolonged half-life, rezafungin (CD101), is currently evaluated in phase III clinical studies.

Mechanism of action: Echinocandins non competitively inhibit the β-1,3-glucan synthase enzyme. This enzyme is responsible of the formation of beta-glucan, a major fungal cell wall component. Loss of beta-glucan causes cell wall damages resulting in growth arrest of the fungus.

Antifungal activity and spectrum: Echinocandins are mainly active against yeasts, such as *Candida* spp. They only have fungistatic activity against *Aspergillus* spp. This antifungal effect is however observed at low concentrations.

Clinical application: Echinocandins are available for parenteral administration only. They exhibit few side effects. Because of their limited antifungal activity against *Aspergillus* spp., these drugs only represent second-line or third-line treatments of IA

in case of intolerance to azoles and/or amphotericin B. Combination of azoles and echinocandins can be used in severe cases, because of their synergistic interaction and possible improved efficacy [14].

IV. Resistance of *Aspergillus fumigatus* to antifungal drugs

Antimicrobial resistance refers to the ability of a microorganism to overcome the effect of an antimicrobial agent and to survive and multiply despite appropriate doses of this drug. A microorganism is defined as “resistant” to an antimicrobial drug, when treatment with this drug is associated with a high prediction of failure of therapy.

The activity of a drug against a microorganism can be expressed by the assessment of the minimal inhibitory concentration (MIC), which is the concentration of the drug required to completely inhibit the growth of the pathogen *in vitro*. For each microorganism-drug interaction, a MIC threshold can be defined to determine “susceptibility” (i.e. MIC below the cut-off) and “resistance” (i.e. MIC above the cut-off), which predicts the probability of success or failure to therapy, respectively.

Resistance of a microorganism to a drug can be natural (i.e. intrinsic) or acquired (by genetic mutations).

“Tolerance” refers to the persistence of residual growth above the MIC threshold, which usually results from adaptive mechanisms of stress adaptation that allow the microorganism to survive despite the inhibitory growth effect of the drug.

1) *Azole resistance*

Over the last decade, emergence of azole resistance has been observed all over the world. First sporadic cases of azole resistance have been described in the United

States in the 80s. Since then, cases of acquired azole resistance have been occasionally reported in patients with prolonged exposure to azoles [15]. More recently, azole resistance has emerged in environmental *A. fumigatus* strains, as a probable result of the widespread use of fungicides in the agriculture. This phenomenon has been initially described in the Netherlands, where the proportion of azole resistant strains has increased to reach 15 to 20% in 2019 [16]. However, it has now been reported in all continents [17]. (Figure 14)

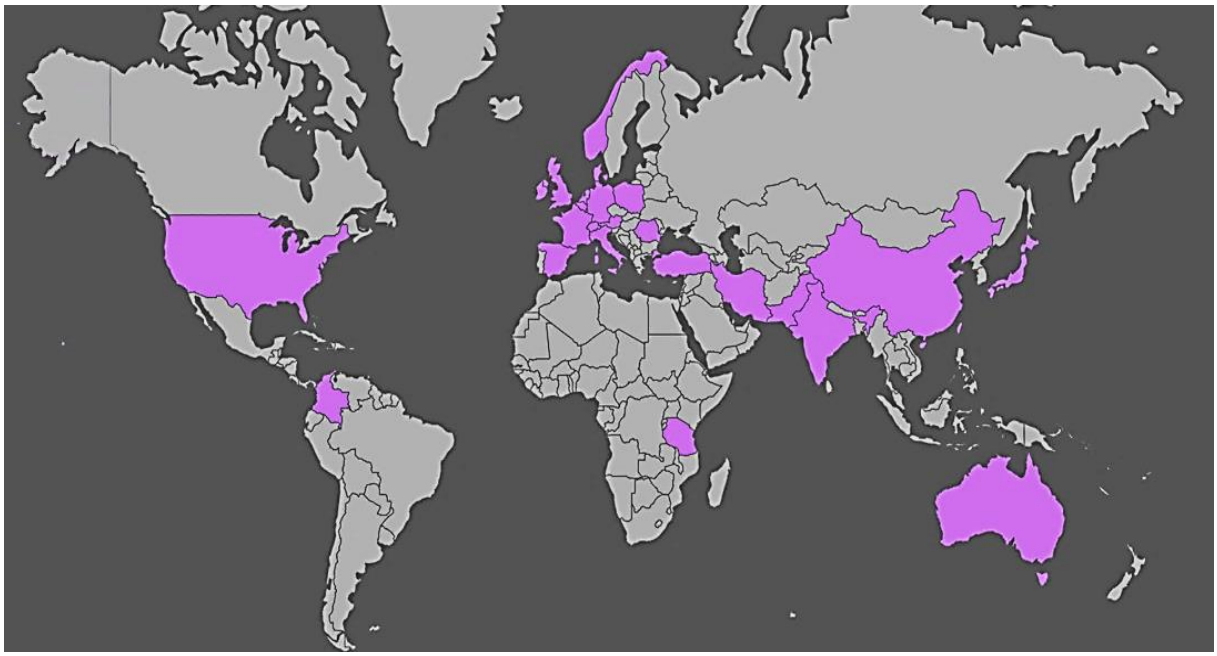


Figure 14: Worldwide repartition of azole resistance
(adapted from Meis Jacques F. *et al*. [18])

Azole resistance resulting from this environmental source has typical signatures in the *cyp51A* gene encoding for the lanosterol 14 α-demethylase, the target gene of azoles, consisting of the combination of a tandem repeat (TR) in the promoter and an amino acid substitution in the gene (TR_{34}/L98H and TR_{46}/Y121F/T289A). These mutations reduce the affinity of azoles for the target enzyme and can also be associated with upregulation of the target gene. *A. fumigatus* strains with these mutations exhibit pan-

azole resistance. They usually infect patients who are naïve of azole treatments causing severe and difficult-to-treat IA associated with high mortality rates [18].

Azole resistance can also develop after prolonged exposure to azoles in patients with chronic pulmonary aspergillosis. These azole resistant *A. fumigatus* strains also exhibit mutations in the *cyp51A* gene, but at different sites (G54, G138, M220 and G448) [19].

Recently, mutations in other genes, such as *hmg1*, encoding for an enzyme involved in the ergosterol biosynthesis pathway (HMG-CoA reductase) and *hapE* (encoding for the CCAAT binding factor complex) were found to play a role in azole resistance [20]. Besides, *A. fumigatus* clinical isolates with phenotypic azole resistance in the absence of mutations in *cyp51A* or other relevant genes have also been identified, which suggests the existence of other mechanisms of stress adaptation or tolerance to azoles. Two transcription factors AtrR and SrbA were found to play a crucial role in azole resistance by overexpressing *cyp51A* and an ABC-transporter (*abcC*) [21]

Upregulation of the efflux pumps (transporters) help to decrease the concentration of drug in the cell and could participate in the azole adaptation. It is a well-known mechanism of azole resistance or tolerance in other fungi, such as the pathogenic yeasts *Candida* spp. [22], but its contribution to azole resistance in *A. fumigatus* is less obvious [23,24]. Transporters are transmembrane proteins that carry molecules in or outside the cell. Two families of transporters are involved in the efflux of azole drugs, the ATP binding cassette (ABC) and major facilitator superfamily (MFS) [23-25]. In *A fumigatus*, the transporters AtrI (ABC) and MdrA (MFS) were found to influence the basal level of azole susceptibility [23]. The ABC transporter encoding gene *cdr1B* (=*abcC*) was also found to be overexpressed in several azole resistant *A. fumigatus* isolates and its deletion resulted in decreased azole resistance [26]. Several other ABC

and MFS transporters were found to be upregulated in *A. fumigatus* wild-type strains exposed to azoles or among *A. fumigatus* resistant isolates [27,28].

2) *Amphotericin B resistance*

While some *Aspergillus* spp. (*A. terreus*) exhibit some natural resistance to amphotericin B, acquired resistance to this drug is extremely rarely observed in *A. fumigatus* and its mechanisms are poorly understood.

3) *Echinocandin resistance*

A. fumigatus exhibits some natural degree of tolerance to echinocandins, with persistent residual growth above the MIC. In addition, a "paradoxical effect", defined as a paradoxical growth recovery at increasing concentrations is usually observed with the echinocandin drug caspofungin (Figure 15). This phenomenon is due to compensatory remodeling of the cell wall resulting in an increased of the chitin content to counteract the effect of the loss of beta-glucan. The calcium-calmodulin-calcineurin pathway and the heat shock protein 90 (Hsp90) were found to play a key role in the paradoxical effect of caspofungin [29].

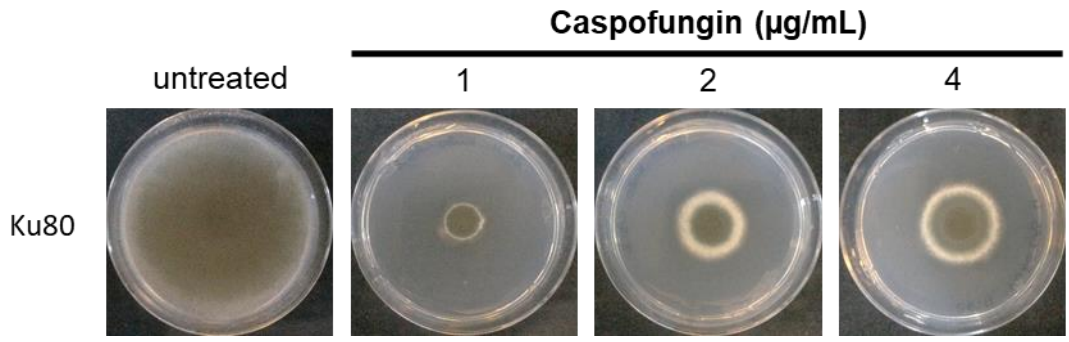


Figure 15: Paradoxical effect of caspofungin observed in a wild type *A. fumigatus* strain (Ku80)

Hsp90 is an essential molecular chaperone controlling the folding, activation, translocation and degradation processes of multiple client proteins (e.g. transcription factors, kinases or key signaling transducers) [30]. This protein is also a trigger of various stress responses. In yeasts, Hsp90 controls about 10% of the whole proteome and plays a role in azole resistance of *Candida albicans.* [31]**.** In *Aspergillus fumigatus*, Hsp90 was shown to have a predominant role in tolerance to caspofungin and in the caspofungin paradoxical effect [32]. The effect of Hsp90 in this stress response is supposed to be mediated via the calcineurin pathway.

Calcineurin is a Ca^{2+}/calmodulin-regulated serine/threonine protein phosphatase, which is a client protein of Hsp90 [33]. Calcineurin was shown to play a role in growth, morphology and pathogenicity of *A. fumigatus* [34]. Moreover, deletion of the catalytic subunit of calcineurin results in abolition of the caspofungin paradoxical effect, which supports its role in caspofungin tolerance [33,35]. Exposure to caspofungin results in an increase of intracellular calcium, which binds to calmodulin. This complex activates calcineurin by phosphorylation. Activated calcineurin then desphosphorylates CrzA, a transcription factor involved in upregulation of chitin synthases encoding genes (*chsA*, *chsC*, *chsG* and *csmB*), which results in an increase of the chitin content of the cell wall in order to compensate β-1,3-glucan decrease (Figure 16). Pharmacologic inhibition of different steps in this pathway (e.g. calcium chelators, anticalcic drugs, anti-calcineurin drugs, or the Hsp90 inhitibor geldanamycin) results in increased susceptibility to caspofungin and abolition of the paradoxical effect in *A. fumigatus* [36,37].

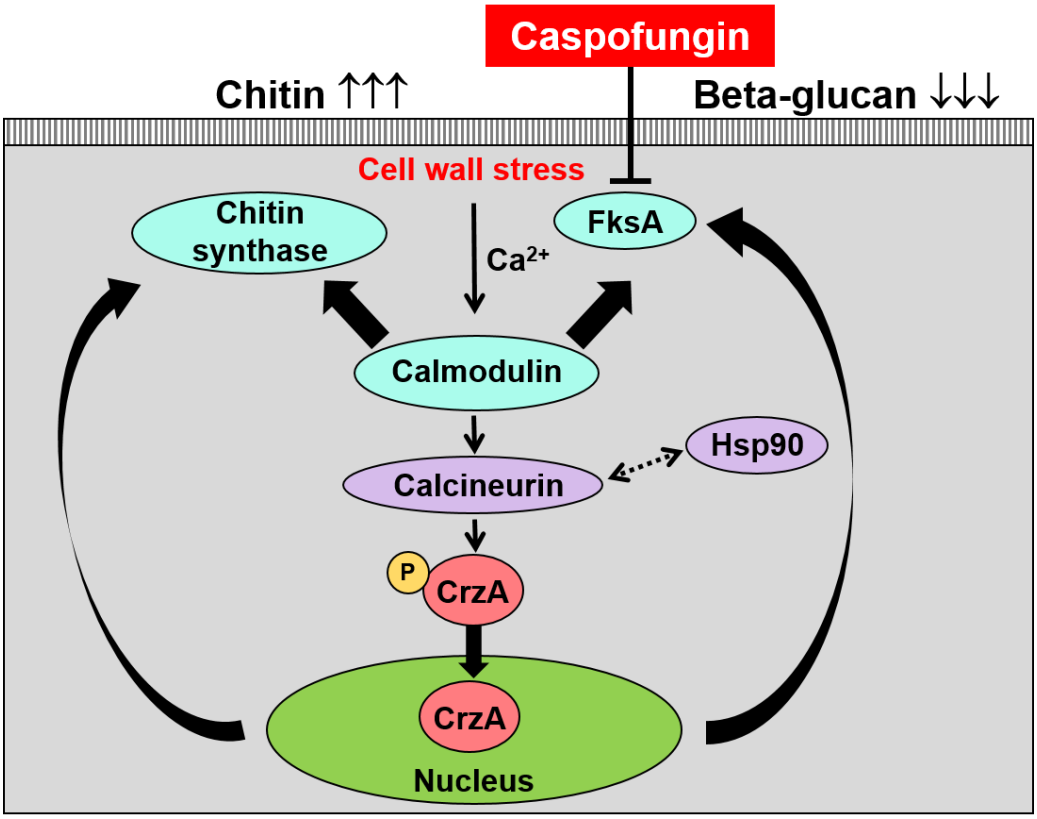


Figure 16: Mechanisms involved in caspofungin paradoxical effect

While both Hsp90 and calcineurin were shown to play a role in caspofungin tolerance and paradoxical effect, the complexity of this network remains to be elucidated.

As part of my thesis work, I have published a mini-review entitled “Echinocandins for the Treatment of Invasive Aspergillosis: from Laboratory to Bedside” *in Antimicrobial Agents and Chemotherapy* [38]. This review describes the mechanisms of resistance/tolerance to echinocandins (in particular caspofungin) in *A. fumigatus* and discusses the role of echinocandins in the treatment of IA **(ANNEX 1)**.

AIMS OF THIS WORK

Mechanisms involved in antifungal stress adaptation of *A. fumigatus*

The overall objective of this work was to get further insights in the adaptive mechanisms of stress response to antifungal drugs in *A. fumigatus*. For this purpose, we have focused on two areas of interest with the following aims:

1) Tolerance to caspofungin

   Aim: to decipher the network of Hsp90 in caspofungin tolerance and paradoxical effect in *A. fumigatus*.

2) Azole resistance resulting from adaptive stress responses (i.e. genes overexpression) independent from *cyp51A* mutations

   Aim: to assess the ability of *A. fumigatus* to adapt *in vitro* to azole stress and to decipher the molecular mechanisms of such stress adaptation.

Deciphering the mechanisms of these stress responses to antifungal drugs is ultimately expected to reveal novel antifungal targets for the treatment of IA.

RESULTS

Project 1: Tolerance to echinocandins mediated by the Hsp90 network

In this work, we investigated the mechanisms of caspofungin tolerance that are dependent of Hsp90. In particular, our objective was to identify the intracellular pathways and client proteins that are under the control of Hsp90 in caspofungin tolerance and paradoxical effect.

Hsp90 was previously shown to play a role in stress adaptation to echinocandins and in caspofungin paradoxical growth. However, the mechanisms and pathways by which Hsp90 controls this stress response remains unclear. In this work, we have revealed a key role of the mitochondrial respiratory chain (MRC) in this Hsp90-dependent pathway of tolerance to caspofungin in *A. fumigatus*.

1- Summary

Introduction

Echinocandins, such as caspofungin, are used as second-line treatment of IA, but their efficacy against *A. fumigatus* is limited. The heat shock protein 90 (Hsp90) orchestrates caspofungin stress response in *A. fumigatus* and is the trigger of an adaptive phenomenon called the paradoxical effect (growth recovery at increasing caspofungin concentrations). It was previously demonstrated that genetic or pharmacologic inhibition of Hsp90 results in increased susceptibility to caspofungin and abolition of the paradoxical effect.

Aim

The aim of this study was to elucidate the Hsp90-dependent mechanisms of caspofungin tolerance and paradoxical effect in order to identify novel therapeutic targets.

Methods

For this work, we used the strain called pthiA-hsp90, where the *hsp90* native promoter has been replaced by a thiamine dependent-promoter [32]. Addition of thiamine to the growth medium results in complete repression of *hsp90* and lack of growth of the fungus. In the absence of thiamine, *hsp90* expression is sufficient to maintain *A. fumigatus* viability. However, under caspofungin exposure, the loss of promoter regulation cripples the stress response and results in abolition of the paradoxical effect.
As a first step, transcriptomic profiles (RNA seq) of the wild-type *A. fumigatus* strain (Ku80 parental strain) were compared to that of pthiA-hsp90 in the absence or presence of caspofungin. Genes that were significantly overexpressed under caspofungin exposure in Ku80 but not in pthiA-hsp90 were considered as candidates for Hsp90-dependent genes in caspofungin stress response.
A subset of these genes of interest were selected according to their functions. Further experiments were performed to characterize their role in caspofungin stress response and paradoxical effect.

Results

We found that caspofungin induced a drastic overexpression of the genes of the mitochondrial respiratory chain (MRC), in particular NADH-ubiquinone oxidoreductases (complex I), in Ku80 but not in pthiA-Hsp90.

These findings suggest that the MRC has a role in the stress response to caspofungin, which is dependent of Hsp90. MRC is composed of several complexes located across the membrane which consume oxygen and by electron transfer reaction produce ATP. To further characterize the role of the MRC in caspofugin tolerance and paradoxical effect, we tested the effect of MRC inhibitors. Rotenone, a MRC complex I inhibitor, could abolish the caspofungin paradoxical effect in Ku80. Fluorescent staining of active mitochondria, measurement of oxygen consumption and of ATP production confirmed the activation of the MRC in Ku80 in response to caspofungin, which was impaired in pthiA-Hsp90. Using aequorin as a bioluminescent reporter for calcium intracellular levels, we demonstrated that inhibition of Hsp90 by geldanamycin or MRC complex I by rotenone prevented the increase of intracellular calcium, which was previously shown to be essential for caspofungin stress response.

Conclusion

In this project, we revealed the unique role of the MRC in caspofungin stress response, which is dependent of Hsp90. Based on these results, we propose the following model to explain the mechanisms of caspofungin tolerance and paradoxical effect in *A. fumigatus* (Figure 17).

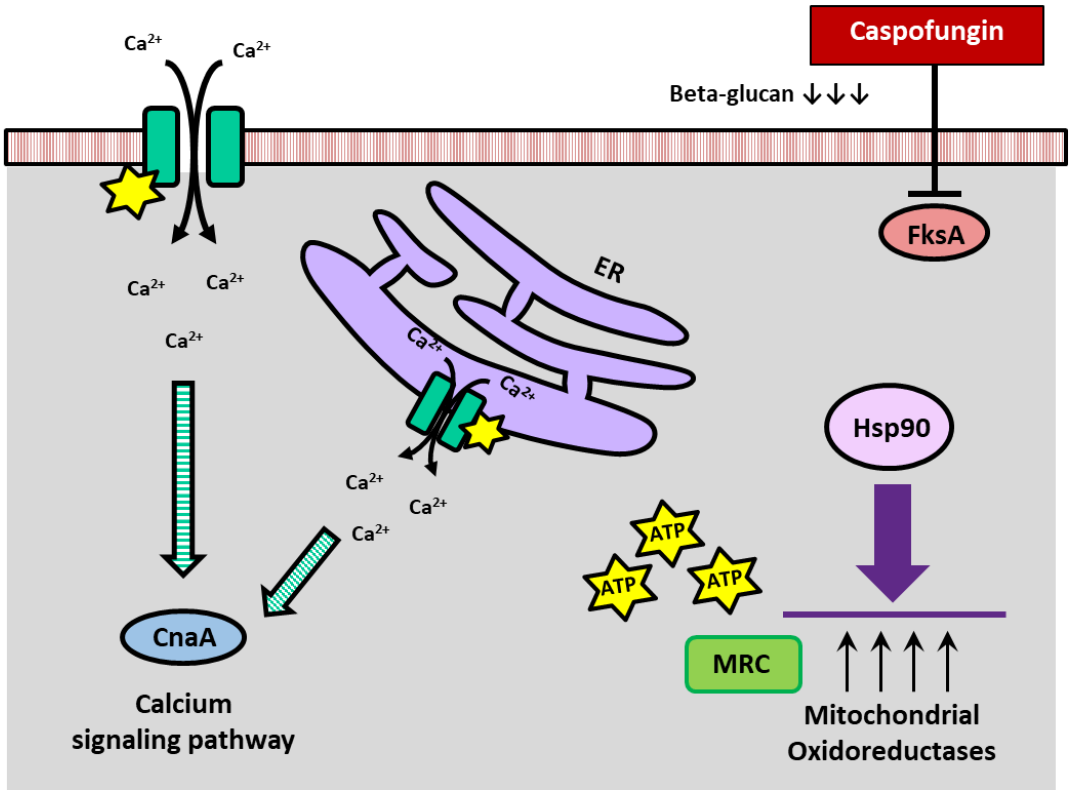


Figure 17: Hypothetical model for caspofungin tolerance / paradoxical effect

Activation of the mitochondrial respiratory chain (MRC) by Hsp90 results in increased oxygen consumption in order to increase ATP production. ATP activates the ATP-dependent calcium-transporters localized on the endoplasmic reticulum or at the membrane to allow intracellular Ca^{2+} release, which activates the calcineurin pathway.

2- Contribution

My contributions to this project are the following:

- Design and optimization of the protocols
- Realization of the experiments
- Data analyses
- Redaction of the first draft of the manuscript and figure formatting.

3- Outputs

Publications in peer-reviewed journals:

- **Aruanno M**, Bachmann D, Sanglard D, Lamoth F. Link between heat shock protein 90 (Hsp90) and the mitochondrial respiratory chain in the caspofungin stress response of Aspergillus fumigatus. *Antimicrob Agents Chemother* 2019, r 63:e00208-19. https://doi.org/10.1128/AAC.00208-19 (original research article).

Full article is presented in **ANNEX 2**

Presentations to national or international conferences:

- **Aruanno M**, Bachmann D, Sanglard D, Lamoth F. Hsp90 controls caspofungin paradoxical effect of Aspergillus fumigatus via the mitochondrial respiratory chain. Advances Against Aspergillosis (AAA), Lisbon 1-3 February 2018. Oral and poster presentation (Abstract #22).

- **Aruanno M**, Bachmann D, Sanglard D, Lamoth F. Hsp90 controls caspofungin paradoxical effect of Aspergillus fumigatus via the mitochondrial respiratory chain. Annual Congress of the Swiss Society of Microbiology (SSM), Lausanne 28-30 August 2018. Oral presentation (S-84*).

Project 2: Mechanisms of azole stress adaptation independent from *cyp51A* mutations

In this work, our goal was to investigate the mechanisms of azole resistance that are related to stress adaptive mechanisms and independent of *cyp51A* mutations.

We generated an *A. fumigatus* pan-azole resistant strain by exposing the wild-type azole-sensitive strain to sub-inhibitory concentrations of voriconazole. We then performed transcriptomic analysis to analyze the azole adaptive mechanisms of this strain. We identified and characterized the role of drug transporters in this adaptive response to azoles. Furthermore, we demonstrated that inhibitors of drug transporters, such as milbemycin oxime, could play a role in potentiating the effect of azoles against *A. fumigatus*.

1- Summary

Introduction

Azole drugs, such as voriconazole, posaconazole and isavuconazole, represent the most active antifungals against *A. fumigatus* and the first-line treatment of IA. Emergence of pan-azole resistance among *A. fumigatus* is concerning and has been mainly attributed to mutations in the *cyp51A* target gene or in other genes of the ergosterol pathway (e.g. *hmg1*). However, the existence of other mechanisms of azole resistance including overexpression of the target gene (*cyp51A*) and/or drug transporters have been described. Their actual role and clinical significance is however unclear.

Aim

To generate *in vitro* an azole-resistant *A. fumigatus* strain in the absence of *cyp51A* (or *hmg1*) mutations and to characterize the mechanisms of azole resistance in this strain.

Methods

We performed microevolution experiment exposing an *A. fumigatus* wild-type strain (Ku80) to infra-minimal inhibitory concentration (MIC) of voriconazole to generate an azole-resistant strain in the absence of *cyp51A* mutations (Ku80R). We then performed transcriptomic analyses (RNA-seq) to identify the groups of genes that were overexpressed in this Ku80R (azole-resistant) strain compared to its parental background Ku80 strain.

Results

After successive subcultures of Ku80 in media containing sub-inhibitory concentrations of voriconazole, we obtained the Ku80R strain exhibiting pan-azole resistance (i.e. increase of MIC above the threshold defining resistance), in the absence of mutations in *cyp51A* or other genes known to be involved in azole resistance (*hmg1*, *atrR*, *srbA*, *hapE*). This resistance was partially reversible after drug relief, which suggests that it is independent from mutations.

Transcriptomic analyses revealed overexpression of the transcription factor *asg1*, several ATP Binding Cassette (ABC) and Major Facilitator Superfamily (MFS) transporters and genes of the ergosterol biosynthesis pathway in Ku80R compared to

Ku80. Sterol analysis of the cell membrane did not show any significant biochemical changes in Ku80R compared to Ku80.

We then investigated to role of drug transporters in azole resistance and the ability of transporters inhibitors, such as milbemycine oxime (MLB) or phenylalanine-arginine beta-naphthylamide (PAβN), to potentiate the effect of azoles against *A. fumigatus*. Using the rhodamine 6G fluorescent marker (a substrate of drug transporters), we showed that MLB could inhibit the transporters. MLB could potentiate the activity of azoles, but this effect was relatively modest. In checkerboard dilutions, criteria of synergism were reached only for MLB and PAβN with posaconazole against Ku80.

The role of the transcription factor *asg1*, which was overexpressed in Ku80R, was also investigated. Deletion of *asg1* in Ku80 resulted in some impact on drug transporters expression (decreased expression of *abcD* and *mfsC*, and increased expression of *mdr1*), but had no impact on azole susceptibility.

Conclusion

This work provided further insights in the mechanisms of azole stress adaptation involving drug transporters in *A. fumigatus*. Targeting drug transporters may represent a novel approach to potentiate azole activity and/or prevent azole resistance. However, currently available drug transporters only have modest effect. Importantly, we showed that azole resistance may rapidly result from continuous exposure to infra-therapeutic azole concentrations in the absence of *cyp51A* gene mutations.

2- Contribution

My contributions to this project were the following:

- Design and optimization of protocols
- Realization of the experiments
- Data analyses
- Redaction of the first draft of the manuscript and figure formatting.

3- Outputs

Publications

- **Aruanno M**, Gozel S, Mouyna I, Parker JE, Bachmann D, Flamant P, Coste AT, Sanglard D, Lamoth F. Insights in the molecular mechanisms of an azole stress adapted laboratory-generated *Aspergillus fumigatus* strain. Paper submitted to *Medical Mycology* with invitation to submit a revision. Revised manuscript submitted on October 16th 2020 and currently under review.

  Full article is presented in **ANNEX 3**

Presentations to national or international conferences:

- **Aruanno M**, Bachmann D, Sanglard D, Lamoth F. Rapid induction of reversible azole resistance in *Aspergillus fumigatus*. *Advances Against Aspergillosis* (AAA), Lisbon 1-3 February, 2018. **Poster presentation** (abstract #23).

- **Aruanno M**, Bachmann D, Sanglard D, Lamoth F. Rapid induction of reversible azole resistance in *Aspergillus fumigatus*. *Annual Congress of the Swiss Society of Microbiology (SSM).* Lausanne, 28-30 August 2018. **Oral presentation** (MY-03*).

- **Aruanno M**, Bachmann D, Sanglard D, Lamoth F. Milbemycin prevents rapid induction of azole tolerance in *Aspergillus fumigatus*. *European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)*. Amsterdam 13-16 April 2019. **Oral presentation** (abstract #2580).
- **Aruanno M**, Gozel S, Bachmann D, Parker JE, Coste A, Sanglard D, Lamoth F. Epigenetic mechanisms of azole stress adaptation in *Aspergillus fumigatus*. *Advances Against Aspergillosis and Mucormycosis (AAA)*. Lugano, Switzerland, 27 – 29 February 2020. **Poster presentation** (abstract #97).
- **Aruanno M**, Gozel S, Bachmann D, Parker JE, Coste A, Sanglard D, Lamoth F. Epigenetic mechanisms of azole stress adaptation in *Aspergillus fumigatus*. Joint meeting *SSI-SSHH*. Geneva, Switzerland, 2-4th september 2020. **Poster presentation** (P-77)**.**

CONCLUSIONS

AND

PERSPECTIVES

Aspergillus fumigatus is the major pathogenic mold in humans and invasive aspergillosis (IA) is increasingly reported, not only in the ever expanding population of immunocompromised patients, but also in new settings, such as ICU patients with severe Influenza or Coronavirus disease 2019 (COVID-19). The limited number of available antifungal drug classes for the treatment of IA and the emergence of acquired azole resistance poses a serious concern. In this context, there is an urgent need for a better understanding of the mechanisms of antifungal drug resistance in *A. fumigatus* and for the identification of novel antifungal targets.

While acquired azole resistance via mutations in the *cyp51A* gene has been well described, other mechanisms of acquired antifungal resistance or stress adaptation (in the absence of mutations) remain underexplored.

The aim of this work was to provide further insights in such mechanisms of stress adaptation to antifungal drugs in *A. fumigatus*.

The limited efficacy of the echinocandin drug caspofungin, due to some degree of tolerance and possibly to a loss of efficacy at higher concentrations (known as the “paradoxical effect”), is a well-known phenomenon. The heat shock protein 90 (Hsp90) and the calcineurin pathway are known to be essential triggers of the caspofungin paradoxical effect. However, the mechanisms underlying this stress response are complex and not entirely elucidated. In this work, we identified the mitochondrial respiratory chain (MRC) as a key player in caspofungin paradoxical effect. This is the first time that the MRC appears to play a role in adaptive responses to antifungal drugs in *A. fumigatus*. This finding opens perspectives for therapeutic strategies targeting the MRC. However, current MRC inhibitors (e.g. rotenone) are not fungal specific and toxic

for humans. Further investigations to define the role of the MRC in mechanisms of antifungal resistance or tolerance in fungi would be warranted, in order to identify more specific targets in this pathway. While we demonstrated that the activation of the MRC was dependent of Hsp90, the nature of this link remains unclear and is supposed to result from an indirect (rather than direct) effect (e.g. activation of transcription factors or other client proteins of Hsp90). This could deserve further analyses. Similarly, it would be interesting to look for downstream effectors in this pathway. As illustrated in our hypothetical model (Figure 17), activation of the MRC results in ATP production. Increase of intracellular calcium is known to be a trigger of the caspofungin paradoxical effect[36]. As the calcium channels (located in the endoplasmic reticulum or the cell membrane) are ATP-dependent, we hypothesize that the MRC may trigger the paradoxical effect by producing ATP for the calcium channels. Indeed, in our work, the MRC inhibitor rotenone could abolish the peak of calcium following caspofungin exposure (see Annex 2). For instance, working on the inositol-1,4,5-trisphosphate receptors, which are the major intracellular calcium release channels, could represent an interesting approach.

Our work on the mechanisms of azole resistance that are independent of *cyp51A* mutations in *A. fumigatus* highlighted some interesting aspects. First, we showed that *A. fumigatus* can rapidly develop a clinically relevant level of resistance by exposure to sub-inhibitory concentrations of azoles. Because achieving therapeutic concentrations is difficult for voriconazole and posaconazole with constant need of monitoring plasma levels in patients with IA, this could represent a significant finding. The pan-azole *A. fumigatus* strain that we generated did not exhibit mutations in

cyp51A or in other genes known to be involved in azole resistance. Transporters overexpression seemed to be a determinant factor of acquired resistance in this strain. Whether this mechanism of resistance represents an under recognized phenomenon should be further investigated by testing transporters expression in clinical *A. fumigatus* isolates with azole resistance in the absence of *cyp51A* mutations.

Transporters inhibition to potentiate the antifungal activity of azoles may represent an interesting therapeutic approach. However, the additive effect of milbemycin on azoles was modest. Only the interaction with posaconazole reached synergistic criteria. Additional research would be warranted to develop more effective transporters inhibitors.

In conclusion, this work highlights the importance of adaptive responses to antifungal drugs in *A. fumigatus* and open perspectives for novel therapeutic approaches.

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ANNEX 1

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MINIREVIEW

Echinocandins for the Treatment of Invasive Aspergillosis: from Laboratory to Bedside

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ABSTRACT Echinocandins (caspofungin, micafungin, anidulafungin), targeting β-1,3-glucan synthesis of the cell wall, represent one of the three currently available antifungal drug classes for the treatment of invasive fungal infections. Despite their limited antifungal activity against *Aspergillus* spp., echinocandins are considered an alternative option for the treatment of invasive aspergillosis (IA). This drug class exhibits several advantages, such as excellent tolerability and its potential for synergistic interactions with some other antifungals. The objective of this review is to discuss the *in vitro* and clinical efficacy of echinocandins against *Aspergillus* spp., considering the complex interactions between the drug, the mold, and the host. The antifungal effect of echinocandins is not limited to direct inhibition of hyphal growth but also induces an immunomodulatory effect on the host's response. Moreover, *Aspergillus* spp. have developed important adaptive mechanisms of tolerance to survive and overcome the action of echinocandins, such as paradoxical growth at increased concentrations. This stress response can be abolished by several compounds that potentiate the activity of echinocandins, such as drugs targeting the heat shock protein 90 (Hsp90)-calcineurin axis, opening perspectives for adjuvant therapies. Finally, the present and future places of echinocandins as prophylaxis, monotherapy, or combination therapy of IA are discussed in view of the emergence of pan-azole resistance among *Aspergillus fumigatus* isolates, the occurrence of breakthrough IA, and the advent of new long-lasting echinocandins (rezafungin) or other β-1,3-glucan synthase inhibitors (ibrexafungerp).

KEYWORDS *Aspergillus*, anidulafungin, calcineurin, caspofungin, heat shock protein 90, ibrexafungerp, micafungin, paradoxical effect, rezafungin

Molds of the genus *Aspergillus* (particularly, *Aspergillus fumigatus*) are the causal agents of invasive aspergillosis (IA), a life-threatening infection affecting immunocompromised hosts, such as hematological cancer or transplant patients. The current antifungal armamentarium for the treatment of IA is limited to three antifungal drug classes. Fungicidal drugs, such as the triazoles (e.g., voriconazole, posaconazole, or isavuconazole) and the polyenes (amphotericin B formulations) represent the first-choice treatments, whereas the fungistatic echinocandins (caspofungin, anidulafungin, and micafungin) represent an alternative and are only marginally used as monotherapy (1, 2). However, use of echinocandins is gaining interest because of the emergence of acquired azole resistance in *A. fumigatus* isolates and the limitations related to drug interactions and/or toxicity with azoles and amphotericin B. The aim of this review is to discuss the role of the echinocandins in the treatment of IA, from the mechanistic point of view of drug-pathogen-host interactions to clinical application and perspectives.

ECHINOCANDINS AGAINST *ASPERGILLUS*

Mechanisms of action. The echinocandin drugs are lipopeptides derived from fungal secondary metabolites. This antifungal class currently consists of three commer-

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cially available drugs, i.e., caspofungin, micafungin, and anidulafungin; and a novel molecule with a prolonged half-life, rezafungin (CD101), is currently in phase 3 evaluation. All echinocandins are available for parenteral (intravenous) administration only. Their antifungal activity relies on inhibition of the biosynthesis of β-1,3-glucan, one of the major polysaccharides of the cell wall in ascomycetous fungi, by targeting β-1,3-glucan synthase (encoded by *fks1*) in a noncompetitive way (3). β-1,3-Glucan is an important structural component of the cell wall, which plays an essential role in protection from the environment, containment of osmotic pressure, morphogenesis of hyphae, and invasive properties in host tissues (4). In addition to its structural role, β-1,3-glucan is an important trigger of the innate immune system, which is recognized by the Dectin-1 receptor at the surface of host immune cells (5). The concept that the antifungal effect of echinocandins may also result from an immunopharmacological effect emerged recently. Lamaris et al. (6) showed that caspofungin exposure was associated with a concentration-dependent increase in β-1,3-glucan exposure in the cell wall of *A. fumigatus* isolates. This effect peaked at a caspofungin concentration of 0.06 μg/ml, with a subsequent decline in β-1,3-glucan exposure at higher concentrations. Preexposure of *A. fumigatus* isolates to caspofungin 0.06 μg/ml also resulted in increased hyphal damage induced by polymorphonuclear neutrophils (PMNs) *in vitro* and increased expression of Dectin-1 by PMNs, which supports the role of echinocandins in triggering the host's immune response against the mold (6). Moretti et al. (7) observed different host response patterns in terms of PMN recruitment and cytokine production in experimental models of IA with escalating doses of caspofungin. Experiments with knockout mice for different innate immune receptors suggested modulatory roles of Dectin-1, TLR-2, TLR-4, and TLR-9 on the net activity of caspofungin.

Whereas echinocandins are fungicidal against the most relevant pathogenic yeasts, such as *Candida* spp., they are fungistatic against *Aspergillus* spp. and some other pathogenic filamentous fungi. *In vitro*, echinocandins induce a hyphal growth arrest with turgescence and blunting of hyperbranched hyphae (Fig. 1A). This effect is usually observed at low concentrations ($<$0.03 μg/ml) for most *Aspergillus* spp., thus defining a minimal effective concentration (MEC), instead of an actual MIC.

Tolerance. The ability of the fungus to maintain residual growth above the threshold of inhibition of the drug is referred to as tolerance. This is an epigenetic phenomenon, which, contrary to resistance, is not the result of acquired mutations but of mechanisms of stress response. Another expression of the ability of *Aspergillus* spp. to generate compensatory mechanisms of adaptation to the stress induced by echinocandins is the paradoxical effect (PE) (8). This phenomenon refers to decreased activity of the drug and recuperation of fungal growth at increasing concentrations above a certain threshold (Fig. 1B). It is comparable to the Eagle effect that was initially described for antibacterial drugs. The PE was first observed in 1988 in *Candida* spp. yeasts with the experimental echinocandin drug cilofungin and described in more detail by Stevens et al. (9, 10) with caspofungin. Subsequently, the phenomenon was reported for different *Aspergillus* species (11, 12).

The PE results from the activation of intracellular signaling pathways, which leads to cell wall remodeling with increases in the chitin content to compensate for the loss of β-1,3-glucan. It is species, strain, and drug specific (13). A PE can be observed in ~60% to 80% of *A. fumigatus* clinical isolates, occurring mainly with caspofungin, whereas this phenotype is usually absent with micafungin and anidulafungin or occurs only at higher concentrations (11, 12, 14). The PE has also been reported among isolates of *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus niger* (11, 15, 16).

The mechanisms behind this phenomenon were first studied in yeasts, revealing roles for protein kinase C, the high osmolarity glycerol response, and the calcineurin pathway (17–19). In *A. fumigatus* isolates, the PE can be suppressed by targeting different steps of the calcineurin pathway (14). The initial trigger of the PE consists of the entry of calcium (Ca^{2+}) and increase in intracellular Ca^{2+}, which binds to calmodulin and activates calcineurin by phosphorylation at its serine-proline-rich region

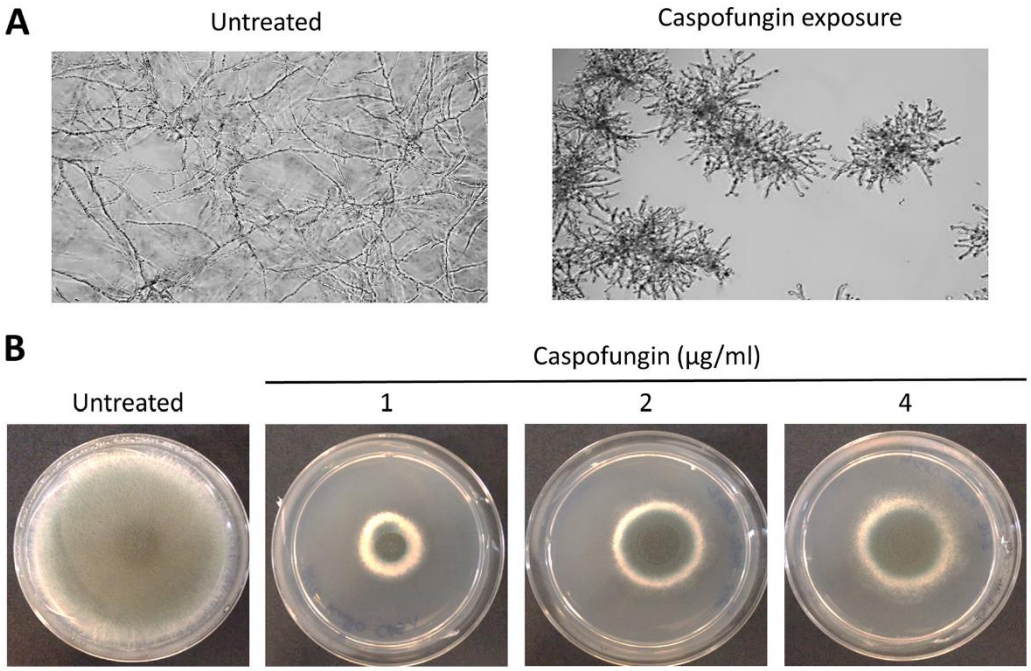

FIG 1 Tolerance of *Aspergillus fumigatus* to caspofungin. (A) Microscopic observation of *A. fumigatus* isolates after 24 h growth at 37°C in liquid RPMI 1640 medium in the absence of drug and in the presence of caspofungin 1 μg/ml. (B) Visualization of the paradoxical effect of caspofungin on *A. fumigatus* isolates. Pictures were taken after 5 days of growth at 37°C on glucose minimal medium agar plates supplemented with caspofungin at increasing concentrations (0, 1, 2, and 4 μg/ml).

concomitantly with increased expression of calmodulin and calcineurin (20, 21). Ca^{2+} deprivation, which can be achieved by Ca^{2+} chelators (BAPTA) or Ca^{2+} channel blockers (verapamil), results in abolition of the PE in *A. fumigatus* isolates (21). Interestingly, caspofungin exposure at PE concentrations resulted in a more important increase in cytosolic Ca^{2+} compared to that with micafungin, which does not induce PE, illustrating the drug specificity of the PE (21).

Activated calcineurin then dephosphorylates the transcription factor CrzA that moves to the nucleus and binds to specific promoter regions (calcineurin-dependent reporter elements) of the chitin synthase-encoding genes (*chsA*, *chsC*, *chsG*, and *csmB*) (22). Both calcineurin and CrzA are necessary to increase expression of chitin synthases and chitin content of the cell wall after caspofungin exposure (14, 22, 23). The *cnaA* and *crzA* deletion mutants also exhibit decreased β-1,3-glucan in the cell wall and lack the PE in response to caspofungin (23).

Heat shock proteins 90 (Hsp90) and 70 (Hsp70) are essential molecular chaperones that are supposed to control the calcineurin pathway in this response. A certain level of Hsp90 is required to generate the PE, which can be abolished by substitution of the native *hsp90* promoter, by Hsp90-inhibitory drugs (geldanamycin), or by compromising Hsp90 function with acetylation-mimetic mutations (K27A) or lysine deacetylase inhibitors (trichostatin A) (24, 25). Affecting the interaction between the chaperones Hsp90 and Hsp70 by mutation of the Hsp70 EELD C-terminal domain also results in suppression of the PE (26). Deletion of the Hsp90-Hsp70 organizing protein (Hop, corresponding to StiA in *A. fumigatus*) results in hypersensitivity to caspofungin (26). Thus, Hsp90 and Hsp70 seem to act in concert to control the calcineurin pathway in caspofungin stress response. However, there may be other downstream effectors in the complex network of Hsp90. Very recently, the mitochondrial respiratory chain was shown to play a role in the PE, and activation of mitochondria in response to caspofungin was dependent on Hsp90 (27).

Analyses of the intracellular trafficking of the different actors of the Hsp90-calcineurin pathway showed that calcineurin and calmodulin localized at the hyphal tips and septa and that Hsp90 moves from the cytosol to the cell wall and sites of septa formation in the presence of caspofungin (27–30). Physical interaction between Hsp90 and calcineurin has been demonstrated in yeasts (31). However, the network of interactions of these stress proteins at the sites of cell wall regeneration remains to be elucidated.

In addition to the calcineurin pathway, a role of the cell wall integrity (CWI) pathway was recently highlighted. Deletions of the CWI MAPK gene *mpkA* and its downstream transcription factor *rlmA* resulted in loss of the PE (22). However, this pathway does not seem to play a role in PE via overexpression of chitin synthases. On the contrary, *mpkA* had a repressive effect on these genes but was also shown to positively impact the expression of other cell wall components, such as β-1,3-glucan or α-1,3-glucans (22, 32). Indeed, Loiko and Wagener (33) demonstrated that the key adaptive mechanism in PE does not seem to be related to the increased expression of chitin synthases but rather to the recovery of β-1,3-glucan synthase activity. Synthesis of β-1,3-glucan takes place at the cell membrane by the β-1,3-glucan synthase complex, consisting of a catalytic subunit, Fks1, and a regulatory subunit, RhoI. The crucial role of this complex in the PE was also supported by a recent study showing distinct localization patterns at low (non-PE) and high (PE) caspofungin concentrations (34). Under caspofungin exposure, Fks1 moves from the hyphal tips to vacuoles. However, continuous exposure to high caspofungin concentrations (4 μg/ml) will induce relocalization of Fks1 to the tips along with the phenotypic appearance of PE, which does not occur at lower concentrations. RhoI remains at the hyphal tips, where it is essential for Fks1 activation and the PE. Indeed, farnesol, which mislocalizes RhoI, abolishes the PE in *A. fumigatus* isolates (34).

Taken together, these results suggest that tolerance to caspofungin in *A. fumigatus* isolates represents a complex and dynamic process occurring in two phases. Whereas calcineurin-dependent overexpression of chitin synthase seems to be essential for initial adaptation and survival in response to caspofungin stress, the PE, representing a delayed adaptive phenomenon, relies on the restoration of β-1,3-glucan synthase activity (Fig. 2).

The clinical relevance of the PE is unclear. Note that the first description of this phenomenon in *Aspergillus* derived from *in vivo* observations in animal models (35–37). In a murine model of IA with escalating doses of caspofungin, Wiederhold et al. (37) initially observed a dose-dependent effect, with optimal efficacy on the reduction of pulmonary fungal burden with doses of 1 mg/kg but a significant loss of efficacy at 4 mg/kg. Interestingly, this transitional margin between the optimal and decreased efficacy observed *in vitro* and in murine models corresponded to the clinical range of therapeutic doses and trough plasma concentrations of caspofungin (38). However, the paradoxical increase in fungal burden observed *in vivo* does not necessarily correlate with the *in vitro* paradoxical growth. Moretti et al. (7) observed that the proinflammatory effect of caspofungin at higher doses was still present with *Aspergillus* strains lacking the *in vitro* PE. This study suggests that the expression of different pattern recognition receptors at the surface of immune cells varies according to different caspofungin concentrations and influences the *in vivo* activity of caspofungin, with Dectin-1, TLR-2, and TLR-9 playing roles in the increased fungal burden and proinflammatory effect at higher doses (7).

Acquired resistance. Besides tolerance, which is an inherent ability of wild-type *A. fumigatus* isolates to adapt to echinocandin stress, resistance to echinocandins can be acquired by mutations in the β-1,3-glucan synthase-encoding gene (*fks1*). Although this mechanism of resistance has been well described in *Candida* spp. with specific mutations occurring in known hot spot regions of *fks1* (39), it seems to be uncommon in *Aspergillus* spp. The potential of *A. fumigatus* to develop such resistance has been demonstrated by the generation of laboratory strains with an S678Y or S678P mutation in *fks1* (corresponding to the S645 site of *Candida albicans*) associated with phenotypic

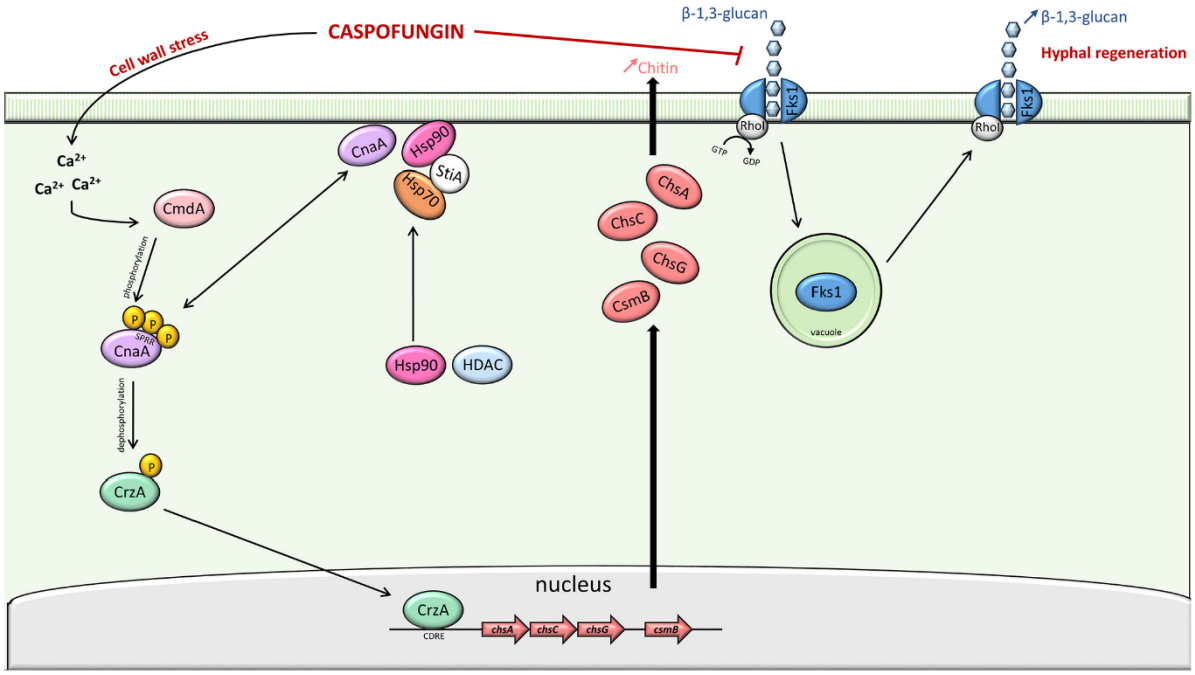

FIG 2 Schematic representation of the caspofungin paradoxical effect in *Aspergillus fumigatus*. The cell wall stress induced by caspofungin results in increased intracellular calcium (Ca^{2+}). Calmodulin (CmdA) binds Ca^{2+} and activates the calcineurin α-catalytic subunit (CnaA) by phosphorylation at the serine-proline-rich region. CnaA dephosphorylates transcription factor CrzA, which moves to the nucleus and binds to specific promoter motifs (calcineurin-dependent reporter elements [CDRE]) to induce expression of the chitin synthase genes (*chsA*, *chsC*, *chsG*, and *csmB*). On caspofungin exposure, the heat shock protein 90 (Hsp90) shifts from the cytosol to the cell wall, where it possibly interacts directly with calcineurin or other client proteins. Hsp90 function relies on its interaction with Hsp70 and the Hsp90-Hsp70 organizing protein StiA. Histone deacetylases (HDAC) are also important for Hsp90 function. During the early phase of caspofungin exposure, the β-1,3-glucan synthase (FksA) in complex with the GTPase RhoI, is inhibited by caspofungin and moves into vacuoles. After prolonged exposure at high caspofungin concentration, FksA relocalizes to the cell wall and recovers its β-1,3-glucan synthesis activity.

resistance to all three echinocandins (40, 41). To date, only one clinical strain harboring an *fks1* mutation (F675S) has been identified in a patient with chronic pulmonary aspergillosis with substantially elevated echinocandin MICs compared with the wild type after prolonged micafungin therapy (42). The loss of fitness resulting from these *fks1* mutations may be the reason it rarely occurs naturally in *Aspergillus* spp. (43).

ECHINOCANDINS IN CLINICAL PRACTICE

Susceptibility testing and interpretation. Testing of *Aspergillus* spp. susceptibility to echinocandins is not routinely recommended and is actually of little utility for clinical management. Because of the fungistatic activity of echinocandins, the threshold of activity is expressed as an MEC. Echinocandins are active *in vitro* against most *Aspergillus* spp. (44, 45). The activity of echinocandins is conserved against azole-resistant *A. fumigatus* isolates, including those harboring *cyp51A* mutations (46). Of the three echinocandins, micafungin and anidulafungin are the most active *in vitro*, with MECs usually one or two dilutions lower than that of caspofungin (44). The novel long-acting echinocandin rezafungin (CD101) demonstrated acceptable *in vitro* activity against *Aspergillus* spp., including azole-resistant *A. fumigatus* and cryptic species (47, 48).

Most studies of large collections of *Aspergillus* isolates showed a narrow range of MEC distribution for echinocandins (44, 49, 50). However, the epidemiological cutoff values (ECVs) obtained in these studies differ. Whereas Pfaller et al. (44, 49) reported an MEC of $\leq$0.06 μg/ml for $>$99% of isolates for all three echinocandins, higher caspofungin ECVs were reported in another study (0.25 to 1 μg/ml) (50). Reader-dependent variability in the appreciation of MEC cutoff may explain these variations, although adhesion to the strict definition of MEC (i.e., transition to compact rounded colonies)

was found to have good reproducibility (51). Because of the lack of correlation between MEC and outcomes, both the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) abstain from any recommendation of interpretation for echinocandins against *Aspergillus* spp. (52, 53). The occurrence of mutations in hot spot regions of the *fks1* gene is the only known mechanism of echinocandin resistance in *Aspergillus* spp. and appears to be a rare event (40–42). Substantially higher MECs (at least 20-fold) are expected in this setting. In general, antifungal susceptibility to echinocandins should not be routinely tested because of the lack of an established correlation between MECs and clinical response.

Efficacy of echinocandins in animal models of IA. Several animal models of IA demonstrated the efficacy of echinocandins. Caspofungin monotherapy at different dosages (1 and 2.5 mg/kg daily) was associated with significantly improved survival and significant reduction of fungal burden in most tissues compared with untreated groups in a guinea pig model of IA (54). Micafungin showed comparable results in murine models, with a 50% effective dose of 0.25 to 0.5 mg/kg (55, 56). Micafungin seemed to exhibit a dose-dependent effect on survival, whereas caspofungin was associated with higher fungal burden in tissues at higher doses than at lower doses, without significant impact on survival (37, 54, 55), which supports some role of the PE with the latter drug. In comparative studies with other antifungal classes, the success rate of echinocandins for the treatment of pulmonary IA tended to be lower than that of voriconazole but comparable to that of amphotericin B (54, 55). In animal models, echinocandins achieved good penetration in most tissues, with the exception of the brain and eye because of their large size and amphipathic properties (57).

Echinocandins for the treatment of IA. Randomized clinical trials comparing the efficacy of echinocandins versus other antifungal drugs for the treatment of IA are lacking. One trial compared the efficacy of caspofungin versus amphotericin B for the empirical antifungal treatment of persistent neutropenic fever (58). The success rates using a composite endpoint were similar. Caspofungin exhibited higher success rates among patients with fungal infections at baseline, but the small number of IA cases (12 in both arms) did not allow for drawing conclusions. Three prospective noncomparative phase 2 studies assessed the efficacy of caspofungin as first-line treatment of proven or probable IA in patients with hematological malignancies and reported success rates (complete or partial response at the end of therapy) of 30% to 55% (59–61). The other studies reporting data about the efficacy of caspofungin for the treatment of IA consisted mainly of retrospective analyses or prospective observational registries. According to a recent review, the overall success rate (complete or partial response after pooling of all cases from individual studies) was 54% and 47% for caspofungin as first-line or second-line/salvage therapy, respectively (62). A wide range of success rates was observed between studies (27% to 92% and 28% to 71% for first- and second-line treatment, respectively) (59). These results may be influenced by multiple factors, including the type of population and underlying diseases, the diagnostic work-up procedure and timing of diagnosis, the timing of the assessment of therapeutic response, and the timing of the switch from first-line antifungal therapy to second-line caspofungin. Most important, few of these studies provided a direct comparison with other antifungals. Only one study reported a significantly higher IA-associated mortality rate for caspofungin than for voriconazole (63). The efficacy of micafungin was also assessed in a few studies with a limited number of cases and comparable results (30% to 50% success rate) (64, 65). Data about anidulafungin monotherapy of IA are scarce. Although echinocandins may be recommended as alternative therapy of pulmonary IA, they should not be used for cerebral aspergillosis because of their poor penetration of the hematoencephalic barrier (1, 2). Data about their efficacy for other extrapulmonary IAs are limited.

Table 1 shows several situations for which echinocandins may be considered first-line or salvage therapy of IA.

TABLE 1 Possible role and indications for echinocandins in the treatment of invasive aspergillosis

Indication	Aim[a]	Situation	Level of evidence (Ref.)
First-line treatment (monotherapy)	To treat IA when no alternative regimen (or potential risks outweighing benefits for other regimens)	Relative contraindications to azoles (underlying liver disease, drug-drug interactions, prolonged QT interval); relative contraindications to AMB (underlying kidney disease, nephrotoxic comedications)	Noncomparative prospective or retrospective studies (overall success rate, 30–90%) (62)
Second-line treatment (monotherapy)	To treat IA when first-line antifungals have failed or need to be discontinued	Toxicity of triazoles (hepatic test disturbances, visual/neurological side effects); toxicity of AMB (acute renal failure); failure of previous antifungal regimens	Noncomparative prospective or retrospective studies (overall success rate, 30–70%) (62)
In combination with triazoles or AMB	To obtain synergistic interactions (triazoles, AMB)	Severe and/or disseminated IA, galactomannan-positive IA; in case of failure of previous regimen or breakthrough IA; for IA due to azole-resistant *A. fumigatus*	One randomized controlled trial (trends, benefit limited to subgroup analyses) (81); expert opinion; murine models (75, 77)
	To palliate potential PK/PD defect until first-line drug achieves appropriate serum level (triazoles)	In severe and/or disseminated IA	Expert opinion
	To palliate potential inefficacy of first-line drug (triazoles)	For empirical treatment, if suspicion or high local prevalence of azole-resistant *A. fumigatus*; breakthrough IA	Expert opinion (82)
	To obtain synergistic interactions on biofilms (triazoles, AMB)	For *Aspergillus* endocarditis or osteomyelitis with presence of prosthetic material	*In vitro* studies (79)

[a]AMB, amphotericin B; IA, invasive aspergillosis; PK/PD, pharmacokinetic/pharmacodynamic.

Echinocandins in combination therapy of IA. *In vitro* and *in vivo* interactions of echinocandins with other antifungal drugs, particularly amphotericin B and triazoles, against *Aspergillus* spp. have shown various results. Importantly, the type of growth medium was found to influence the *in vitro* interactions and serum attenuated the synergistic effect, suggesting that these observations may not necessarily have the same significance *in vivo* (66). Moreover, differences in drug exposure were found to influence these interactions (67). Both synergistic and indifferent interactions have been described for combinations of echinocandins and amphotericin B *in vitro* and in murine models of IA, which were strain dependent and possibly drug dependent (66, 68–70). Similar observations were reported for the interactions between echinocandins and triazoles with the presence of synergism for most but not all isolates (54, 66, 71, 72). The combination of anidulafungin and voriconazole also demonstrated synergism against azole-resistant *A. fumigatus* isolates (73, 74). However, the synergism was decreased among isolates harboring mutations in the tandem repeats of *cyp51A* (i.e., the most frequently observed) compared with wild-type isolates or those harboring other types of mutations (74). These observations are supported by a murine model of pulmonary IA in which the combination of voriconazole and anidulafungin was synergistic against an azole-susceptible *A. fumigatus* isolate but only additive against the azole-resistant isolate harboring the TR_{34}/L98H mutation, with higher doses required for the latter (75). The combination of posaconazole and caspofungin was synergistic in most cases (76). Interestingly, the synergism was also present against azole-resistant *A. fumigatus* isolates and was more pronounced for those harboring the tandem repeat of the promoter region or the M220 mutation in *cyp51A* compared with other mutations of resistance. This positive interaction was confirmed *in vivo* in a murine model of pulmonary IA demonstrating improved survival among mice infected with azole-resistant *A. fumigatus* strains and treated with posaconazole plus caspofungin compared with monotherapies (77). Interaction of echinocandins with the new mold-active

triazole isavuconazole against *Aspergillus* spp. was described as indifferent for most cases in one study (78).

One study suggested that both voriconazole and amphotericin B may interact synergistically with caspofungin on *Aspergillus* biofilms (79).

Assessment of the efficacy of antifungal drug combinations in clinical practice is difficult due to multiple potential confounding factors. The combination of liposomal amphotericin B (standard dosage) and caspofungin was found to be superior to liposomal amphotericin B alone (high dosage) in a small pilot study (80). A large randomized double-blind placebo-controlled trial failed to demonstrate the superiority of the combination therapy of voriconazole and anidulafungin over voriconazole alone for the treatment of IA for the primary outcome of 6-week mortality, despite a trend in favor of the combination (81). However, a *post hoc* analysis in the subgroup of patients with IA diagnosis relying on positive galactomannan showed a significant benefit of the combination. The reason the combination therapy demonstrated superiority over voriconazole monotherapy only in the restricted population of galactomannan-positive IA is unclear. One possible explanation proposed by the authors is that this subgroup represents a more homogeneous population with fewer potential confounding factors for the outcome analysis.

Clinical experience is lacking to assess the efficacy of the triazole-echinocandin combination for the treatment of IA due to azole-resistant *A. fumigatus*, but expert opinions support the use of this combination as an alternative to liposomal amphotericin B monotherapy for the empirical treatment of IA in areas with a high prevalence of azole resistance (i.e., >10%) or in cases of documented azole resistance (82).

Echinocandins may also be combined initially with triazoles with the goal to rapidly achieve therapeutic levels in severe IA cases, considering that steady state for azoles is only reached after 5 to 7 days of therapy. Albeit theoretical and not supported by evidence, this approach is recommended by some experts.

Overall, evidence for a clear benefit of the combination of echinocandins with other antifungal drugs is limited but sufficient to recommend its use in particular situations, such as severe and/or disseminated IA, salvage therapy, or treatment of suspected or documented azole-resistant *A. fumigatus* (Table 1).

On the laboratory side, several compounds with modest antifungal activity *per se* demonstrated their ability to potentiate the activity of echinocandins, particularly caspofungin. Targeting the Hsp90-calcineurin axis can be achieved by different methods and results in hypersensitivity to caspofungin and abolition of the PE at higher concentrations. However, because this intracellular pathway is highly conserved in eukaryotes, use of these compounds in humans is limited by a lack of specificity and toxicity. Calcineurin inhibitors, such as tacrolimus (FK506) or cyclosporine, are strong immunosuppressive drugs that favor the occurrence and progression of IA. The discovery of a key serine-proline-rich region specific to the fungal calcineurin and essential for its function presents perspectives for the development of novel fungal-specific calcineurin inhibitors (83). Targeting Hsp90 is a difficult challenge because of the highly conserved structure of this chaperone among eukaryotes. Several Hsp90 inhibitors have passed the different stages of clinical development for an application in cancer therapy (84). However, the antifungal activity of these compounds, e.g., geldanamycin, is limited, and their positive interaction with echinocandins is observed only at toxic concentrations (85). A more promising approach consists of targeting the histone deacetylases (HDACs) to indirectly cripple Hsp90 function. Trichostatin A demonstrated *in vitro* synergistic interaction with caspofungin against *Aspergillus* spp. at concentrations that were well tolerated in mice (25, 85, 86). Novel HDAC inhibitors that are more stable are currently being contemplated for anticancer therapy, and their antifungal activity should be investigated. Finally, there may be an interest in investigating the role of some Ca^{2+} channel inhibitors used for the treatment of hypertension (e.g., verapamil), which can potentiate caspofungin activity and inhibit the PE (21).

Echinocandins for IA prophylaxis. Because of the hepatotoxicity of azole compounds and their multiple drug interactions, echinocandins are increasingly considered for use in prophylaxis of invasive fungal infections. However, data to demonstrate their efficacy in this setting are lacking. Breakthrough invasive fungal infections (consisting mainly of IA) are usually observed at a frequency of 5% to 7% during echinocandin prophylaxis, but a wide range of occurrence (1% to 28%) has been reported in the literature (87). This is usually higher than the rate reported during mold-active azole prophylaxis. Indeed, one study found that echinocandin prophylaxis was an independent risk factor of breakthrough invasive fungal infections compared with voriconazole or posaconazole prophylaxis during chemotherapy of acute leukemia (88). The role of echinocandin prophylaxis for the prevention of IA seems to be limited to situations in which a mold-active azole is contraindicated.

Other β-1,3-glucan synthase inhibitors. Ibrexafungerp (previously referred to as SCY-078 or MK-3118) is a semisynthetic β-1,3-glucan synthase inhibitor derived from the natural product enfumafungin (isolated from the fungus *Hormomema* spp.) and belonging to the triterpenoids (structurally different from the echinocandins). Ibrexafungerp has the advantage of bioavailability by the oral route and an antifungal spectrum similar to that of echinocandins with fungistatic activity against *Aspergillus* spp. *In vitro* testing showed MECs ranging from 0.06 to 0.25 μg/ml for the most relevant pathogenic *Aspergillus* spp., including azole-resistant *A. fumigatus* (89, 90). Because ibrexafungerp targets a different region of the β-glucan synthase than echinocandins, its activity is not affected by the common hot spot mutations described in *Candida* spp. and *A. fumigatus* isolates (91, 92). Ibrexafungerp demonstrated synergistic *in vitro* activity in combination with mold-active azoles (voriconazole, isavuconazole) or amphotericin B against azole-susceptible *A. fumigatus* (90), and a phase 2 clinical trial that will test the efficacy of the ibrexafungerp-voriconazole combination versus voriconazole monotherapy for the treatment of IA is forthcoming.

CONCLUSIONS AND PERSPECTIVES

Of the three antifungal drug classes, echinocandins are the least active against *Aspergillus* spp. However, echinocandins also have incontestable advantages, such as their quasi-absence of related toxicity and lack of drug-drug interactions, which represent frequent limitations for triazoles and amphotericin B. A new niche for echinocandins may arise from increasing reports of azole-resistant *Aspergillus* spp. This perspective should, however, be moderated with the warning that increased echinocandin use has also been associated with changes in the epidemiology of candidemia and emergence of echinocandin resistance among *C. albicans* and *C. glabrata* isolates (93, 94).

Recent advances in drug development increase the spectrum of this antifungal drug class, with the advent of a long-lasting echinocandin (rezafungin). Moreover, novel types of β-glucan synthase inhibitors are under development, such as ibrexafungerp, a compound structurally different from echinocandins but with comparable activity against *Aspergillus* spp. and availability for oral administration (47, 89). With the frequent need of prolonged antifungal therapy for IA, these new formulations may represent an alternative in the future. The widespread use of mold-active azole prophylaxis also raises the challenge of the therapeutic management of breakthrough IA, for which echinocandins may be considered in combination with other antifungals. Echinocandins display positive interactions with the two other existing antifungal drug classes and some experimental drugs. Moreover, their antifungal effect is not limited to their fungistatic activity against *Aspergillus* hyphae but also involves an important immunomodulatory effect on the host immune response, which plays a determinant role in the outcome of IA. Therefore, the story of echinocandins illustrates the complex and dynamic interactions between the antifungal drug, the fungus, and the host immunity during the course of invasive mycosis, which is a fascinating topic. Further studies should focus on better defining the role of the different echinocandins when used alone or in combination in the treatment of IA. An alarming observation is that

despite the extended spectrum of marketed antifungal drugs in the last decade, we still have only three drug classes. Moreover, resistance in *Aspergillus* isolates is usually observed across all drugs within a class. Therefore, drug combinations may become a cornerstone to combat IA with more-resistant *Aspergillus* spp. in the future, and echinocandins appear as the optimal candidates to act synergistically with other antifungals.

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ANNEX 2

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MECHANISMS OF RESISTANCE

Link between Heat Shock Protein 90 and the Mitochondrial Respiratory Chain in the Caspofungin Stress Response of *Aspergillus fumigatus*

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ABSTRACT *Aspergillus fumigatus* is an opportunistic mold responsible for invasive aspergillosis. Triazoles (e.g., voriconazole) represent the first-line treatment, but emerging resistance is of concern. The echinocandin drug caspofungin is used as second-line treatment but has limited efficacy. The heat shock protein 90 (Hsp90) orchestrates the caspofungin stress response and is the trigger of an adaptive phenomenon called the paradoxical effect (growth recovery at increasing caspofungin concentrations). The aim of this study was to elucidate the Hsp90-dependent mechanisms of the caspofungin stress response. Transcriptomic profiles of the wild-type *A. fumigatus* strain (KU80) were compared to those of a mutant strain with substitution of the native *hsp90* promoter by the *thiA* promoter (*pthiA*-hsp90), which lacks the caspofungin paradoxical effect. Caspofungin induced expression of the genes of the mitochondrial respiratory chain (MRC), in particular, NADH-ubiquinone oxidoreductases (complex I), in KU80 but not in the *pthiA*-hsp90 mutant. The caspofungin paradoxical effect could be abolished by rotenone (MRC complex I inhibitor) in KU80, supporting the role of MRC in the caspofungin stress response. Fluorescent staining of active mitochondria and measurement of oxygen consumption and ATP production confirmed the activation of the MRC in KU80 in response to caspofungin, but this activity was impaired in the *pthiA*-hsp90 mutant. Using a bioluminescent reporter for the measurement of intracellular calcium, we demonstrated that inhibition of Hsp90 by geldanamycin or MRC complex I by rotenone prevented the increase in intracellular calcium shown to be essential for the caspofungin paradoxical effect. In conclusion, our data support a role of the MRC in the caspofungin stress response which is dependent on Hsp90.

KEYWORDS NADH-ubiquinone oxidoreductases, echinocandins, invasive aspergillosis, mitochondria, paradoxical effect, rotenone, transcriptomics

Aspergillus fumigatus is a ubiquitous mold which can cause a broad spectrum of diseases, including the devastating invasive aspergillosis (IA), in patients with impaired immunity, such as transplant recipients or cancer patients (1, 2). The treatment of IA remains a challenge, as only three drug classes are available (azoles, polyenes, and echinocandins), and emergence of resistance to azoles, the first-line treatment, is increasingly reported (3). Echinocandins, such as caspofungin, micafungin, and anidulafungin, can be used as second-line therapy for IA or in combination with voriconazole for refractory cases or when azole resistance is suspected (4–6). Echinocandins inhibit the synthesis of (1-3)-β-D-glucan, a major cell wall component. However, their *in vitro* activity against *A. fumigatus* is limited and only fungistatic with persistent growth above the MIC threshold. Moreover, a paradoxical effect, defined as a return to growth at increasing concentrations, can be observed with caspofungin, which may

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have some clinical relevance (7). This phenomenon of tolerance indicates the existence of compensatory mechanisms of the cell wall which are mediated by the heat shock protein 90 (Hsp90) and the calcium-calcineurin pathway (7, 8). Hsp90 is a molecular chaperone playing a key role in the mechanisms of stress adaptation, including the development of antifungal drug resistance or tolerance in *A. fumigatus* and other pathogenic fungi (9, 10). The essential role of Hsp90 in the caspofungin stress response of *A. fumigatus* has been previously highlighted (8, 11). However, Hsp90-dependent pathways in this response remain partly unknown. We identified a yet-unrevealed role of the mitochondrial respiratory chain (MRC) in the caspofungin stress response, which was dependent on Hsp90.

RESULTS

Caspofungin stress results in overexpression of genes of the MRC, which is dependent on Hsp90. Our first objective was to determine which genes are involved in the caspofungin stress response in the wild-type *A. fumigatus* isolate KU80. In order to identify which of them are dependent on Hsp90, we used the *pthiA*-hsp90 mutant with substitution of the *hsp90* promoter by the *thiA* promoter (8). Exposure to thiamine results in *hsp90* repression and complete growth inhibition. However, in the absence of thiamine, this strain has sufficient Hsp90 levels to maintain normal basal growth, but the lack of the native *hsp90* promoter does not allow the achievement of appropriate Hsp90 levels for stress adaptation when exposed to caspofungin (8). As a result, the *pthiA*-hsp90 mutant strain has no basal growth defect but cannot generate tolerance and a paradoxical effect to caspofungin (Fig. 2).

Transcriptomic analyses (RNA sequencing [RNA-seq]) were performed in three biological replicates of whole-RNA extracts of KU80 and the *pthiA*-hsp90 mutant (without the addition of thiamine) under basal conditions and after 2 h of exposure to caspofungin at 2 μg/ml (i.e., the concentration required to induce the paradoxical effect of caspofungin). As previously demonstrated (8), the expression of *hsp90* was significantly decreased (3.1-fold, $P = 0.04$) in the *pthiA*-hsp90 mutant compared to KU80 in the presence of caspofungin.

Genes for which a significant increase (fold change $\geq$2 and $P \leq 0.05$) was observed upon caspofungin exposure in KU80 were selected. The transcriptional response of the *pthiA*-hsp90 mutant in the absence and presence of caspofungin was analyzed for these genes (see Table S1 in the supplemental material). We found that the mitochondrion-carried genes of the mitochondrial respiratory chain (MRC) were strongly induced by caspofungin in KU80 but not in the *pthiA*-hsp90 mutant (Fig. 1A and Table S1). Although the basal expression of some of these genes was somewhat higher in the *pthiA*-hsp90 mutant than in KU80, no increase was observed upon caspofungin exposure. The MRC genes of *A. fumigatus* were identified and classified in their respective complexes (I to IV) by nBlast with other fungi (*Aspergillus oryzae* and *Neurospora crassa*). A majority of the genes exhibiting the highest induction of expression in the wild type belonged to complex I (NADH-ubiquinone oxidoreductases) (Fig. 1A).

Because MRC gene expression can be influenced by fungal growth, we have looked for possible variations in the mycelial mass between KU80 and the *pthiA*-hsp90 mutant in the presence or absence of caspofungin under the same experimental conditions (22 h growth with an additional 2 h with or without caspofungin). Our data show that there was no statistically significant difference in fungal growth between KU80 and the *pthiA*-hsp90 mutant in the presence or absence of caspofungin at the time of the analysis (Fig. 1B).

As a next step, we wanted to know if the induction of MRC gene expression in KU80 was unique to caspofungin or could result from a nonspecific effect of any fungal growth inhibitory drug using a potent antifungal drug, such as voriconazole. The transcriptomic profile of KU80 upon 2 h of voriconazole exposure was analyzed and did not show any significant increase in expression of the MRC genes compared to the untreated KU80 strain (Fig. S1).

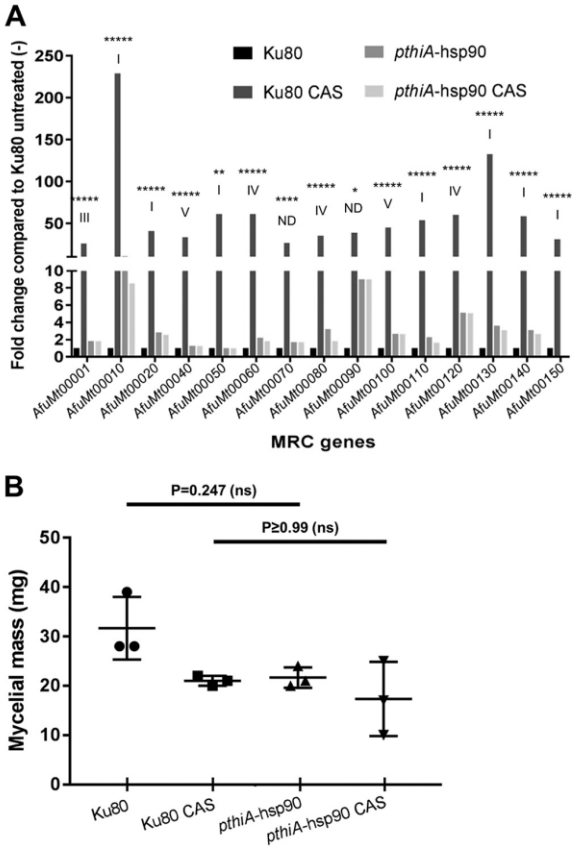

FIG 1 Transcriptomic analyses of MRC genes in *A. fumigatus* KU80 (parental strain) and the *pthiA*-hsp90 mutant under basal conditions (untreated) and after 2 h of caspofungin (CAS) exposure. (A) Gene expression in fold change compared to the reference condition (KU80, untreated). Black, KU80 untreated (KU80); dark gray, KU80 with caspofungin (KU80 CAS); gray, *pthiA*-hsp90 mutant untreated (*phtiA*-hsp90); light gray, *pthiA*-hsp90 mutant with caspofungin (*phtiA*-hsp90 CAS). The *P* values are expressed as *, ≤0.01; **, ≤0.001; ****, ≤0.00001; *****, ≤0.000001. Numbers I to V correspond to the MRC complex to which the genes were assigned according to nBlast. ND, not determined. (B) Dried mycelial mass (in milligrams) of the different strains (KU80 and *pthiA*-hsp90 mutant) under the experimental conditions of the transcriptomic analyses (24 h untreated and 22 h untreated with an additional 2 h of caspofungin exposure). Error bars represent standard deviation of the results from experiments in triplicate. ns, not significant.

We thus hypothesized that the MRC could play a unique role in the caspofungin stress response, as illustrated by the significant increase in expression of the MRC genes in KU80 upon caspofungin exposure but not upon voriconazole exposure. Furthermore, the lack of induction of MRC genes in the *pthiA*-hsp90 mutant suggests that MRC induction in the caspofungin stress response may be dependent on Hsp90 and that this effect is not a consequence of a growth defect in the *pthiA*-hsp90 mutant compared to the parental KU80 strain.

MRC complex I activation is required for the paradoxical effect of caspofungin. In order to further investigate the potential role of the MRC in the caspofungin stress response and paradoxical growth, we assessed the effect of various MRC inhibitors on KU80 growth with increasing concentrations of caspofungin. Exposition to rotenone, a MRC complex I inhibitor, resulted in a loss of paradoxical growth at increased caspofungin concentrations, an effect similar to that observed in the *pthiA*-hsp90 mutant (Fig. 2). Rotenone was also able to abolish the paradoxical effect of caspofungin in other *A. fumigatus* strains, such as the wild-type AF293 strain (data not shown). However,

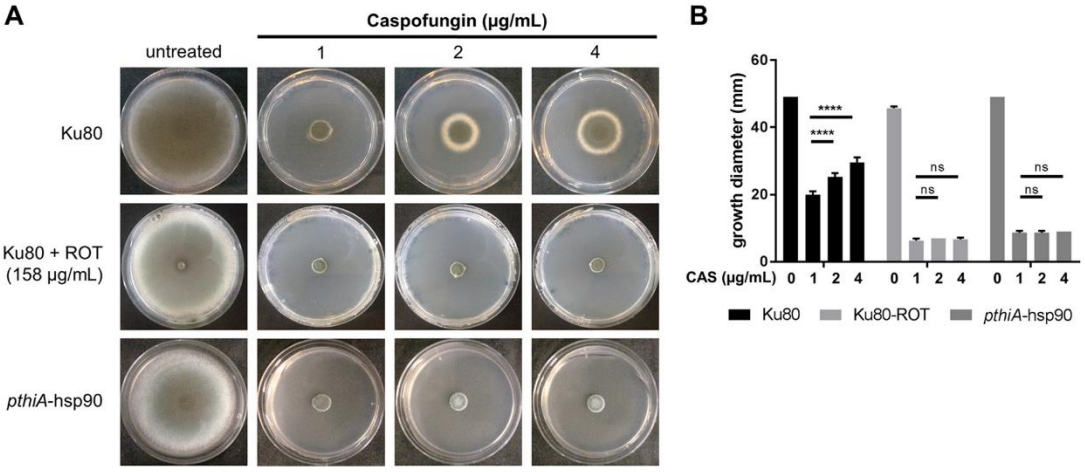

FIG 2 Effect of *hsp90* repression (*pthiA*-hsp90 mutant strain) and MRC complex I inhibition (rotenone) on the caspofungin paradoxical effect of *A. fumigatus*. (A) Pictures were taken after 5 days of growth at 37°C on glucose minimum medium (GMM) agar plates supplemented with caspofungin (CAS) at increasing gradient concentration. Rotenone (ROT) was added at a fixed concentration of 158 μg/ml. (B) Graphs represent the mean diameters of the colonies. Error bars represent standard deviations of the results from experiments in triplicate. *P* values are represented for comparisons of the diameters of the colonies exposed to caspofungin 1 μg/ml versus 2 and 4 μg/ml in order to demonstrate the paradoxical effect (significant recovery of the growth at concentrations above 1 μg/ml). ****, $P \leq 0.0001$; ns, not significant.

paradoxical growth at high caspofungin concentrations was conserved in the presence of other MRC inhibitors, such as antimycin A (complex III inhibitor), oligomycin (ATPase inhibitor), and azide (complex IV inhibitor), or under hypoxic growth conditions (Fig. S2). These results show that MRC complex I is important for the caspofungin stress response and paradoxical effect.

Mitochondrial activity is impaired in the *pthiA*-hsp90 mutant under caspofungin stress. As a next step, we attempted to demonstrate the functional effect of *hsp90* repression and downregulation of MRC genes on the activity of the mitochondria in response to caspofungin stress. Staining of mycelia with MitoTracker Deep Red FM (staining all mitochondria irrespective of their activity) did not show any difference between KU80 and the *pthiA*-hsp90 mutant (data not shown). We then used MitoTracker Red CM-H_2XRos, which fluoresces only upon oxidative activity of mitochondria. Exposure to caspofungin (2 μg/ml) for 2 h induced fluorescence in KU80 but not in the *pthiA*-hsp90 mutant (Fig. 3).

Mitochondria use oxygen to produce ATP via the MRC. In order to measure ATP production, we used a luciferase assay (CellTiter-Glo luminescent cell viability assay), which produces a luminescent signal proportional to ATP quantity. A significant increase in ATP production was observed in KU80 upon 2 h of caspofungin exposure. Although the *pthiA*-hsp90 mutant exhibited a higher basal level of ATP production, no increase was observed in the presence of caspofungin (Fig. 4). Finally, analyses with an oximeter showed that the consumption of oxygen by KU80 increased upon caspofungin exposure compared to the untreated condition. However, the *pthiA*-hsp90 mutant strain exhibited a basal defect in oxygen utilization and was unable to increase oxygen consumption under caspofungin stress (Fig. 5). This impairment of oxygen consumption was not related to a growth defect in the *pthiA*-hsp90 mutant strain, as illustrated by the comparisons of mycelial mass with the parental KU80 strain, showing no significant differences (Fig. 1B). We concluded that activation of the mitochondria leading to ATP generation in response to caspofungin was impaired in the *pthiA*-hsp90 mutant.

Both MRC and Hsp90 inhibition prevent the increase of intracellular calcium in response to caspofungin stress. An increase in intracellular calcium (Ca^{2+}) triggers the calcineurin pathway and was shown to be essential for caspofungin stress response

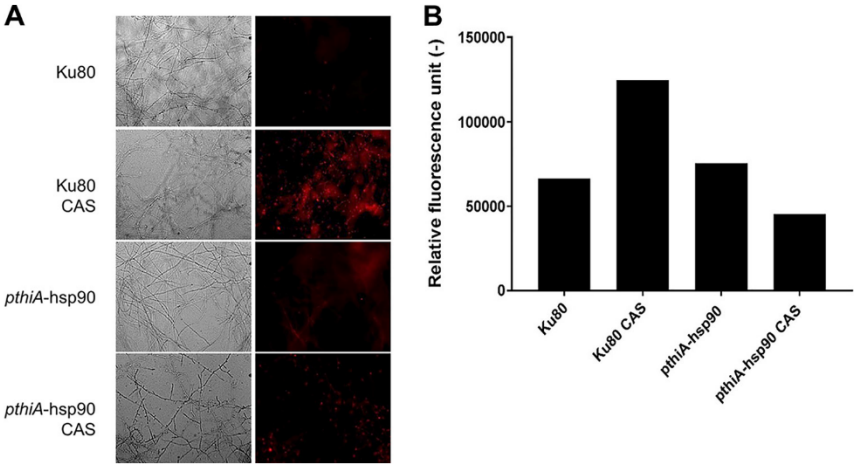

FIG 3 Visualization of active mitochondria in KU80 and *pthiA*-hsp90 mutant in the absence or presence of caspofungin (CAS). (A) Cultures performed on coverslips in GMM broth at 37°C for 24 h in the absence or presence of caspofungin (2 μg/ml after 22 h). Left, light microscopy; right, fluorescence microscopy stained with MitoTracker Red CM-H_2XRos. (B) Graphs represent fluorescence quantification for each condition, measured using the ImageJ software. Fluorescence is expressed in relative fluorescent units (RFU).

and paradoxical growth in *A. fumigatus* (12). ATP is required for the uptake of extracellular Ca^{2+} by ATP channels of the cell membrane and also for the release of Ca^{2+} stores from the endoplasmic reticulum (13). We hypothesized that Hsp90 and the MRC are essential for caspofungin stress response by generating the ATP required for the increase in cytoplasmic Ca^{2+}. For this purpose, we used a KU80 strain harboring the bioluminescent Ca^{2+} reporter aequorin ($AEQ^{\Delta akuB}$) to measure intracellular calcium (14). As previously reported (12), we observed an increase in intracellular Ca^{2+} upon caspofungin exposure (Fig. 6A). However, this response was abolished in the presence of geldanamycin (Hsp90 inhibitor) or rotenone (MRC complex I inhibitor), since no Ca^{2+} increase was observed (Fig. 6A). The peak of Ca^{2+} could also be abolished in the presence of the Ca^{2+} chelator BAPTA [1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] (Fig. 6B), which suggests that it results from an external calcium source,

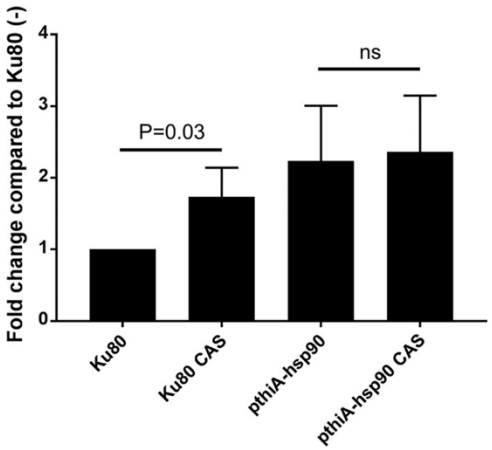

FIG 4 Measurement of ATP production by CellTiter-Glo in KU80 and *pthiA*-hsp90 mutant cell lysates after 24 h growth in GMM broth at 37°C in the absence or presence of caspofungin (CAS; 2 μg/ml added after 22 h). Bars represent means with standard deviations of the results from three biological replicates, with results expressed as fold change in luminescence compared to the untreated KU80 strain. ns, not significant.

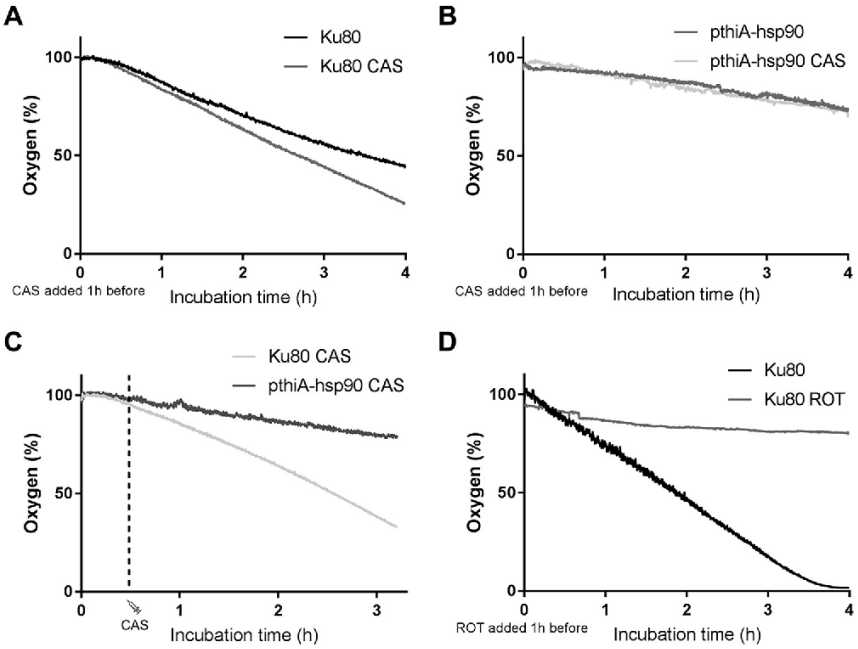

FIG 5 Oxygen measurements in KU80 and the *pthiA*-hsp90 mutant in the absence or presence of caspofungin and rotenone. Graphs represent percentage of oxygen (*y* axis) in the chambers over time (*x* axis). (A) KU80 in the absence or presence of 2 μg/ml caspofungin (CAS) added 1 h before start of measurement. (B) *pthiA*-hsp90 mutant in the absence or presence of 2 μg/ml CAS added 1 h before start of measurement. (C) KU80 and *pthiA*-hsp90 mutant with addition of 2 μg/ml CAS after 30 min (dashed line). (D) KU80 in the absence or presence of 158 μg/ml rotenone (ROT) added 1 h before the start of measurement.

as previously reported (12). These results further support the link between Hsp90 and the MRC in the caspofungin stress response, which could be essential for ATP production and extracellular Ca^{2+} uptake by ATP-dependent Ca^{2+} channels.

DISCUSSION

Echinocandins are gaining interest as salvage or combination therapy for IA since resistance to azoles is emerging. Their limited *in vitro* antifungal activity against *A. fumigatus* is the consequence of a compensatory stress response, as illustrated by persistent growth above the MIC and the so-called paradoxical effect, with a loss of efficacy at increasing concentrations. In this study, we provide further insights into the mechanisms of this tolerance phenomenon, highlighting a previously unknown role of the mitochondrial respiratory chain (MRC) which is dependent on the molecular chaperone Hsp90. Exposure of the wild-type *A. fumigatus* to caspofungin resulted in activation of the MRC, with increased oxygen consumption and ATP production. This stress response seems to be unique to caspofungin, as we did not observe an increase in the expression of MRC genes in the presence of voriconazole. Compromising Hsp90 function (by loss of the native *hsp90* promoter) resulted in a lack of activation of the MRC genes, in particular, the NADH-ubiquinone oxidoreductases (complex I). This coincided with mitochondrial dysfunction, as illustrated by an impaired use of oxygen and ATP production in the presence of caspofungin. Furthermore, inhibition of MRC complex I by rotenone resulted in effects similar to those of *hsp90* repression, with abolition of the paradoxical effect of caspofungin and the absence of an increase in intracellular calcium, which is known to be essential for the paradoxical effect (8, 12).

Mitochondria are important for many cellular processes in eukaryotes. In *A. fumigatus*, genes involved in mitochondrial dynamics and in the endoplasmic reticulum-mitochondria encounter structure (ERMES) were shown to play a role in virulence and

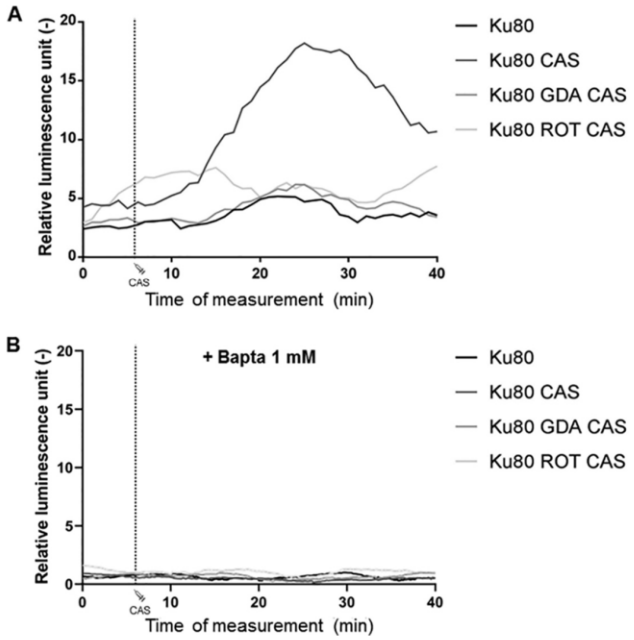

FIG 6 Intracellular calcium (Ca^{2+}) measurement using the bioluminescent reporter aequorin expressed in KU80. (A) The $AEQ^{\Delta akuB}$ strain was preincubated in the absence or in the presence of 4 μg/ml geldanamycin (GDA) or 158 μg/ml rotenone (ROT), added 1 h before start of measurement at room temperature. Caspofungin (CAS; 2 μg/ml) was injected 6 min after start of the measurement. (B) Same experiment as in panel A with the addition of BAPTA (1 mM) for 1 h at room temperature before the start of measurement. Results represent mean curves of triplicates and are expressed in relative luminescence units (RLU) over time.

antifungal resistance (15, 16). However, little is known about the role of MRC. The MRC is composed of four electron transfer complexes (I to IV) located in the inner membrane of mitochondria. The transfer of electrons generates a proton gradient and allows ATP production by an ATPase (complex V) (17). Complex I (NADH-ubiquinone oxidoreductases) plays an important role in energy conversion, and the loss of its activity results in mitochondrial dysfunction (18). In the model mold *Neurospora crassa*, complex I consists of 35 subunits, of which 7 are encoded by mitochondrial DNA (ND1, ND2, ND3, ND4, ND4L, ND5, ND6) (18). An alternative pathway involved in respiratory activity exists and consists of a single polypeptide located in the mitochondrial inner membrane that oxidizes NADPH and is not coupled to proton pumping (18). In our transcriptomic data, only the mitochondrion-carried genes of the MRC were overexpressed in the presence of caspofungin, while no significant changes were observed for the chromosomally carried genes of the MRC and the *aox* gene of the alternative pathway.

Grahl et al. demonstrated a role of mitochondrial respiration in the oxidative stress response and virulence, which was compromised after the deletion of cytochrome *c* (*cycA*, complex III) (17). However, we did not observe an increase in *cycA* expression in the presence of caspofungin in our study. Bromley et al. showed that mitochondrial complex I enzymes are involved in resistance to azoles (19). Deletion or mutation of the chromosomal gene encoding the 29.9-kDa subunit of this complex (Afu2g10600) resulted in azole resistance. The same effect was achieved by pharmacological inhibition by rotenone. While the role of MRC in echinocandin resistance of *A. fumigatus* has not been previously investigated, Chamilos et al. showed that inhibition of the MRC by antimycin A (complex III inhibitor) and benzohydroxamate (BHAM; an alternative pathway inhibitor) could increase susceptibility to caspofungin in the pathogenic yeast

Candida parapsilosis (20). In the present study, enhanced caspofungin activity against *A. fumigatus* was not observed with the addition of antimycin A or azide, only with rotenone (complex I inhibitor). Taken together, these data suggest that the MRC may have crucial and distinct roles in modulating antifungal drug stress responses.

While our data indicate a key role of the MRC in caspofungin stress adaptation, the link between the MRC and Hsp90 is more complex to elucidate. Because both Hsp90 and the MRC are important for fungal growth and morphogenesis, an alternative hypothesis could be that the impact of Hsp90 impairment on MRC function is an epiphenomenon resulting from fungal growth defect. To minimize this effect, we used our *pthiA*-hsp90 mutant, which has sufficient Hsp90 levels to maintain basal growth but cannot achieve appropriate Hsp90 levels under caspofungin stress. Indeed, our measurements of mycelial mass under the experimental conditions of this study confirmed that there were no statistically significant differences in fungal growth between KU80 and the *pthiA*-hsp90 mutant in the absence or presence of caspofungin. However, we observed some basal alterations in oxygen consumption, ATP production, and MRC gene expression in the *pthiA*-hsp90 mutant, suggesting some basal impairment of MRC function with potential impact on growth and morphogenesis, albeit not phenotypically apparent. While caspofungin has a more pronounced inhibitory effect on the rate of fungal growth over time in the *pthiA*-hsp90 mutant, this effect is manifest only after a few days (loss of paradoxical growth in the *pthiA*-hsp90 mutant) but is not apparent at the early time point of this analysis (e.g., 2 h of caspofungin exposure). This cannot explain the drastically opposite response of MRC gene expression observed immediately after caspofungin exposure with strong MRC induction in the wild-type KU80 and complete lack of MRC activation in the *pthiA*-hsp90 mutant. Moreover, we did not observe any increase in MRC gene expression in KU80 with another antifungal drug, such as voriconazole, which suggests that MRC activation does not simply reflect the nonspecific effect of growth inhibition but is rather a specific response to caspofungin stress.

The mechanism by which Hsp90 can influence mitochondrial activity remains unclear. We know from our previous work that Hsp90 does not move to the mitochondria upon caspofungin exposure (21). We thus hypothesize that the impact of Hsp90 on MRC function is probably indirect. As an essential molecular chaperone, Hsp90 controls the activation of multiple client proteins, including transcription factors, which may induce MRC gene expression. However, the mechanisms of regulation of mitochondrial genes remain largely unknown. The role of calcium and calcineurin pathway in caspofungin tolerance and paradoxical effect has been previously established (12, 22). Here, we demonstrate that both pharmacologic inhibition of Hsp90 and MRC resulted in a lack of increase in cytosolic Ca^{2+} in response to caspofungin. Indeed, ATP produced by the MRC may be required for the activity of the Ca^{2+} channels and Ca^{2+} homeostasis in stress responses.

We conclude that mitochondria play an important role in the mechanisms of stress response and tolerance to caspofungin in *A. fumigatus* and that appropriate Hsp90 levels are required for activation of the mitochondrial respiratory chain in this response. These results may open perspectives for identifying novel antifungal targets in this pathway, in particular, the MRC, which remains largely unexplored in *A. fumigatus*.

MATERIALS AND METHODS

Strains and growth conditions. Three *A. fumigatus* strains were used in this study, as follows: the *akuB*^{KU80} strain (here referred to as KU80), used as the reference strain (23); the *pthiA*-hsp90 mutant, in which the native *hsp90* promoter was replaced by the *thiA* promoter in the KU80 background (8); and the AEQ$^{\Delta akuB}$ strain (a gift from Nick Read, Manchester, UK), with the aequorin luminescent reporter expressed in the KU80 background, which was used for measurement of intracellular calcium (14).

Cultures were performed on glucose minimal medium (GMM) with supplementation of 1.5% agar for solid plates (24).

The antifungal drugs used in this study were obtained as powder suspensions (Sigma-Aldrich, St. Louis, MO, USA) and dissolved in sterile water (caspofungin) or dimethyl sulfoxide (DMSO) (voriconazole), for a stock concentration of 5 mg/ml.

Transcriptomic analyses. Transcriptomic analyses were performed with the parental KU80 strain and the *pthiA*-hsp90 mutant in the absence of any drug and in the presence of caspofungin or voriconazole. For untreated conditions, a suspension of about 4.10^5 spores/ml was inoculated in 250 ml GMM broth and incubated for 24 h at 37°C under constant agitation (225 rpm). For the treated conditions, the spore suspension was grown for 22 h in the absence of any drug, and the antifungal drug (caspofungin or voriconazole) was then added at a concentration of 2 μg/ml for an additional 2 h of incubation. The mycelial mass was then washed with cold sterile distilled water, filtrated, immediately frozen with liquid nitrogen, and reduced to a fine powder. Total RNA was extracted with the RNeasy plant kit (Qiagen, Inc., Venlo, The Netherlands) and purified with the Turbo DNA-free kit (Thermo Fisher Scientific, Reinach, Switzerland). RNA concentration was measured with NanoDrop 1000 spectrophotometer (Witec AG, Switzerland) and adjusted to a concentration of 9 ng/μl in RNA-free water. Tubes were kept frozen at −80°C until analysis.

The quality of the total RNA samples was checked with a Fragment Analyzer (Advanced Analytical Technologies) prior to preparation of the RNA libraries with the TruSeq stranded mRNA library prep kit (Illumina), according to the manufacturer's instructions. Samples were sequenced in an Illumina HiSeq 2000 sequencing platform using the 100-nucleotide (nt) single-end protocol with all the samples on same lane (25).

The analysis was performed with three biological replicates for each condition. RNA-seq data were processed using CLC Genomics Workbench version 10.1.1 (Qiagen). Reads were aligned to the *A. fumigatus* genome Af293 and read counts normalized by the quantile approach method. All conditions were compared with each other and filtered according to a false-discovery rate (FDR) cutoff of ≤0.05.

Only genes with significantly increased expression levels ($P \leq 0.05$) in the caspofungin-exposed strain compared to the untreated condition were considered.

Quantification of mycelial mass. Conidia of KU80 and the *pthiA*-hsp90 mutant were harvested from fresh GMM agar plates, counted with a hemocytometer, and adjusted for a quantity of 10^7 spores in flasks containing 25 ml of GMM liquid medium. Both strains were incubated at 37°C for 22 h and an additional 2 h in the absence or presence of 2 μg/ml caspofungin for the untreated and caspofungin-treated conditions, respectively. The mycelial mass was filtered and completely dried overnight at 60°C before weighing. The experiment was performed in triplicate.

Measurement of caspofungin paradoxical effect. A 10-μl aqueous suspension with 10^4 conidia of the tested *A. fumigatus* strain (KU80, AF293, or *pthiA*-hsp90 mutant) was inoculated on GMM agar plates supplemented with caspofungin at a concentration of 1, 2, or 4 μg/ml. The paradoxical effect of caspofungin was defined as a significant increase in fungal growth between caspofungin concentrations of 1 and 2 or 4 μg/ml. Different inhibitors of the MRC were tested at different concentrations for their ability to abolish the paradoxical effect, as follows: rotenone (MRC complex I inhibitor), antimycin A (MRC complex III inhibitor), oligomycin (ATPase inhibitor), and sodium azide (MRC complex IV inhibitor). The effect of hypoxic conditions on paradoxical growth was also tested in the GENbox anaerobic generator (bioMérieux, France). Pictures were taken after 5 days of incubation at 37°C. Experiments were performed in triplicate.

Quantification of intracellular ATP. The KU80 and *pthiA*-hsp90 mutant strains were cultured at a concentration of 10^6 conidia/ml in GMM broth at 37°C under constant agitation (225 rpm) for 24 h. Caspofungin (2 μg/ml) was added at 22 h for an additional 2 h for the treated conditions. Cultures were filtered and washed with sterile water using a Büchner funnel. The mycelial mass was immediately frozen with liquid nitrogen, reduced to fine powder with mortar and pestle, and kept at −20°C. Proteins were extracted with lysis buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 0.1% Triton, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1× protease inhibitory cocktail). Protein concentration was measured by the Bradford method (26) and adjusted to a concentration of 30 μg/ml for each sample. Lysates of 100 μl of each strain were incubated with 100 μl of CellTiter-Glo one solution (Promega, Fitchburg, WI, USA) in a 96-well plate (black/clear flat-bottom) for 10 min before measurement of luminescence using a LUMIstarOmega microplate reader (BMG Labtech, Ortenberg, Germany). A standard curve was made using ATP at a concentration range of 0 to 1 μM. The experience was performed in three biological replicates, and the mean of the final results was expressed as the fold change compared to KU80 under basal conditions.

Visualization of active mitochondria. A total of 10^4 conidia of KU80 and the *pthiA*-hsp90 mutant were incubated on microscope coverslips in GMM broth at 37°C for 24 h. Caspofungin (2 μg/ml) was added after 22 h for an additional 1-h incubation. Cultures were incubated with 1 μM MitoTracker Deep Red FM or MitoTracker Red CM-H_2XRos (Thermo Fisher, Waltham, MA, USA) for 45 min at 37°C and then observed with a fluorescence microscope (Axioplan 2; Zeiss, Oberkochen, Germany). Fluorescence quantification was determined using the ImageJ software.

Oxygen measurement. Oxygen measurement was performed with the Dual Digital Model 20 oximeter (Rank Brothers, Cambridge, England). KU80 and *pthiA*-hsp90 mutant strains were cultured at a concentration of 10^7 conidia/ml in GMM broth at 37°C for 7 h under constant agitation (225 rpm) to allow the start of germination and then transferred to the oximeter chambers that were saturated in oxygen, sealed with Parafilm for 4 h, and maintained at 37°C. Caspofungin (2 μg/ml) was added with a syringe 1 h before measurement or 30 min after the beginning of measurement. The effect of rotenone (158 μg/ml) was also tested. Oxygen consumption was measured every 15 s for 3 to 4 h.

Calcium measurement. A total of 10^6 conidia of KU80-AEQ were incubated in calcium-free medium (GMM supplemented with 50 mM uridine and 25 mM uracil) in a white 96-well plate for 18 h at 37°C, washed with PGM [20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.7), 50 mM glucose, 1 mM $MgCl_2$], and incubated for 4 h at 4°C in PGM supplemented by 1.06 μg/ml water-soluble coelen-

terazine (Sigma-Aldrich, St. Louis, MO, USA) for protein reconstitution (27). Cells were then incubated at room temperature for 1 h in the presence of the inhibitors geldanamycin (4 μg/ml; Sigma-Aldrich) and rotenone (158 μg/ml; Sigma-Aldrich). Caspofungin at 2 μg/ml was added 6 min after the start of luminescence measurement using a LUMIstarOmega microplate reader (BMG Labtech, Ortenberg, Germany). The experiment was repeated with the addition of 1 mM Ca^{2+} chelator BAPTA (Sigma-Aldrich) 1 h before measurement to remove all source of extracellular Ca^{2+}.

Statistical analyses. For transcriptomic data (RNA-sequencing), statistical analyses were performed in R (version 3.1.1) using the edgeR Bioconductor package implemented in the CLC software (28). This software implements the exact test for two-group comparisons accounting for overdispersion caused by biological variability (29). Nonparametric tests were performed using the software GraphPad Prism 7.03. *P* values were calculated by multiple comparisons using a Kruskal-Wallis test and considered significant at $\leq$0.05.

Data availability. Raw sequence reads of the current RNA-seq data can be found under BioProject number PRJNA486252.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00208-19.

SUPPLEMENTAL FILE 1, XLSX file, 1.1 MB.

SUPPLEMENTAL FILE 2, PPTX file, 8.7 MB.

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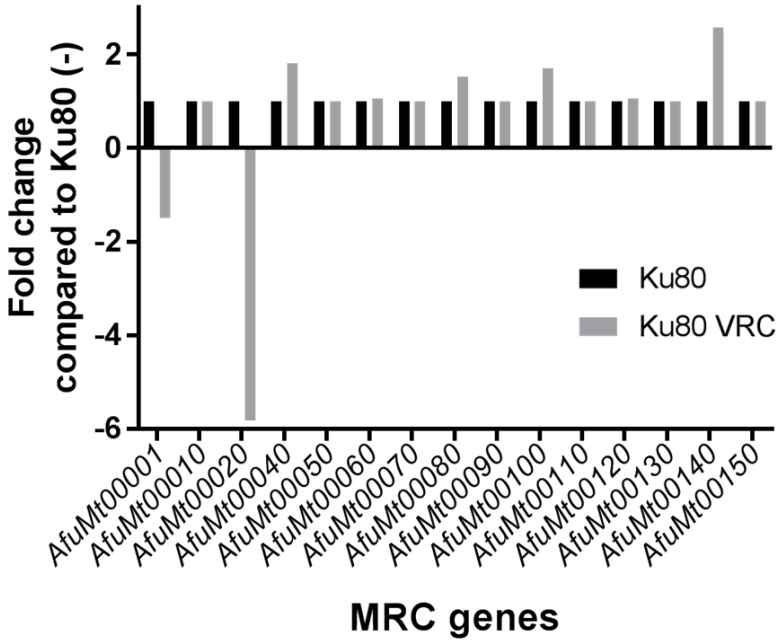
Supplementary data 1

Fig. S1



Transcriptomic analyses of mitochondrial respiratory chain (MRC) genes in *A. fumigatus* (Ku80) untreated and after 2h voriconazole exposure at 2 µg/mL.

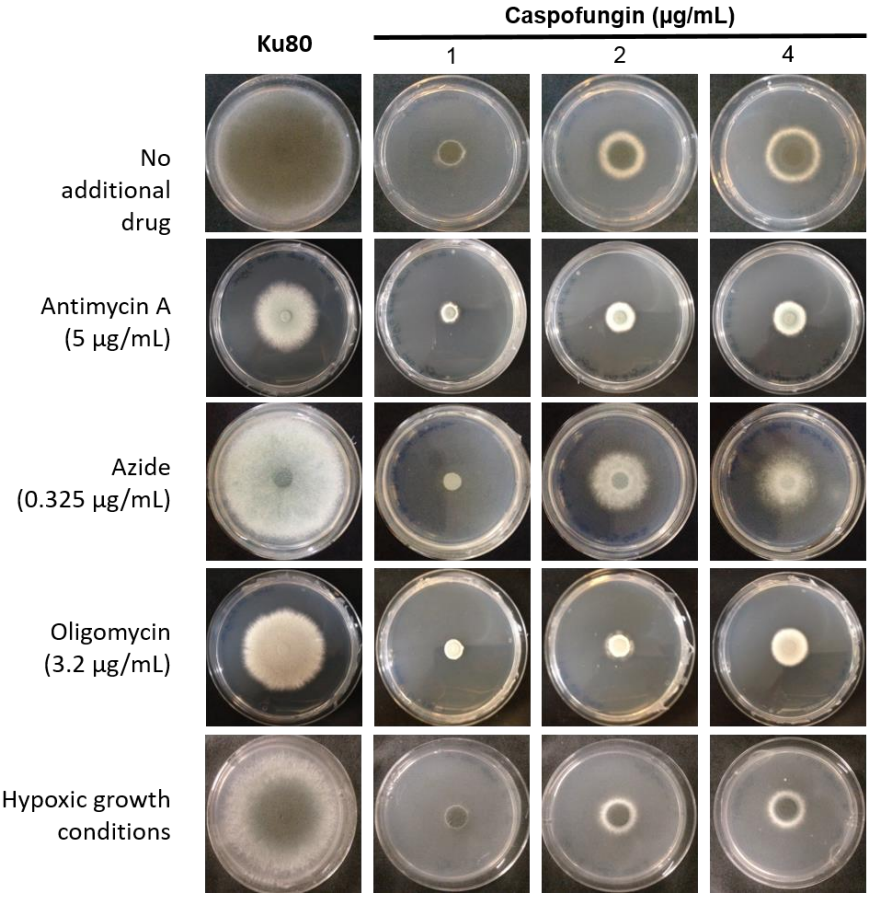
Gene expression in fold-change compared to the reference condition (Ku80, untreated). For all comparison, p-value ≥0.05 (not significant).

Black: Ku80 untreated (Ku80), **Grey**: Ku80 treated with voriconazole (VRC).

Supplementary data 2

Fig. S2

Effect of different mitochondrial respiratory chain (MRC) inhibitors and hypoxic growth condition on the caspofungin paradoxical effect of *A. fumigatus* (Ku80).
Pictures were taken after 5 days of growth at 37°C on glucose minimum medium (GMM) agar plates supplemented with caspofungin at increasing gradient concentration. Antimycine A was added at a fixed concentration of 5 µg/mL, Azide at 0.325 µg/mL, Oligomycin at 3.2 µg/mL. Hypoxic conditions were performed in GENbox Anaerobic (Biomérieux, France)



Supplementary data 3

Transcriptomic analyses can be found on NCBI under BioProject number PRJNA486252.

ANNEX 3

Insights in the molecular mechanisms of an azole stress adapted laboratory-generated *Aspergillus fumigatus* strain

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ABSTRACT

Aspergillus fumigatus is the main cause of invasive aspergillosis, for which azole drugs are the first-line therapy. Emergence of pan-azole resistance among *A. fumigatus* is concerning and has been mainly attributed to mutations in the target gene (*cyp51A*). However, azole resistance may also result from other mutations (*hmg1*, *hapE*) or other adaptive mechanisms. We performed microevolution experiment exposing an *A. fumigatus* azole-susceptible strain (Ku80) to sub-minimal inhibitory concentration (MIC) of voriconazole to analyze emergence of azole resistance. We obtained a strain with pan-azole resistance (Ku80R), which was partially reversible after drug relief, and without mutations in *cyp51A*, *hmg1* and *hapE*. Transcriptomic analyses revealed overexpression of the transcription factor *asg1*, several ATP Binding Cassette (ABC) and Major Facilitator Superfamily (MFS) transporters and genes of the ergosterol biosynthesis pathway in Ku80R. Sterol analysis showed a significant decrease of the ergosterol mass under voriconazole exposure in Ku80, but not in Ku80R. However, the proportion of the sterol compounds was similar between both strains. To further assess the role of transporters, we used the ABC transporter inhibitor milbemycine oxime (MLB). MLB inhibited transporter activity in both Ku80 and Ku80R and demonstrated some potentiating effect on azole activity. Criteria for synergism were reached for MLB and posaconazole against Ku80. Finally, deletion of *asg1* revealed some role of this transcription factor in controlling drug transporter expression, but had no impact on azole susceptibility.

This work provides further insight in mechanisms of azole stress adaptation and suggests that drug transporters inhibition may represent a novel therapeutic target.

LAY SUMMARY

A pan-azole resistant strain was generated *in vitro*, in which drug transporter overexpression was a major trait. Analyses suggested a role of the transporter inhibitor milbemycin oxime in inhibiting drug transporters and potentiating azole activity.

INTRODUCTION

The filamentous fungus *Aspergillus fumigatu*s is the main cause of invasive aspergillosis, a life-threatening disease among hematologic cancer and transplant patients, for which mold-active azoles, such as voriconazole, posaconazole or isavuconazole, are the mainstay of prevention and therapy.[11] Recent emergence of acquired pan-azole resistance among *A. fumigatus* clinical and environmental isolates has been reported in all continents, which poses a serious public health concern and has been linked to the massive use of azole-containing fungicides in agriculture.[17,39] Mutations at well-defined regions of the promoter and/or coding sequence of the *cyp51A* gene, which encodes the lanosterol 14 α-demethylase, the target of azole drugs in the ergosterol biosynthesis pathway, are considered as the predominant mechanism of azole resistance in *A. fumigatus*.[40] The role of mutations affecting the genes encoding HMG-CoA reductase (*hmg1*) and the CCAAT binding factor (*hapE*) has also been demonstrated.[41,42] In addition, mechanisms of upregulation of the target gene *cyp51A,* which is under the control of the transcription factors *atrR* and *srbA*, as well as overexpression of drug transporters can play a role in azole stress adaptation.[21,24,26,43] The objective of this work was to investigate these mechanisms of azole stress adaptation by *in vitro* generation of a pan-azole resistant *A. fumigatus* strain.

METHODS

Strains, growth conditions and drugs

The *A. fumigatus* akuB^{KU80} strain (here referred as Ku80) was used in this study as the parental strain for transformation.[44]

Cultures were performed on glucose minimal medium (GMM) solid or liquid medium.[34] For transformation, spores were prepared for electroporation in yeast extract-glucose medium (YG) and in Yeast extract dextrose medium (YED).[45]

Escherichia coli DH5α competent cells were used for plasmid production. Cells were grown in Luria-Bertani broth (LB) liquid medium at 37°C under constant agitation at 200 rpm and plated on LB agar supplemented with 50 µg/ml carbenicillin for selection of plasmid-containing bacteria. Plasmids were extracted with the Plasmid Mini Kit (Qiagen Inc, Venlo, The Netherlands).

The antifungal drugs (voriconazole, posaconazole and isavuconazole) and transporter inhibitors (milbemycine oxime, magnolol, clorgyline, and Phenylalanine-Arginine Beta-Naphthylamide) used in this study were obtained as a powder suspension (Sigma-Aldrich, Saint-Louis, MO) and dissolved in dimethyl sulfoxide (DMSO), for a stock concentration of 5 mg/mL.

Generation of the Ku80R pan-azole resistant strain

The *A. fumigatus* Ku80 strain was exposed to sub-minimal inhibitory concentrations (MIC) of voriconazole on successive subcultures on agar plates. The drug was dissolved in agar, starting at 0.125 µg/mL with gradual increase (doubling concentration) up to 2 µg/mL when residual growth was observed on agar plates. Single colonies were picked and reinoculated on 10 successive agar plates to generate the Ku80R strain.

In order to assess the reversibility of azole resistance, the Ku80R strain was inoculated on 10 consecutive subcultures in the absence of voriconazole.

Construction of the asg1 deletion mutant

The Ku80 strain, which is deficient for non-homologous end joining and possesses a high rate of homologous recombination, was used for transformation.[44] The 600 bp *asg1* gene (Afu1g16160) was replaced by the approximately 4000 bp hygromycin resistance cassette used as selection marker. For this purpose, the approximately 1000 bp upstream and downstream sequences of *asg1* were amplified by the primers described in Supplementary Material S1 and cloned into the plasmid pUCGH[46] to flank the hygromycin resistance cassette at BamHI/NotI and HindIII/SbfI sites, respectively. Transformation in the Ku80 recipient strain was performed by electroporation according to methods previously described.[45] Briefly, the Ku80 strain was incubated for 4 hours in liquid YG, then in YED in order to prepare the spores for electroporation. The electroporation was performed following these parameters: 1 KV, 400 Ohms, 25µfarads, in 1mm electroporation cuvettes, using the Gene Pulser Xcell Electroporation Systems (BioRad Laboratories, Hercules, CA). After electroporation, spores were incubated for 1h30 in YG medium and finally inoculated on GMM agar plates. The day after, a hygromycin overlay (50 mg/ml dissolved in 10 ml for a final concentration of 150 µg/ml in GMM agar plates) was added on the top of each plate to select the hygromycin resistant clones. Genetic deletion and substitution by the construct was demonstrated by PCR as shown in Supplementary Material S2.

Antifungal susceptibility testing

MICs were determined by microbroth dilution method according to the Clinical and Laboratory Standards Institute (CLSI)[47] using ranges of drug concentrations from 0.0625 to 8 µg/mL. Drug interactions were assessed by the checkerboard dilution

method, as previously described.[48] The fractional inhibitory concentration index (FICI) was calculated for each combination with synergy, indifference and antagonism defined as an FICI ≤0.5, >0.5 to 4, and >4, respectively.

Drug susceptibility was also assessed by spotting assay. Spores were collected in sterile water, counted with a hemocytometer and adjusted to a concentration of 10^7 spores/mL. Serial dilutions were performed and a drop of 5 µL containing 10^2 to 10^5 spores was spotted on glucose minimal medium (GMM) agar plates. Cultures were grown for 48h at 37°C with a gradient of azole concentrations (voriconazole, posaconazole and isavuconazole) adapted to the MIC of the strain (i.e. higher gradient for the Ku80R strain) and in the presence or absence of a fixed dose of milbemycine oxime (MLB).

Sequencing

Strains were cultured overnight in GMM liquid medium at 37°C under constant agitation (220 rpm). Mycelia were washed with sterile water, filtrated, frozen with liquid nitrogen and reduced to a powder with a mortar and pestle. DNA was extracted from the powder using the DNeasy Plant Mini Kit (Qiagen Inc, Venlo, The Netherlands). The entire *cyp51A* (including promoter), *hmg1*, *hapE*, *atrR*, and *srbA* genes were amplified using the primers described in Supplementary Material S1. Sequencing was performed by Sanger method by Microsynth AG (Balgach, Switzerland). All sequences have been deposited in GenBank (National Center for Biotechnology Information [NCBI], Bethesda, MD) under accession numbers MT468485 to MT469492 and MT642598, MT642599.

Transcriptomic analyses

Transcriptomic analyses were performed with the parental Ku80 strain and Ku80R strain in the absence of any drug and in the presence of voriconazole. A suspension of $4x10^5$ spores/mL was inoculated in 250 ml of GMM broth and incubated for 24h at 37°C under constant agitation (220 rpm). For the voriconazole-treated conditions, the spore suspension was grown for 22h in the absence of any drug and voriconazole was then added at the concentration of 2 µg/mL for an additional 2h of incubation. The mycelial mass was then washed with cold sterile water, filtrated, immediately frozen with liquid nitrogen and reduced to a powder. Total RNA was extracted with the RNeasy plant kit (QIAGEN Inc, Venlo, The Netherlands) and purified with the Turbo DNA free kit (Thermo Fisher Scientific, Reinach, Switzerland). RNA concentration was measured with Nanodrop-1000 (Witec AG, Switzerland) and adjusted to the concentration of 9 ng/µl in RNA-free water. Tubes were kept frozen at -80°C until analysis.

Transcriptomic analyses were performed as previously described.[49,50] In brief, quality of RNA was checked with a Fragment AnalyzerTM (Advanced Analytical Technologies) and RNA libraries were performed with with TruSeq Stranded mRNA Library Prep Kit (Illumina). Sequencing was performed in Illumina HiSeq 2000 platform using the 100-nt single-end protocol with all the samples on the same lane. The experiment was repeated in three biological replicates for each condition. RNA-seq data were processed using CLC Genomic Workbench Version 10.1.1 (Qiagen). Reads were aligned to the *A. fumigatus* Af293 genome. Read counts were normalized by the quantile approach method. The different conditions were compared using the edgeR Bioconductor package implemented in the CLC software calculating the Exact Test for

two-group comparisons, which accounts for overdispersion caused by biological variability.[51,52] The statistical cut-off used in differential gene expression was a false discovery rate (FDR) ≤ 0.05.

The complete transcriptomic dataset has been deposited at the NCBI under bioproject name PRJNA631905 (available at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA631905/).

Real-time reverse transcription polymerase chain reaction (RT-PCR)

The RNA was extracted for each strain/condition as above described. RNA was then converted into cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Switzerland). RT-PCR was performed in 96-well plates using a mastermix (Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix, USA) with 15 ng of cDNA and the primers of the gene of interest (listed in Supplementary Material S1) and supplemented with nuclease-free water up to 20 µl for each reaction. Experiments were performed in technical triplicates and biological duplicates. The $2^{-\Delta\Delta Ct}$ analytical method normalized to beta-tubulin was used for calculation of expression fold changes.[53] P values were calculated with GraphPad Prism and considered as significant if ≤0.05, with a one-way ANOVA statistical test.

Analysis of sterol components by mass spectrometry

Spore suspensions of Ku80 and Ku80R were grown in GMM broth as above described in the absence or presence of voriconazole (2 µg/mL for the last 2h). Frozen-dried powder of mycelia was weighed and resuspended in 1mL ddH_2O and sonicated 6 times for 30s at 50% power (Branson SFX150) to disrupt the cells. Sterols were

extracted and derivatized as previously described.[54] An internal standard of 10 µg of cholesterol was added to each sample and lipids were saponified using alcoholic KOH and non-saponifiable lipids extracted with hexane. Samples were dried in a vacuum centrifuge and were derivatized by the addition of 0.1mL N,O-bis(Trimethylsilyl)trifluoroacetamide with Trimethylchlorosilane (BSTFA TMCS, 99:1, Sigma) and 0.3 mL anhydrous pyridine (Sigma) and heating at 80°C for 2 hours. Trimethylsilyl (TMS)-derivatized sterols were analysed and identified using GC/MS (Thermo 1300 GC coupled to a Thermo ISQ mass spectrometer, Thermo Scientific) and Xcalibur software (Thermo Scientific). The retention times and fragmentation spectra for known standards were used to identify sterols. Integrated peak areas were determined to calculate the percentage of total sterols. Ergosterol quantities were determined using standard curves of peak areas of known quantities of cholesterol and ergosterol.

Measurement of transporter activity

The fluorescent dye rhodamine 6G (Sigma-Aldrich, Saint-Louis, MO), a substrate of ABC drug transporters, was used to assess the effect of MLB on the activity of the ABC drug transporters in Ku80 and Ku80R. The protocol was derived from that previously described in yeasts,[55] which was adapted to *A. fumigatus* on the basis on previous publications.[56,57] A total of 10^9 spores of Ku80 and Ku80R were incubated in GMM+0.5% yeast extract for 8h at 37°C under constant agitation (220 rpm). Germinating cells were centrifuged, washed twice with cold phosphate buffered saline (PBS) and resuspended in glucose-free PBS for 1h at 37°C with rhodamine 6G (Sigma-Aldrich, Saint-Louis, MO) at 2.4 µg/mL to allow rhodamine 6G accumulation.

Cells were then washed twice with PBS and resuspended in glucose-free PBS for a final concentration of 10^8 spores/mL in the presence or absence of MLB at a final concentration of 8 µg/mL. A total of 180 µL of the final suspension was immediately distributed in the wells of a 96-well black plate and the measurement was started immediately. Each condition was tested in triplicates. The instrument used for fluorescence measurement was the LUMIstarOmega microplate reader (BMG LABTECH, Ortenberg, Germany). Fluorescence ($\lambda_{emission}$=540nm and $\lambda_{excitation}$=580 nm) was measured in the supernatant every minute during 2 hours. Before each measurement, the plate was automatically shaken for one second to homogenize the sample. A well containing PBS only served as the blank. The signal was expressed in relative fluorescence unit (RFU), where the fluorescence of the blank sample was subtracted to the absolute fluorescence of the sample (mean of the triplicates for each condition). After initial equilibration phase (time for the curve to stabilize), the baseline measurement was set up at the value of 1 for each condition and further measurements expressed as a fold-change compared to the baseline measurement of each sample. Experiments were performed in the absence of glucose and following injection of glucose (for a final concentration in the wells of 1% glucose) 45 min after the beginning of measurement to re-energize transporters.

RESULTS

In vitro induction of pan-azole resistance in A. fumigatus

The *A. fumigatus* wild-type strain Ku80 was exposed to voriconazole by consecutive subcultures on GMM agar plates containing sub-MIC concentrations of voriconazole. After 10 subcultures, the MIC of voriconazole was increased from 0.5 µg/mL in the

non-exposed Ku80 strain to 4 µg/mL in the exposed strain (further referred to as Ku80R). This strain exhibited cross-resistance to posaconazole (MIC increase from 0.25 to 1 µg/mL) and isavuconazole (MIC increase from 0.5 to 4 µg/mL). Resistance was partially reversible after consecutive subcultures on voriconazole-free GMM agar plates (from 4 to 1 µg/mL). Entire sequencing of the *cyp51A* (including the promoter), *hmg1*, *atrR*, *srbA* and *hapE* genes did not reveal any mutation in the Ku80R strain compared to the parental Ku80 strain. We thus obtained a laboratory-generated *A. fumigatus* strain (Ku80R) with pan-azole resistance according to the clinical breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) definitions,[58] in the absence of mutations in *cyp51A* (or other genes known to be involved in azole resistance) and with a potential of reversibility.

Transcriptomic profile of the laboratory-generated azole-resistant A. fumigatus strain

The transcriptomic profile (RNA-seq) of the laboratory-generated pan-azole resistant Ku80R strain was analyzed and compared to the parental wild-type azole-sensitive Ku80 strain in the absence or presence of voriconazole (2 µg/mL for 2h exposure) in three biological replicates. Data were obtained for 9787 genes, of which 810 displayed significant overexpression in Ku80R compared to Ku80 in the absence or presence of voriconazole (Supplementary Material S3). Under basal conditions, a significant increase of expression of *cyp51A* and other genes of the ergosterol biosynthesis pathway (*erg1*, *erg3*, *erg3A*, *erg24*, *erg24B*, *erg25, erg25B*) was observed in Ku80R compared to Ku80 (Figure 1A). Under voriconazole exposure, a significant

overexpression of *erg1*, *erg3*, *erg24*, *erg24B* and *erg25* was also observed in Ku80R compared to Ku80 (Figure 1A).

Significant overexpression of genes encoding ATP Binding Cassette (ABC) and Major Facilitator Superfamily (MFS) transporters was also observed in Ku80R compared to Ku80 under basal conditions (*abcB*, *abcD*, *mdr1*, *mfsC*, *mdrA*) and under voriconazole exposure (*abcB*, *abcD*, *atrI*, *mdr1*, *mfsC* and *mdrA*) (Figure 1B).

Among known transcription factors, we observed significant overexpression of *asg1* (*Afu1g16160*) in Ku80R compared to Ku80 under basal conditions and following voriconazole exposure (Figure 5A), while the expression of *atrR* and *srbA* was similar between both strains.

We concluded that upregulation of genes encoding drug transporters and/or the ergosterol biosynthetic pathway could be a predominant mechanism of azole resistance in Ku80R.

Analysis of sterol components of the cell membrane

In order to assess the impact of overexpression of ergosterol biosynthetic genes in Ku80R, we quantified by mass spectrometry the sterol composition of Ku80 and Ku80R in the presence or absence of voriconazole (2 µg/mL for 2h exposure). Analyses of the proportion of sterols is shown in Figure 2A. The comparison between Ku80 and Ku80R (in the absence or presence of voriconazole) did not show any significant difference. In both strains, the proportion of ergosterol was decreased under voriconazole exposure (compared to the untreated condition), while the proportion of the intermediate components lanosterol and eburicol was increased. Results of the quantification of the total ergosterol mass is shown in Figure 2B. The comparison

between Ku80 and Ku80R showed no significant difference in the absence or in the presence of voriconazole. However, voriconazole exposure resulted in a significant decrease of the ergosterol mass in Ku80, which was not the case in Ku80R.

These results may suggest a possible adaptive response in Ku80R, which is capable of maintaining its ergosterol mass upon voriconazole exposure.

Effect of drug transporter inhibition

Because activation of drug transporters was supposed to be involved in azole resistance of Ku80R based on transcriptomic data, we tested the effect of drug transporter inhibition on Ku80 and Ku80R. We tested the ability of milbemycine oxime (MLB), an ABC transporter inhibitor having demonstrated some antifungal activity and synergism with azoles against *Candida* spp.,[55] to inhibit drug transporter activity in Ku80 and Ku80R. For this purpose, we measured the efflux of the fluorescent dye rhodamine 6G, which is a substrate of the ABC transporters, in the absence or presence of MLB at a concentration of 8 µg/ml. As shown in Figure 3, MLB was effective in inhibiting the efflux of rhodamine 6G in both Ku80 and Ku80R.

As a next step, we tested the *in vitro* activity of MLB alone and in combination with triazoles against Ku80 and Ku80R. Results of MIC values and fractional inhibitory concentration index (FICI) are shown in Table 1. MLB had no antifungal activity *per se* against Ku80 and Ku80R. By checkerboard microdilution method, a synergistic effect was observed between MLB and posaconazole against Ku80 (FICI 0.25), but not with voriconazole and isavuconazole (FICI 0.62). In the Ku80R strain, the interaction of MLB and triazoles was classified as indifferent, despite some additive effect with posaconazole (FICI 0.62). Spotting assays confirmed some potentiating effect of MLB

on triazoles with distinct effects according to the drugs and strains (Figure 4). Against Ku80, MLB had a clear potentiating effect with posaconazole and isavuconazole, but its impact on voriconazole effect was very modest. Against Ku80R, MLB could also potentiate the effect of posaconazole at higher concentrations, However, its impact was absent or very modest for isavuconazole and voriconazole.

Other molecules with a known inhibitory effect on drug transporters were tested for their interactions with azoles in checkerboard microdilution. The broad-spectrum inhibitor of efflux pumps phenylalanine-arginine beta-naphthylamide (PAβN)[59] showed a positive interaction, which was comparable to MLB, with synergistic criteria achieved only for posaconazole against Ku80 (FICI 0.31) (Table 1). No positive interaction were observed between azoles and magnolol (a putative ABC transporter inhibitor having demonstrated some synergistic effect with azoles against *Candida* spp.[60] and the monoamine oxydase A inhibitor clorgyline, a broad-spectrum ABC and MFS transporter inhibitor in potentiating azole activity against *Candida* spp.[61] (data not shown).

Role of the transcription factor asg1 in controlling drug transporters

As a next step, we intended to investigate the role of the putative transcription factor *asg1* (*Afu1g16160*), which was significantly overexpressed in Ku80R (35-fold increase and 51-fold increase in Ku80R compared to Ku80 in the absence and presence of voriconazole, respectively. Figure 5A). In *Candida albicans*, *asg1* was found to be able to restore expression of the ABC transporter PDR5 and azole resistance when complementing a *Saccharomyces cerevisiae* strain lacking PDR1 and PDR3.[22] However, its deletion in *C. albicans* did not alter fluconazole susceptibility.[22]

In order to investigate the role of *asg1* in azole resistance of *A. fumigatus*, we generated a deletion of this gene in the Ku80 background (Ku80Δ*asg1* strain). Expression of selected ABC and MFS drug transporters (i.e. *abcD*, *mfsC* and *mdr1* which showed the highest overexpression in Ku80R in transcriptomic analyses, Figure 1B) was assessed by real-time reverse transcription PCR (RT-PCR) in Ku80Δ*asg1* and Ku80 in the presence or absence of voriconazole (2 µg/mL for 2h exposure). As shown in Figure 5B, the loss of *asg1* resulted in lack of overexpression of *abcD* under voriconazole exposure in Ku80Δ*asg1* compared to Ku80. Albeit conserved in Ku80Δ*asg1*, the overexpression of *mfsC* under voriconazole exposure was significantly lower compared to that observed in Ku80. In contrast, the expression of *mdr1* under voriconazole exposure was higher in Ku80Δ*asg1* compared to Ku80. While *asg1* deletion resulted in some modifications in drug transporters expression, we did not observe any impact on voriconazole susceptibility. MICs of Ku80Δ*asg1* were similar to that of Ku80 within +/- one dilution (0.25, 0.5 and 1 µg/mL, for voriconazole, posaconazole and isavuconazole, respectively) and we did not observe any difference in spotting assays with voriconazole as shown in Figure 5C.

DISCUSSION

Acquired azole resistance in *A. fumigatus* is a complex phenomenon and multiple mechanisms can be involved. While most previous studies have highlighted the pivotal role of mutations in *cyp51A* or other genes (*hmg1*, *hapE*),[40-42] azole resistance can be observed in the absence of these mutations, suggesting other mechanisms of stress

adaptation, such as upregulation of the target gene (*cyp51A*) and/or genes encoding drug transporters.

In the present work, we generated *in vitro* a pan-azole resistant *A. fumigatus* strain by continuous azole exposure (Ku80R). Azole resistance was partially reversible and not associated to any mutations in *cyp51A*, *hmg1*, *atrR*, *srbA* and *hapE*. Our transcriptomic analysis supported the role of overexpression of genes of the ergosterol biosynthesis pathway (including *cyp51A*) and of ABC and MFS drug transporters in the acquired azole resistance of this strain (Figure 1). It is unclear whether this effect results from upregulation of the genes or an increased in the number of transporters, as we have not checked for gene copy number.

Sterol analysis did not reveal any significant difference between Ku80 and Ku80R (Figure 2). However, in contrast to Ku80, Ku80R did not exhibit a significant decrease in ergosterol mass upon voriconazole exposure. This result, coupled with the overexpression of *cyp51A* in Ku80R in response to voriconazole, suggests that this strain might display some adaptive response of the cell membrane and the ability to maintain its ergosterol production despite azole stress. However, the actual significance of these results and their contribution to azole resistance of this Ku80R strain is unclear.

We then hypothesized that overexpression of drug transporters could represent the main trigger of rapid acquisition of azole resistance in Ku80R.

High expression levels of ABC and MFS transporters have already been reported in clinical and environmental azole resistant *A. fumigatus* isolates.[23,26,28,62] Inactivation of *atrI* and *mdrA in A. fumigatus* affected the basal level of azole susceptibility and their complementation in a *Saccharomyces cerevisiae* strain lacking the ABC transporter

PDR5 could restore azole resistance.[23] Deletion of the ABC transporter encoding gene *cdr1B* (here referred as *abcC*) also resulted in decreased azole resistance.[26]

Little is known about the transcription factors controlling drug transporter expression. Previous studies have demonstrated the role of *atrR* and *srbA* in azole resistance, which could be mainly related to their role in regulating *cyp51A* expression.[21,43] Only *atrR* was also found to be involved in drug transporter expression of *cdr1B* (*abcC* in the present study).[21] Interestingly, *abcC* was not significantly overexpressed in our Ku80R strain (Figure 1). In the present study, we found no mutations or expression changes of the *atrR* and *srbA* genes in Ku80R. However, we observed a significant overexpression of the transcription factor *asg1* in this azole resistant strain (Figure 5A). Further analyses showed that *asg1* could be involved in the induction of expression of the ABC transporter *abcD* in Ku80 under voriconazole exposure, as this effect was absent in Δ*asg1* (Figure 5B). Deletion of *asg1* also altered the expression of *mdr1* and *mfsC*, which were respectively higher and lower upon voriconazole exposure compared to the wild-type strain (Figure 5B). We did not further investigate the role of *asg1* in controlling other drug transporters. Effectively, deletion of *asg1* had no impact on azole susceptibility (Figure 5C), which suggests that his regulatory role on transporters expression is not of primary importance for azole resistance. These findings are consistent with those previously observed in *C. albicans* about the role of *asg1*.[22]

Following the postulate that drug transporters may have an important role in azole resistance of Ku80R, we assessed the potential of drug transporter inhibitors for preventing or abolishing azole resistance. The ABC transporter inhibitor MLB was able to reduce transporter activity in both Ku80 and Ku80R (Figure 3). However, the

potentiating effect of MLB on azoles was modest and, interestingly, was mainly observed with posaconazole (Figure 4). Only the interaction of MLB and posaconazole against Ku80 was characterized as synergistic. MLB also displayed some potentiating effect with posaconazole against Ku80R, as illustrated by the spotting assay (Figure 4), but this interaction was not characterized as synergistic in checkerboard assay. It could be that the considerable overexpression of both ABC and MFS transporters in Ku80R overwhelms the inhibitory capacity of MLB affecting mainly ABC transporters. We did not observe a direct antifungal effect of MLB against *A. fumigatus*, as previously reported by Silva et al. against *Candida* spp.[55] In this latter study, MLB displayed intrinsic fungicidal activity and synergistic interaction with fluconazole against *C. albicans* and *C. glabrata in vitro* and in a murine model of invasive candidiasis. Finally, we found a similar interaction between azoles and another transporter inhibitor, PAβN, that displayed synergistic interaction with posaconazole against Ku80 only (Table 1). The actual mechanisms by which MLB and PAβN cripple drug transporters remains poorly elucidated.

In conclusion, we showed that *A. fumigatus* can rapidly respond *in vitro* to azole stress achieving MIC corresponding to the definition of clinical resistance,[58] which was a reversible effect and not associated with any previously described mutation. Because infra-therapeutic concentrations of voriconazole and posaconazole are frequently observed in clinical practice, it is possible that such phenomenon may be clinically relevant. Overexpression of drug transporters seems to play a role in this phenomenon and our results provide further insights in the role of drug transporters and their transcription factors in azole resistance of *A. fumigatus*. However, our results highlight

that the mechanisms of azole resistance in *A. fumigatus* are multiple and complex, reflecting the ability of this ubiquitous fungus to adapt to many environmental stress conditions in the course of its evolution.

Transporter inhibition could represent an interesting adjuvant therapeutic approach. However, the effect of ABC transporter inhibitors (e.g. MLB, PAβN) on potentiating azoles was only modest, suggesting compensatory effects mediated by other transporters or other mechanisms. Further investigations of potential broad-spectrum drug transporter inhibitors are warranted.

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DISCLOSURE OF INTERESTS

None to declare (all authors)

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TABLES

Table 1. Results of antifungal susceptibility testing and checkerboard dilution assays

	MIC [µg/ml]					FICI (MLB)			FICI (PAβN)		
Drugs	VRC	POS	ISA	MLB	PAβN	VRC	POS	ISA	VRC	POS	ISA
Ku80	0.5	0.25	0.5	32	32	0.62	0.25	0.62	0.62	0.31	1.03
Ku80R	4	1	4	32	32	1	0.62	1.12	0.62	0.62	0.75

MIC: minimal inhibitory concentrations according to the CLSI protocol [47], FICI: fractional inhibitory concentration, VRC: voriconazole, POS: posaconazole, ISA: isavuconazole, MLB: milbemycin oxime, PAβN: phenylalanine-arginine beta-naphthylamide

Interpretation of FICI results: ≤0.5 = synergistic, >0.5 to 4 = indifferent, and >4 = antagonistic

Note: for MLB and PAβN, displaying no *per se* antifungal activity against *A. fumigatus*, the highest value of the tested range of concentrations (i.e. 32 µg/ml) was used for FICI calculation.

FIGURES

Figure 1

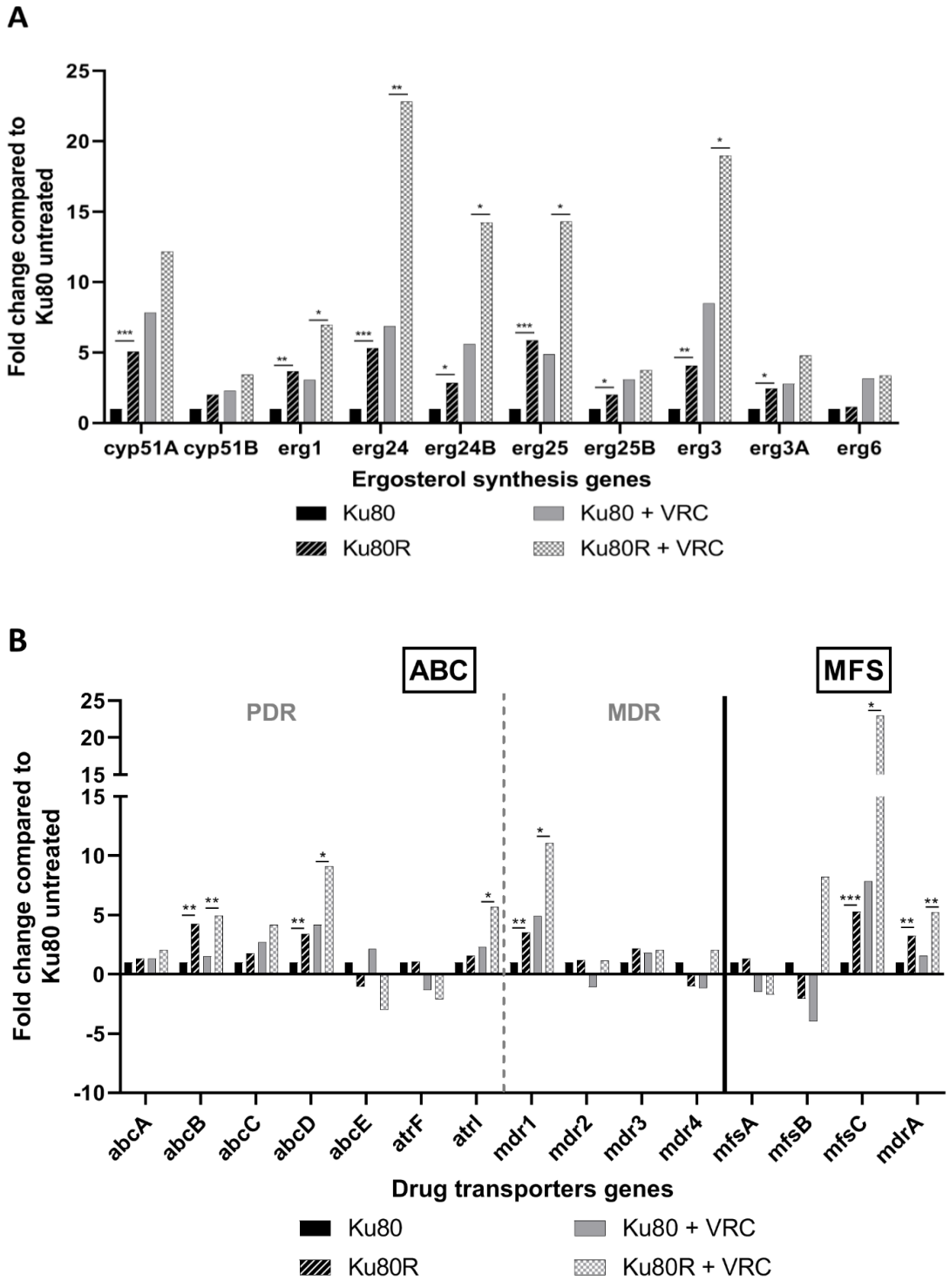


Figure 1. Transcriptomic analyses of (A) genes encoding the ergosterol synthesis pathway and (B) genes encoding drug transporters in *A. fumigatus* Ku80 parental strain and Ku80R azole-resistant strain in basal conditions (untreated) and after 2h exposure to voriconazole (VRC) at 2 µg/mL.

Gene expression in fold-change compared to the reference condition (Ku80, untreated). Black: Ku80 untreated (Ku80), striped black: Ku80 with voriconazole (Ku80 + VRC), grey: Ku80R untreated (Ku80R), dotted grey: Ku80R with voriconazole (Ku80R + VRC).

The p-values are expressed as: * ≤0.05, **≤10^{-5}, *** ≤10^{-9}

ABC: ATP binding cassette, MFS: major facilitator superfamily, PDR: pleiotropic drug resistance, MDR: multidrug resistance.

Figure 2

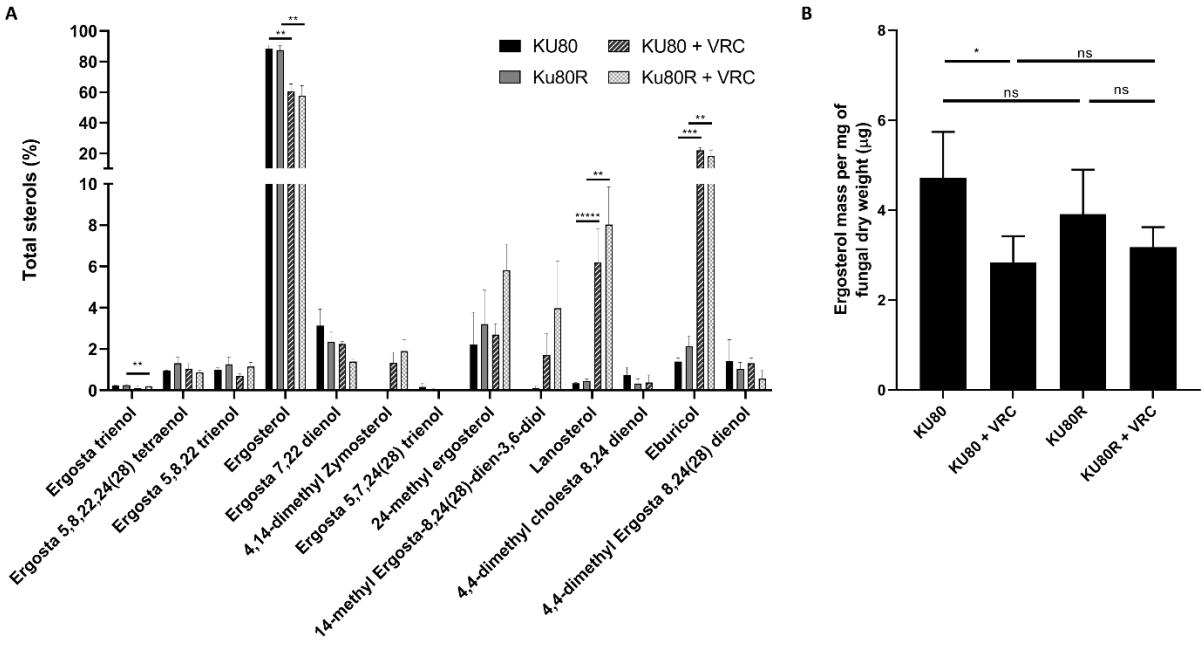


Figure 2. Analysis of sterol components by mass spectrometry. (A) Proportions (percentages) of total sterol content for each individual sterol component and (B) ergosterol weight per fungal dry mass. Analyses were performed for the *A. fumigatus* Ku80 parental strain and the Ku80R azole-resistant strain in basal conditions (untreated) and after 2h exposure to voriconazole (VRC) at 2 µg/mL.

Black: Ku80 untreated (Ku80), grey: Ku80R untreated (Ku80R), striped black: Ku80 with voriconazole (Ku80 + VRC), dotted grey: Ku80R with voriconazole (Ku80R + VRC).

Results represent means of triplicates, bars represent standard deviation.

The p-values are expressed as: * ≤0.05, **≤0.01, *** ≤0.001, ***** $\leq 10^{-5}$.

Significant p-values are shown for the comparisons of Ku80 vs Ku80-VRC and Ku80R vs Ku80R-VRC. P-values for the comparisons for Ku80 vs Ku80R and Ku80-VRC vs Ku80R-VRC were not significant.

Figure 3

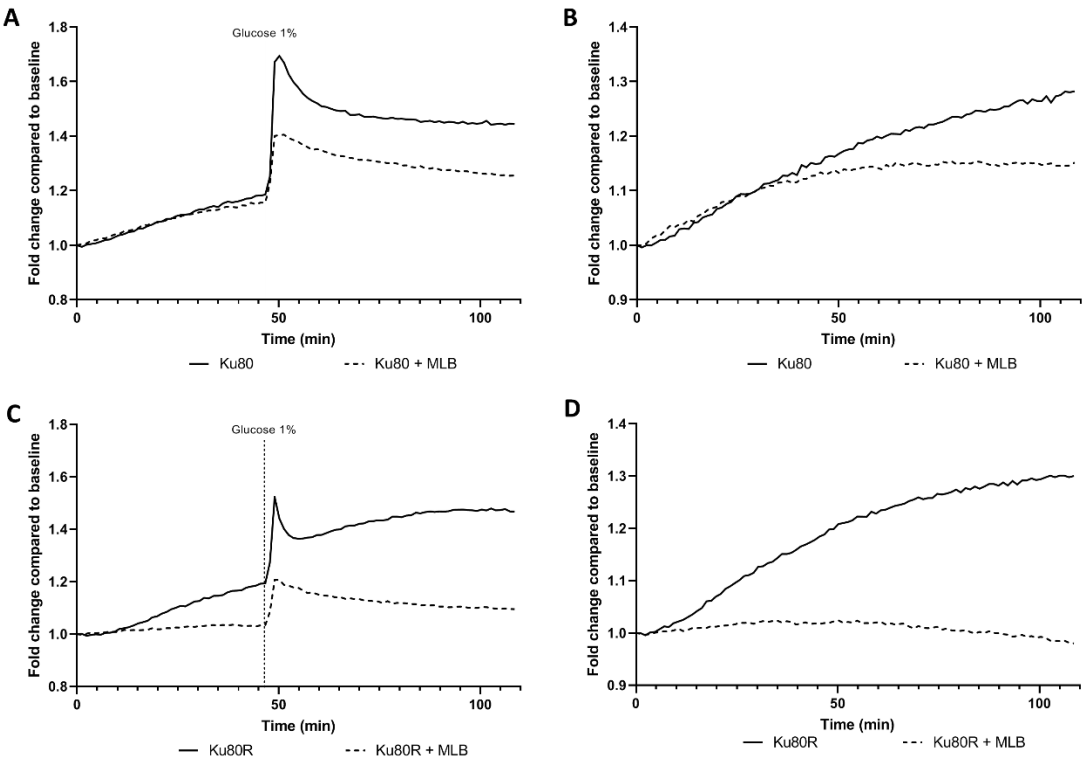


Figure 3. Measurement of drug transporter activity by the fluorescent dye rhodamine 6G. (A) *A. fumigatus* Ku80 parental strain in basal conditions (untreated) and exposed to Milbemycine oxime (MLB) 8 µg/mL with injection of glucose (1% final concentration) after 45 min. and (B) in the absence of glucose. (C) Azole-resistant strain, Ku80R in basal conditions (untreated) and exposed to MLB 8 µg/mL with injection of glucose (1% final concentration) after 45 min. and (D) absence of glucose.

Measurements represent mean of triplicates and are expressed in fold-change of fluorescence compared to the point at T0 (start of measurement =1) for each condition.

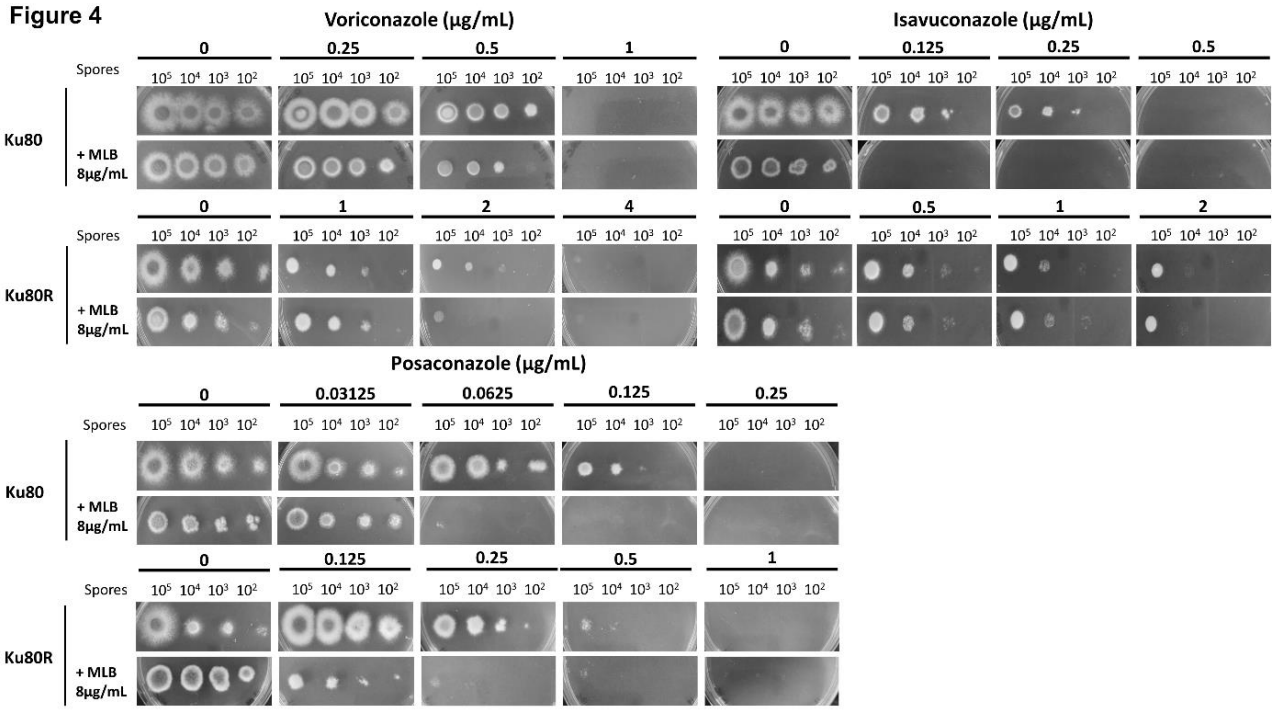


Figure 4. The drug interaction between azoles and milbemycine oxime (MLB) was assessed by spotting assay with serial spore dilutions of the Ku80 parental strain and the Ku80R azole-resistant strain spotted on GMM agar plates containing a range of azole drug concentrations and a fixed dose of MLB (8 µg/mL). Pictures were taken after 48h at 37°C.

Ranges of concentrations were defined according to the minimal inhibitory concentration (MIC) of the tested strain (i.e. higher range for Ku80R).

Figure 5

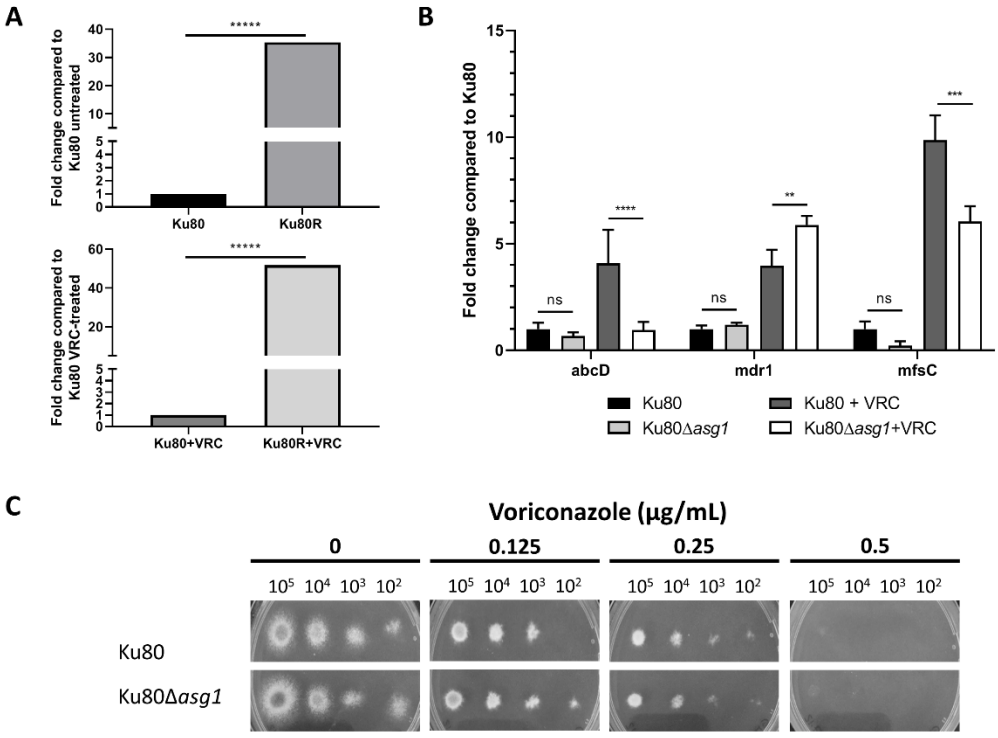


Figure 5. Characterization of the role of the transcription factor *asg1* in azole resistance.

(A) Expression of the transcription factor *asg1* (*afu1g16160*) in *A. fumigatus* Ku80 parental strain and Ku80R azole-resistant strain in basal conditions (untreated) and after 2h exposure to voriconazole (VRC) at 2 µg/mL by transcriptomic analyses (RNA seq).

Gene expression of Ku80R expressed in fold-change compared to Ku80 (=1) in the absence or presence of voriconazole. The p-values is expressed as: *****$\leq 10^{-30}$.

(B) Expression levels of the drug transporter encoding genes *abcD, mdr1* and *mfsC*, in the Ku80△*asg1* strain expressed in fold-change compared to the parental Ku80 strain untreated (=1) in the absence or presence of voriconazole 2 µg/mL (2h exposure).

The p-values are expressed as: ** ≤0.01, *** ≤10^{-3}, ****≤10^{-4} .

(C) Voriconazole susceptibility of the Ku80△*asg1* deletion strain compared to Ku80 assessed by spotting assay with serial spore dilutions spotted on GMM agar plates containing a range of voriconazole concentrations. Pictures taken after 48h at 37°C.

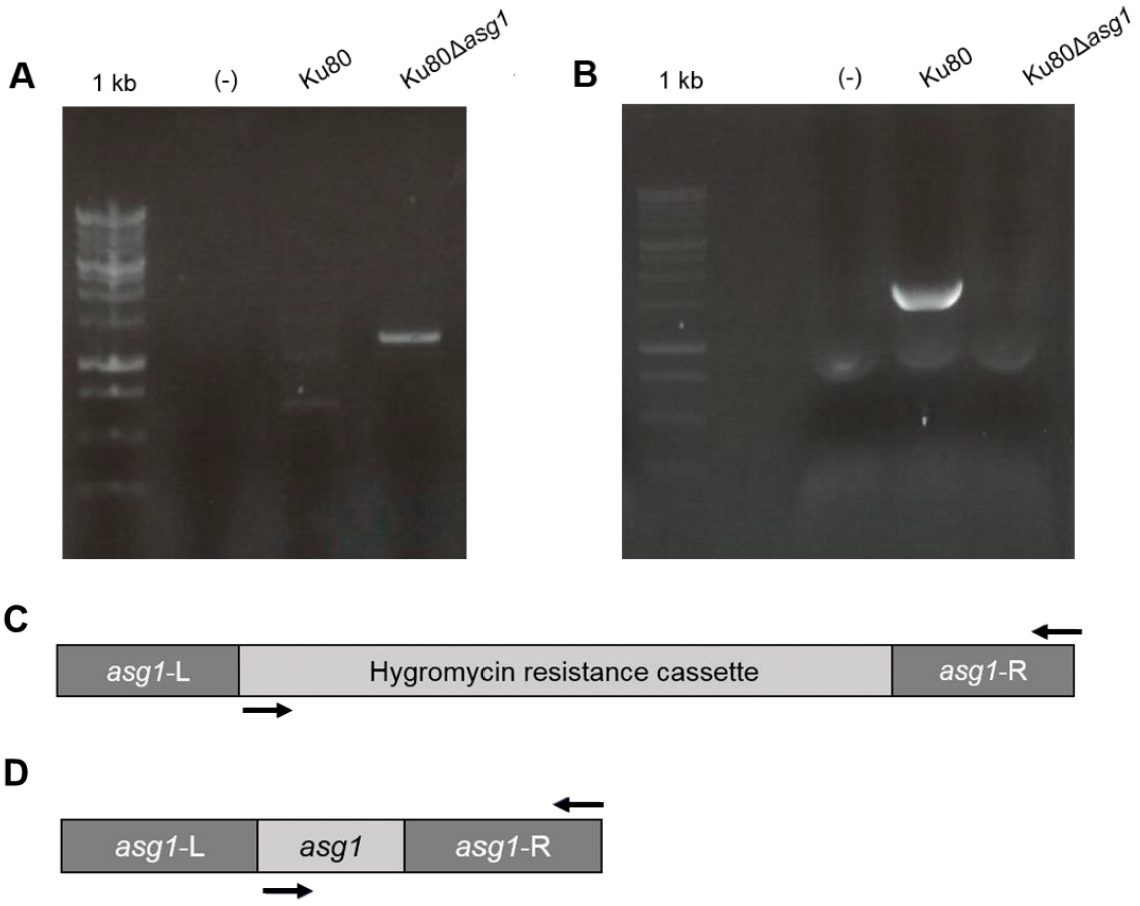
Supplementary data 1

Supplementary material S1: List of primers used in this study for sequencing and cloning

Gene	Primer forward	Primer reverse	Description
PCR			
cyp51A	5′- GCATAGCAGTGTCGGAATCAG -3′	5′- CAACACTTCAGGGCCAGTAA -3′	PCR primers of *cyp51A*
srbA	5′- GGTCTTGACAGGTATGCAGTCT -3′	5′- TGAAGACTGGGCAGCCA -3′	PCR primers of *srbA*
atrR	5′- AAAAGGTGAGCAAGTGGAGTG -3′	5′- CGCCTCTTCCAGCGATTTC -3′	PCR primers of *atrR*
hapE	5′- CCTCTCGCGATACATTACTGTTG-3′	5′- AATTGGATGGCTCGAGATTTCTC -3′	PCR primers of *hapE*
hmg1	5′- TCGAGAGAATTTGAGACGGTACTAAG -3′	5′- TACGAACATGCGATCACCTTTTCTA -3′	PCR primers of *hmg1*
***asg1* (right)**	5′- GAAGACCTGCAGGCCTTTAGCATAGGCGGCATAG -3′	5′- CAACCAAGCTTCACCACCTTCTGAAGAAGAAGC -3′	Downstream region of *asg1* (1000bp)
***asg1* (left)**	5′- TCGATGGATCCCACGTTCTTATTCCCGAACACTTATT -3′	5′- AACCAGCGGCCGCGTCTGTTCGAGTGGATGATGATG -3′	Upstream region of *asg1* (1000bp)
asg1 gene	5′- AGGTTGCTATCCCTCGGATTG -3′	5′- GGTTTGCTCGCCGATTGTTT -3′	PCR primers of *asg1*
hygromycin	5′- AATGCTCCGTAACACCCAATACGCCG -3′	5′- AACTGGCTCTTAATGAGCTGGCGGA -3′	PCR primers of hygromycin cassette resistance

Supplementary data 2

Supplementary Material S2



Demonstration of the deletion of the gene *asg1* in Ku80 by PCR.
(-) represents the negative control without DNA, Ku80 is the parental strain that contains the *asg1* gene, and Ku80Δ*asg1* the mutant strain, in which *asg1* has been substituted by the hygromycin resistance cassette (selective marker). A and B represent the pictures of the electrophoresis gel; C and D are the schematic representation of the PCR.
A and C: amplification of a fragment using primers (black arrows) in the hygromycin cassette (forward) and in the downstream region of *asg1* (reverse). Expected size: 1200 bp (present in Ku80Δ*asg1* and absent in Ku80). B and D: amplification of a fragment using primers (black arrows) in the *asg1* gene (forward) and in the downstream region of *asg1* (reverse). Expected size: 1570 bp (present in Ku80 and absent in Ku80Δ*asg1*).

Supplementary data 3

Transcriptomic data are available on NCBI under bioproject name PRJNA631905 (available at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA631905/).