In humans, *Aspergillus fumigatus* is the most common and life-threatening aerial fungal pathogen, especially among immunocompromised patients, with an overall mortality ranging between 30 to 88%. Azole antifungals, such as voriconazole and posaconazole, are recommended first choice drugs to manage aspergillosis diseases. However, acquired azole-resistance in *A. fumigatus* is an emerging problem that compromises the clinical efficacy of azole antifungals. Although azole-resistance may emerge during antifungal therapy of individual azole-treated patients, selection of resistance may also occur in the environment. Given the prominent role of azoles in the management of aspergillosis diseases, successful management of azole-resistant aspergillosis diseases in patients with chronic pulmonary aspergillosis and invasive aspergillosis is a challenge. Therefore, it is important to explore alternative therapeutic approaches. The research described in this thesis is aimed to provide some experimental evidence that will help to guide physicians in the treatment of patients with azole-resistant aspergillosis diseases.
Strategies for treating aspergillosis due to azole-resistant *Aspergillus fumigatus*:
From the bench to the bedside

Seyed Mojtaba Seyed Mousavi Tasieh
Microscopic morphology of *Aspergillus fumigatus* showing typical columnar, uniseriate conidial heads. Conidiophores are short, smooth-walled and have conical shaped terminal vesicles, which support a single row of phialides on the upper two thirds of the vesicle. Image is adopted with permission from courtesy of Ton Rijs and Hein van der Lee, Department of Microbiology, Radboudumc, Nijmegen, The Netherlands.
Strategies for treating aspergillosis due to azole-resistant *Aspergillus fumigatus*: From the bench to the bedside

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ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het college van decanen
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Strategies for treating aspergillosis due to azole-resistant *Aspergillus fumigatus*: From the bench to the bedside

An academic essay in

Medical Sciences

Doctoral Thesis

to obtain the degree of doctor
from Radboud University Nijmegen

on the authority of the Rector Magnificus prof. dr. S.C.J.J. Kortmann,

according to the decision of the Council of Deans

to be defended in public on Friday, April 25, 2014

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Dr. J.F. Meis
To my parents, my wife and my daughter
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Chapter 1

General introduction, aim and outline of the thesis
Introduction

1. Aspergillus fumigatus and aspergillosis

In humans, *Aspergillus fumigatus* is the most common and life-threatening aerial fungal pathogen, especially among immunocompromised hosts, followed by many other species. *A. fumigatus* was described by Fresenius in 1863 from the bronchi and alveoli of a great bustard (*Otis tarda*). It has been recognized for most of this century as a pathogen, capable of invading human lungs, eyes, pharynx, skin and open wounds (1-5). The potential of aspergillus to cause severe disease in humans was recognized by Young and colleagues in 1966 when a series of 98 patients was described with invasive aspergillosis (6). Aspergillosis is an umbrella term coined by Hinson, Moon, and Plummer in 1952, covering a range from localized conditions to fatal disseminated infections in humans and various animals, caused by fungi belonging to the genus *Aspergillus* (7-10).

Inhalation of *A. fumigatus* spores (conidia) into the lungs can cause multiple diseases in humans, depending on the immunological status of the host, including invasive pulmonary aspergillosis, aspergilloma, and different forms of hypersensitivity diseases such as allergic asthma, hypersensitivity pneumonitis, and allergic bronchopulmonary aspergillosis (7, 11). In addition, *A. fumigatus* can also cause acute community-acquired pneumonia in immunocompetent hosts. This is a rare and much less common infection that carries a uniformly fatal prognosis (12). Underlying lung disease and systemic illness are factors predisposing to this entity. Generally, high concentrations of spores are necessary for infection. Healthy hosts are able to ward off infections, so that severe illness usually results only from massive or long-term exposure (9, 13).

Notably, the population at risk for invasive aspergillosis (IA) is expanding due to recent advances in human medicine and myeloablative therapies, such as: patients on steroids and chemotherapy treatment resulting in severe neutropenia, stem cell and solid organ transplantation for end organ disease, advances in the development of immunosuppressive and myeloablative therapies for autoimmune and neoplastic disease, later stages of AIDS, and a hereditary diseases such as chronic granulomatous disease (14, 15).

Approximately 300,000 people are estimated to develop invasive aspergillosis annually, 1.5%–10% of the millions of highly immunocompromised patients at risk worldwide (14). The global burden of chronic pulmonary aspergillosis (CPA) has recently been estimated at 3 million patients (14, 16).
In animals, aspergillosis is primarily a respiratory infection that may become generalized; however, tissue predilection varies among species. The most common forms are pulmonary infections in poultry and other birds, (9) mycotic abortion and mammary gland infections in cattle (17-27), guttural pouch (auditory tube diverticulum) mycosis in horses (28-36), infections of the nasal and paranasal tissues, intervertebral sites, and kidneys of dogs (37-47), pulmonary and intestinal infections in domestic cats (10, 48-54), and pneumonia associated with disseminated infections in marine mammals (55-57).

2. Phylogeny and subgeneric taxonomy of *Aspergillus* spp.

*Aspergillus* spp. are ubiquitous fungi, usually found almost everywhere on earth, notably more common in the tropics. The great majority of species are saprophytes, commonly or occasionally found in soil, decaying vegetation, seeds and grains. Only a few well-known species are considered as important pathogens of humans or animals (13, 58). The taxonomy of genus *Aspergillus* has been recently reclassified (59, 60), as shown in figure1.

Polyphasic taxonomy has had a major impact on the species concept of the genus *Aspergillus*, which has classified the genus *Aspergillus* into 8 distinct subgenera, including *Aspergillus, Fumigati, Circumdati, Terrei, Nidulantes, Ornati, Warcupi,* and *Candidi* (59). These subgenera are further divided into 22 sections, each of which includes a number of related species (59). Although there are more than 200 known species in the genus, only a small percentage is associated with infection. Among them, *A. fumigatus* (subgenus *Fumigati*, section *Fumigati*), *A. flavus* (subgenus *Circumdati*, section *Flavi*), and *A. niger* (subgenus *Circumdati*, section *Nigri*) are the most frequently encountered species (11, 59, 61). Others, such as *A. terreus* (subgenus *Terrei*, section *Terrei*), *A. versicolor* (subgenus *Nidulantes*, section *versicolor*), *A. nidulans* (subgenus *Nidulantes*, section *Nidulantes*) are occasionally isolated from clinical specimens (62).
Figure 1. The phylogenetic relationships of 10 gene regions of *Aspergillus* species. (Adapted from Aspergillus in genomic era. Varga and Samson. Wageningen Academic Publishers, The Netherlands, 2008.)
3. Antifungal drugs

Antifungals can be grouped into three classes based on their site of action in pathogenic fungi (Fig. 2). Depending on the strategy chosen, different drugs can be used (63, 64).

The triazole agents exert their antifungal activity by blocking the demethylation of lanosterol, thereby inhibiting ergosterol synthesis. They have an expanded-spectrum with fungicidal activity against a wide spectrum of moulds as well as enhanced activity against *Candida* spp. and other yeasts.

The polyenes exert their antifungal activity via binding to ergosterol in the fungal cell membrane. This disrupts cell permeability and results in rapid cell death. The echinocandins represent the newest class of antifungals.

The mechanism of activity of the echinocandins is inhibition of the production of (1,3)-β-d-glucan, an essential component in the fungal cell wall. The spectrum of activity is therefore limited to pathogens that rely on these glucan polymers and is less broad than the spectrums of polyenes or azole agents. The echinocandins exhibit fungicidal activity against many *Candida* spp., making this drug class a desirable alternative to the azole agents, which exhibit only static activity against yeasts. Because mammalian cells have no cell wall, the echinocandins have very few toxic adverse effects in humans [7].
Figure 2. Targets of systemic antifungal agents. Adapted from Mukherjee et al. Clinical Microbiology Reviews. 2005; 18(1):163-94. Echinocandins destroy cell wall, allowing other antifungals (polyenes, azoles, and 5-FC) to enter. Azoles and polyenes can inhibit or bind to ergosterol in cell membrane in cell membrane, cause cell lysis and allowing 5FC to enter the cell and inhibit nucleic acid synthesis.
4. Treatment of *Aspergillus* diseases in humans

Triazole antifungals play an important role in the management of *Aspergillus* diseases (64, 65). Three triazole compounds (itraconazole, voriconazole, and posaconazole) have been clinically licensed and are currently in wide use for the prevention and treatment of invasive aspergillosis (66, 67). A fourth triazole, isavuconazole, is expected to be licensed in the near future. Itraconazole, voriconazole, posaconazole and isavuconazole have been shown to be fungicidal against *Aspergillus* spp. (68-70).

Itraconazole is commonly used for the treatment of chronic and allergic conditions (66, 67). Voriconazole is recommended first choice treatment of invasive aspergillosis with a label indication in adults and children aged 2 and above (67). In addition, voriconazole is the drug of choice for treatment of central nervous system aspergillosis (71). Posaconazole is licensed for patients aged 18 years or older (66); for prophylaxis in patients receiving remission-induction chemotherapy for acute myelogenous leukemia (AML) or myelodysplastic syndromes (MDS) expected to result in prolonged neutropenia and who are at high risk of developing invasive fungal infections; for prophylaxis of invasive fungal infections in hematopoietic stem cell transplant (HSCT) recipients who are undergoing high-dose immunosuppressive therapy for graft versus host disease and who are at high risk of developing invasive fungal infections; and for salvage therapy of invasive aspergillosis in patients with disease that is refractory to amphotericin B or itraconazole or in patients who are intolerant of these medicinal products (64, 65, 72, 73).

Besides to azoles, only liposomal amphotericin B and the echinocandins (caspofungin, micafungin, anidulafungin) have been shown better evidence supporting useful clinical activity against aspergillus diseases and therefore considered as alternative primary therapy of invasive aspergillosis (64).

5. Problem of azole resistance in *Aspergillus* spp

(Intrinsic vs. Acquired)

Although *Aspergillus* spp. are generally susceptible to the above mentioned compounds, intrinsic and acquired resistance has been documented. In general there are two types of resistance; microbiological versus clinical. Microbiological resistance relates to an *in vitro* susceptibility test, which indicates that the activity of a certain drug against the pathogen is low or absent and corresponds with a high probability of treatment failure. *In vitro* resistance can be primary (intrinsic) or secondary (acquired).
Primary resistance occurs naturally, without prior exposure to the drug. Secondary resistance is generated following exposure to an antifungal and may be associated with an altered gene expression (74, 75). Clinical resistance, however, is when a patient fails to respond to antimicrobial therapy despite the administration of an adequate antifungal, which might be due to microbiological resistance of the pathogen but could also be attributed to other factors related to the host or the drug (74).

Notably, recent changes in the taxonomy of *Aspergillus* spp. have had major implications on our understanding of drug susceptibility profiles (76). New sibling species of *A. fumigatus* exhibit *in vitro* susceptibility profiles that differ significantly from that of *A. fumigatus*. While acquired azole-resistance is an emerging problem in *A. fumigatus*, (77, 78) other *Aspergillus* spp may be intrinsically resistant to specific classes of antifungal agents (Table 1). Minimum inhibitory concentrations (MICs) of amphotericin B and azoles for some of the non-*fumigatus* *Aspergillus* spp. are elevated compared to *A. fumigatus* (76). The MICs of *A. flavus* clinical isolates to amphotericin B are consistently two-fold dilution steps higher than those of *A. fumigatus* (79). Using Clinical Laboratory Standards Institute methodology (CLSI) (80), *A. nidulans* was shown to have MIC values of 1 to 2 mg/L of amphotericin B, which is higher than commonly observed with *A. fumigatus* (81). In the section Usti, the azoles are not active against *A. calidoustus* with MICs of ≥8 mg/L, and the other classes of antifungal drugs also appear less active compared with their activity against *A. fumigatus*. For instance, the MICs of amphotericin B were shown to be 1 to 2 mg/L, which is relatively high (82). Resistance of *A. terreus* to amphotericin B is well recognized (83). Based on susceptibility to the azoles three different susceptibility patterns were distinguished in the black aspergilli; *Aspergillus* section Nigri. Some isolates show low azole MICs, others high MICs, and a third group showed an uncommon paradoxical effect. However, these groups did not coincide with species boundaries, making it difficult to interpret as an intrinsic or acquired property of these molds in invertebrates (84).

In *A. fumigatus* two routes of resistance selection have been reported; Azole resistance has been reported in patients with chronic cavitating *Aspergillus* diseases, such as aspergilloma, that receive long-term azole therapy (85). In these patients the initial infection is caused by an azole-susceptible isolate, but through therapy azole-resistant isolates may be cultured. A second route of resistance selection is believed to occur through exposure of *A. fumigatus* toazole 14α-demethylase inhibitors (DMIs) in the environment (75, 86-88), although still controversial (89). Azole fungicides inhibit fungal Cyp51A activity and are abundantly used for crop protection and material preservation. *A. fumigatus*, which is a saprophytic fungus, is believed to become resistant in the environment through exposure to azole fungicides that exhibit activity against this species (75).
Five DMIs, from the triazoles fungicides were identified with a molecule structure that is highly similar to that of the medical triazoles (75, 88) A. fumigatus may develop resistance mechanisms against these azole fungicides and, due to the molecule similarity; the medical triazoles are inactive as well.

A wide range of mutations in A. fumigatus have been described conferring azole resistance commonly involving modifications in the cyp51A-gene, the target of antifungal azoles. Specific mutations correspond with various phenotypes characterized by complete loss of activity of a specific azole, and with decreased activity of others (78). Cyp51A mutations in A. fumigatus commonly affect the activity of all mold-active antifungal azoles.

Notably, case series have been published including both patients with azole resistant chronic Aspergillus diseases and azole-resistant invasive aspergillosis that show the recovery of an azole-resistant isolate is associated with a high probability of azole treatment failure (85, 86, 90-96). In addition, a number of single cases have been described, in which patients with infection due to an azole-resistant isolate failed to azole therapy (85, 86, 90-97).

6. Azole resistance phenotypes in A. fumigatus

Antifungal drug resistance is normally quantified using the MIC. Both the CLSI and European Committee on Antimicrobial susceptibility Testing-subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST) have developed and standardized phenotypic methods that enable the reliable and reproducible determination of the MIC for conidia-forming molds or Aspergillus spp (80, 98).

The MIC is a central component of the PK/PD of antifungals indicating an appropriate antifungal treatment, which represents the lowest drug concentration resulting a notably reduction or complete lack of fungal growth (99). Particularly, in long-term treatment with antifungal agents, it is very important to prevent the evolution and development of resistance by the same strain or replacement by a new strain (96).

There are currently three sets of breakpoints and epidemiological cut-off values available. The first was published in 2009 by Verweij et al. based on clinical experience and the available knowledge at that time (77). Since then breakpoints have been published by the CLSI (100) and the EUCAST-AFT (101, 102), in which <2 mg/L is considered susceptible for itraconazole and voriconazole and ≥2 mg/L resistant; and for posaconazole; ≤ 0.25 and >0.5 mg/L, respectively.
Table 1. Examples of intrinsic resistance against antifungals in *fumigatus* and non-*fumigatus* Aspergillus species.


### 7. Azole resistance genotypes in *Aspergillus* spp

Several mechanisms of resistance have been described in Aspergillus spp. Azole resistance has most commonly been associated with alterations in cyp51A, which represents the target enzyme of the azoles (78). The corresponding phenotype depends on the particular base substitution and often the activity of more than one triazole is affected.
Azole-resistant *A. fumigatus* isolates have been reported as multidrug resistant (93), multi-azole resistant (103), azole cross-resistant (104) and multiple-triazole resistant (78, 86) isolates. The most frequently characterized hot spots are at codons 54, 98, 138, 220 and 448, although several other single nucleotide polymorphisms (SNPs) have been reported (78, 86, 103, 105-107). In addition to SNPs, a combination of genetic changes has been described in azole-resistant *A. fumigatus* isolates which increases cyp51A expression (78, 86, 105, 106).

Up until now, three mechanisms have been described: a 34 base pair tandem repeat combined with a L98H substitution in the Cyp51A-gene (TR\textsubscript{34}/L98H) (105), a 53 bp tandem repeat without substitutions in the Cyp51A-gene (TR\textsubscript{53})(108), and recently a 46 bp tandem repeat with two substitutions in the cyp51A-gene (TR\textsubscript{46}/Y121F/T289A)(81).

In addition, Camps et al. recently reported a novel resistance mechanism, consisting of a mutation in the CCAAT binding transcription factor complex subunit HapE (109). The substitution was found in P88L within the exonic region of HapE gene causing the resistance phenotype. Unlike cyp51A-mediated resistance mechanisms, HapE was associated with a fitness cost (110) as is the case for *A. fumigatus*, azole resistance in other species of *Aspergillus* such as *A. flavus* (111), and *A. terreus* (112), may be also caused by alterations and over-expression of the azole target 14a-demethylase (113).

Cyp51B overexpression is also considered a possible azole resistance mechanism in *A. fumigatus* (113). This indicates that acquired azole resistance is a clinical challenge that is not restricted to *A. fumigatus*, and evidence of non-target resistance is increasing.

### 8. Clinical implications of azole resistance and impact of underlying diseases

There are currently no randomized controlled trials that show azole resistance is associated with an increased probability of treatment failure compared to infection due to wild type isolates. However, case series have been published including both patients with azole-resistant chronic *Aspergillus* diseases and azole resistant invasive aspergillosis that show the recovery of an azole resistant isolate is associated with a high probability of azole treatment failure (85, 86, 90-96).

In the study of Howard et al. a wide range of mutations was found in azole-resistant *Aspergillus* spp. isolates that were cultured from clinical samples in patients underlying non-invasive *A. fumigatus* infections treated with azoles (85).
Two case series of patients with azole-resistant invasive aspergillosis were reported from the Netherlands (81, 114). In one study, eight patients with proven or probable, culture-positive invasive aspergillosis due to A. fumigatus harboring the TR\textsubscript{34}/L98H resistance mechanism were described. Overall, seven of eight (88%) patients had died at 12 weeks.

In a second study the emergence of a voriconazole highly resistance mechanism was described, associated with the TR\textsubscript{46}/Y121F/T289A resistance mechanism (81). At 12 weeks after recovery of the TR\textsubscript{46}/Y121F/T289A isolate, 4 of 8 patients with invasive aspergillosis had died and 2 patients had a persisting infection.

In addition, a number of single cases have been described harboring TR\textsubscript{34}/L98H (85, 86, 90-96) or TR\textsubscript{46}/Y121F/T289A resistance mechanisms (97). In all cases, patients with infection due to an azole-resistant isolate failed to azole therapy. Primary invasive infections due to resistant A. fumigatus isolates have been reported involving the lung (78, 85), bone (90) and brain (85, 91) as well from respiratory isolates in allergic bronchopulmonary aspergillosis (103). However, there is no apparent risk of spread of azole-resistant isolates to other patients.

Notably, it should be considered that there are numerous factors that impact on treatment outcome. Patients with refractory underlying malignancy are prone to fail to azole therapy, even if the infection is caused by an azole-susceptible isolate. Azole exposure might have been insufficient in patients failing therapy and as most patients were culture-positive, treatment might have been initiated relatively late in the course of the infection. Furthermore, azole-resistant infection might occur predominantly in patients in poor clinical condition, compared to wild type isolates. In the absence of robust clinical evidence, experimental models of Aspergillus infection can help us to understand the implications of MIC elevation on treatment efficacy.

The clinical observations are also supported by animal models of IA, where the MIC was shown to have major implications for the efficacy of voriconazole and posaconazole (115, 116). Recently the efficacy of voriconazole and posaconazole was evaluated in a murine model of aspergillosis against three A. fumigatus isolates with different cyp51A substitutions, conferring different in vitro susceptibilities to the drug (115, 117). There was a clear association between the MIC and efficacy in the animal model, with increasing MIC corresponding with decreasing efficacy (115, 117).
9. Epidemiology of azole resistance in *A. fumigatus*

Acquired azole resistance among *Aspergillus* spp. to triazoles is considered to be an emerging phenomenon (104, 118, 119), reported in different continents (85, 86, 95, 96, 114, 120-123). The number of studies that report azole-resistance in *A. fumigatus* has increased in recent years, for which both intrinsic and acquired resistance has been documented depending on the geography and the patient group. Therefore, knowledge of the local epidemiology of azole-resistant *Aspergillus* diseases is important with respect to the development of management strategies.

Notably, the prevalence and local epidemiology of azole-resistance varies depending on the geography and the patient group. Reports from the Netherlands and Manchester display an alarming increase of azole-resistance in *A. fumigatus* since 1998 (Figure 3) (77). In Manchester, the first published case of itraconazole resistance in *A. fumigatus* appeared in 1997 (the isolate originated from the late 1980) (124), then in 2000, epidemiological surveys showed a 2% prevalence of itraconazole-resistance (125), and in 2007 the percentage of patients with an azole-resistant *A. fumigatus* increased up to 15% (78, 104).

In the Netherlands azole resistance increased dramatically from 2.5% in 2000, to 4.9% in 2002, to 6.6% in 2004 and 10% in 2009 (114). In most azole-resistant isolates a specific *Cyp51A* gene-mediated resistance mechanism was reported (TR34/L98H) both in clinical and environmental isolates (86). TR34/L98H first emerged in clinical *A. fumigatus* isolates from Netherlands in 1998 and this resistance mechanism is now endemic in Dutch hospitals (126). *Aspergillus*-related diseases due to TR34/L98H included non-invasive infections and invasive aspergillosis, and infections were found to occur both in azole-treated as well as in azole-naive patients (86, 114).

Importantly, the geographic area where TR34/L98H is reported coincides with the region with the most intensive use of fungicides, therefore, an environmental source is very likely (86, 87). Azole resistance, due to the TR34/L98H resistance mechanism, was also reported in clinical *A. fumigatus* isolates from other European countries, and more recently also in China and India (85, 86, 95, 96, 120-122, 127-131). From a global perspective, fungicide use is second highest in the Asia–Pacific regions (24%), preceded only by western Europe (37%) (132). By contrast, Howard et al. suggest that the widespread increase of azole-resistance in Manchester is related to long-term azole treatment in patients (85).
Figure 3: The percentage of patients with azole-resistant *A. fumigatus* strains in Manchester (United Kingdom), and Nijmegen (the Netherlands) between 1998 to 2007. Adapted from Verweij et al. Drug Resist Updat. 2009;12(6):141-7.
Outline of this thesis

Azole resistance is an emerging problem in hospitals and it remains unclear how patients with azole-resistant aspergillus diseases are best managed. This thesis uses \textit{in vitro} and \textit{in vivo} studies to explore alternative approaches aimed at optimizing the treatment of azole-resistant aspergillus diseases in humans.

\textbf{Chapter 1} encompasses the introduction.

\textbf{Chapter 2} discusses the development and standardization of experimental models evaluating pharmacodynamic and pharmacokinetics of antifungals.

\textbf{Chapter 3} describes the pharmacokinetic(PK)-pharmacodynamic(PD) properties of anidulafungin monotherapy in a non-neutropenic murine model of invasive aspergillosis.

\textbf{Chapter 4.1} investigates the \textit{in vitro} antifungal activity of voriconazole either alone or in combination with anidulafungin against a collection of clinical \textit{A. fumigatus} isolates, including voriconazole-resistant isolates with various substitutions in the cyp51A gene and voriconazole-susceptible isolates, to determine the interaction between these two agents.

\textbf{Chapter 4.2} determines the \textit{in vivo} efficacy of voriconazole and anidulafungin in a non-neutropenic murine model of invasive aspergillosis using a voriconazole-susceptible and a voriconazole-resistant \textit{A. fumigatus} clinical isolate.

\textbf{Chapter 5} focuses on the pharmacodynamics and dose-response relationships of liposomal-amphotericin B against wild-type and three clinical azole-resistant \textit{A. fumigatus} isolates harboring different resistance mechanisms in an immunocompetent murine model of disseminated aspergillosis.

\textbf{Chapter 6.1} provides an overview of our current understanding of azole resistance and the potential role of voriconazole and posaconazole in treatment of patients with azole-resistant aspergillus diseases.

\textbf{Chapter 6.2} focuses particularly on target concentrations of voriconazole and posaconazole, and the utility of therapeutic drug monitoring as an approach to ensure adequate drug exposure.

\textbf{Chapter 7} encompasses the general discussion and future prospectives.
References


Assessment of efficacy of antifungals in experimental models of invasive aspergillosis in an era of emerging resistance: the value of real-time quantitative PCR

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Abstract

Experimental models of invasive aspergillosis (IA) have been used to explore pharmacokinetic and pharmacodynamic (PK/PD) properties of antifungal agents. Survival is still considered the golden standard effect measure but has the disadvantage that a large number of animals are needed to determine the dose–response relationships and PK/PD of antifungals. The feasibility of using fungal load by real-time quantitative PCR (qPCR) as an effect measure has been explored recently. The majority of studies reported convincingly demonstrate a larger dynamic range for qPCR compared to conventional assays. However interpretation and translating the results to guidance in clinical decision making need further study. It is expected that the use of qPCR will become the primary outcome measure for assessment of PK/PD relationships of antifungals in experimental models of IA.

Keywords

Invasive aspergillosis, Experimental model, Fungal load, Quantitative PCR, Therapeutic efficacy
Introduction

Whereas invasive aspergillosis (IA) remains an infection with significant mortality and morbidity ranging from 30 to 80%, the use of clinically licensed azoles such as voriconazole and posaconazole has improved the outcome of patients [1]. However, optimizing antifungal therapy in patients still needs to be addressed. Clinical trials in humans provide valuable evidence for the use of antifungal agents, but these studies are limited due to ethics, time and cost.

Experimental models of IA have become a cornerstone to explore pharmacokinetic and pharmacodynamic (PK/PD) relationships of antifungal agents as well as the comparative utilities of diagnostic markers. In addition, animal models allow predicting the impact of resistance on outcome for IA [2, 3]. This is of particular importance, since resistance, in particularazole resistance in *Aspergillus fumigatus* is increasing [4].

In contrast to investigations evaluating the exposure–response relationships of antibacterials where colony forming units (CFU) have become the mainstay of effect measurements [5], the most commonly used efficacy measures for antifungals are prolongation of survival and various parameters of reduction in tissue burden [6].

However, measurements of tissue burden in IA suffer from a significant number of problems and non-culture based methods in particular qPCR, are rapidly becoming the new gold standard tool for the diagnosis, detection and evaluation of tissue burden of *A. fumigatus*. We here discuss applications and limitations of qPCR for assessment of therapeutic efficacy of antifungal agents in experimental models of IA.

Benefits and limitations of conventional parameters to monitor therapeutic efficacy in IA

At present survival is considered the most reliable effect measure to assess therapeutic efficacy of antifungals in IA animal models infected by both azole susceptible and resistant *A. fumigatus*. For example, in the recent study of Mavridou et al. in an immunocompetent non-neutropenic murine model of disseminated IA, increased MICs correspond with reduced in vivo efficacy [7]. Overall, there was a good relationship between the area under the concentration–time curve (AUC)/MIC ratio and survival (Figure 1).
AUC/MIC was a better predictor than dose/MIC because of the non-linear pharmacokinetics of voriconazole resulting in a disproportional increase in AUC by dose. Such PK/PD relationships can subsequently be used to help deducing dosing regimens and clinical breakpoints in humans.

Although survival studies are still considered the gold standard method to assess the efficacy of antifungals in IA, it has the disadvantage that a large number of animals is needed [8]. Tissue burden studies can be completed more rapidly than survival studies and thus provide some impetus to the development and indications of drugs and also enable significant reduction in the number of animals required for experimental design [9,10]. However, measurements of tissue burden in IA suffer from a significant number of problems.

Choosing the best quantification method has been the major problematic issue. The first parameter used was CFU quantitation in selected organs, mostly in the kidneys, liver, lungs, or brain. However, due to the filamentous nature of *A. fumigatus*, a large fungal mass composed of hundreds of cells may be recorded only as a single unit by the traditional CFU methodology and CFU counts do not accurately reflect the number of viable cells for filamentous fungi such as *A. fumigatus* [11]. 1-3, β-D- Glucan has shown promise as a diagnostic adjunct; however, this marker has a limited detection range and more research is required to define the utility of this assay, in particular in non-neutropenic models of IA [12]. The galactomannan (GM) assay has moderate accuracy for diagnosis of invasive aspergillosis [13]. Recently several studies have shown the increased sensitivity and precision of *A. fumigatus* qPCR over CFU and biomarkers measurement that will be discussed in the next section.
Figure 1. (a) Voriconazole dose–survival and (b) AUC/MIC–survival relationships for four *A. fumigatus* isolates with different MICs. Increased voriconazole exposure was required to obtain maximum efficacy in mice infected by isolates with attenuated susceptibility. From reference [7].

Quantitative PCR as an outcome parameter for evaluating antifungal therapy

A number of studies have indicated that a real-time quantitative PCR assay could be used to measure the fungal burden in organs and thus monitor the progression of infection and efficacy of antifungal therapy (Table 1). To that purpose, it is essential that the load of fungal DNA in blood or tissue specimens corresponds to tissue burden and/or survival [14,15,16] in various experimental situations, including acute and chronic infection models. The rationale is that PCR-based quantification of *A. fumigatus* tissue burden can detect every cell in a filamentous fungal mass, and therefore significantly better than CFU counts. Apart from homogenization issues (see below) the dynamic range of CFU determination is too narrow for filamentous fungi [11] and it may underestimate the absolute fungal burden in an established infection compared to qPCR and GM [17].
<table>
<thead>
<tr>
<th>Reference</th>
<th>Animal model</th>
<th>Drug used for efficacy</th>
<th>Route of infection</th>
<th>Type of specimen</th>
<th>Tissue homogenization</th>
<th>Quantitation method</th>
<th>Correlation between qPCR and CFU</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bowman et al [18]</td>
<td>Mice</td>
<td>Caspofungin, Amphotericin-B</td>
<td>IV</td>
<td>Kidney, Brain, Lung, Spleen, liver</td>
<td>Mechanical pressure, mixing with glass beads and centrifugation</td>
<td>CFU, qPCR</td>
<td>0.80</td>
<td>18S rRNA</td>
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<td>Osullivan et al [22]</td>
<td>Rabbit</td>
<td>Amphotericin-B</td>
<td>IV</td>
<td>BAL, lung</td>
<td>Mechanical disruption by high speed fast prep</td>
<td>CFU, real time FRET</td>
<td>0.72</td>
<td>ITS1, 5.8S, ITS2  rRNA</td>
</tr>
<tr>
<td>Wiederhold et al [32]</td>
<td>Mice</td>
<td>Caspofungin</td>
<td>Sinopulmonary</td>
<td>Lung</td>
<td>Mechanical pressure, mixing with glass beads and centrifugation</td>
<td>qPCR</td>
<td>-</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>Singh et al [24]</td>
<td>Mice</td>
<td>Caspofungin, Amphotericin-B</td>
<td>Intracerebrally</td>
<td>kidney, Brain</td>
<td>Rolling a large bottle over the whirl-pak bags</td>
<td>CFU, GM-EIA, qPCR</td>
<td>0.87* 0.32*</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>MacCallum et al [19]</td>
<td>Guinea pig</td>
<td>Caspofungin, Voriconazole</td>
<td>IV</td>
<td>Kidney</td>
<td>Mechanical pressure, mixing with glass beads and centrifugation</td>
<td>qPCR</td>
<td>0.65** 0.43**</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>Sheppard et al [23]</td>
<td>Mice</td>
<td>-</td>
<td>Aerosol</td>
<td>Lung</td>
<td>Blunt crushing in whirl-pak bags</td>
<td>CFU, GM-EIA, qPCR</td>
<td>0.05</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>Francesconi et al [21]</td>
<td>Rabbit</td>
<td>Ravuconazole, Micafungin, Amphotericin-B</td>
<td>Intratracheal</td>
<td>BAL, Lung</td>
<td>Mechanical disruption by high speed fast prep</td>
<td>CFU, GM-EIA, qPCR</td>
<td>0.80</td>
<td>ITS1, 5.8S, ITS2  rRNA</td>
</tr>
<tr>
<td>Gomez-Lopez et al [20]</td>
<td>Rabbit</td>
<td>-</td>
<td>Intratracheal</td>
<td>Blood, Serum, Lung, Brain</td>
<td>Wizard SV genomic DNA purification system</td>
<td>CFU, GM-EIA, real time FRET, Panfungal PCR assay</td>
<td>-</td>
<td>18S rRNA, ITS1, ITS2  rRNA</td>
</tr>
<tr>
<td>Van Vianen et al [28]</td>
<td>Rat</td>
<td>Caspofungin</td>
<td>Intratracheal</td>
<td>Lung</td>
<td>Mechanical pressure, mixing with glass beads and centrifugation</td>
<td>GM-EIA, real time PCR</td>
<td>-</td>
<td>18S rDNA</td>
</tr>
<tr>
<td>Wiederhold et al [33]</td>
<td>Mice</td>
<td>Amphotericin-B deoxycholate</td>
<td>Sinopulmonary</td>
<td>Lung</td>
<td>Mechanical pressure, mixing with glass beads and centrifugation</td>
<td>qPCR</td>
<td>-</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>Study</td>
<td>Species</td>
<td>Antifungal(s)</td>
<td>Route</td>
<td>Tissue(s)</td>
<td>Sample Preparation</td>
<td>Methodology</td>
<td>Assay Type</td>
<td>rDNA Type</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>---------</td>
<td>------------------------</td>
<td>-------</td>
<td>-------------------</td>
<td>--------------------</td>
<td>----------------------------------</td>
<td>---------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Dennis et al [31]</td>
<td>MIC</td>
<td>Amphotericin-B deoxycholate, Micafungin</td>
<td>Intratracheal</td>
<td>Serum, Lung</td>
<td>Mechanical pressure, mixing with glass beads and centrifugation</td>
<td>GM-EIA, qPCR</td>
<td>-</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>Van de Sande et al [29]</td>
<td>Rat</td>
<td>Caspofungin</td>
<td>Intratracheal</td>
<td>Serum, Lung</td>
<td>Mechanical pressure, mixing with glass beads and centrifugation</td>
<td>GM, real time PCR</td>
<td>-</td>
<td>18S rDNA</td>
</tr>
<tr>
<td>Vallor et al [25]</td>
<td>Guinea pig</td>
<td>Voriconazole, Aerosol</td>
<td>Serum, Lung</td>
<td>Bead beater homogenizer</td>
<td>CFU, GM-EIA, qPCR</td>
<td>-0.86</td>
<td>FKS, 18S rRNA</td>
<td></td>
</tr>
<tr>
<td>Petraitis et al [10]</td>
<td>Rabbit</td>
<td>Anidulafungin, Voriconazole</td>
<td>Endotracheal</td>
<td>BAL, Serum</td>
<td>Mechanical disruption by high speed fast prep</td>
<td>CFU, GM-EIA, qPCR</td>
<td>-</td>
<td>ITS1, 5.8S, ITS2 rRNA</td>
</tr>
<tr>
<td>Zhao et al [27]</td>
<td>Rat</td>
<td>-</td>
<td>IV</td>
<td>BAL, lung, whole blood</td>
<td>Adding PBS to tissue and spin the primary homogenates for using the pellet</td>
<td>CFU, qPCR, real time NASBA assay</td>
<td>BAL -0.40, Lung -0.50</td>
<td>18S rRNA, 28S rDNA</td>
</tr>
<tr>
<td>Arendrup et al [30]</td>
<td>Mice</td>
<td>Posaconazole, Anidulafungin</td>
<td>IV</td>
<td>Kidney</td>
<td>Automated tissue Lyser</td>
<td>CFU, qPCR</td>
<td>-</td>
<td>28S rDNA^4</td>
</tr>
</tbody>
</table>

**Table 1.** Studies describing fungal load quantification to assess therapeutic efficacy of antifungals in experimental models of IA.

* Assays to detect drug efficacy.
** Assays to detect progression of infection.
1^Correlation between qPCR and quantitation methods (Spearman's rank correlation coefficient).
2^Immediately post infection when organisms remain as conidia.
3^Later time-points when organisms have formed hyphae.
4^In these studies infection has been caused by *Aspergillus fumigatus* and *Aspergillus sp.*
The majority of studies that have been performed to date indicate an increased sensitivity and precision of real-time qPCR over CFU measurement and biomarker assays.

For instance, in a mouse model of invasive aspergillosis treated with amphotericin-B and caspofungin, Bowman et al. observed a 4 log10 decrease in conidial equivalent (CE) counts, while in the same animals a 1 log10 decrease in the number of CFU counts, substantiating the problems stated above. Recovery of CFU did not reflect progression or increasing numbers of A. fumigatus in the infected tissues [18]. Similar results have been shown using qPCR for the assessment of kidney fungal burden in a guinea pig model IA [19].

The performance of 2 quantitative polymerase chain reaction (PCR) assays was compared with quantitative cultures and GM antigen detection in a rabbit model of invasive aspergillosis using blood, serum, lung, and brain specimens [20]. The authors concluded that specific real-time PCR assay is a reliable technique to detect A. fumigatus DNA in vivo comparable to cultures and GM determination. These results are also in agreement with the observation of Francesconi et al., Osullivan et al. and Petriatis et al. [10,21,22].

A similar study was conducted by Sheppard et al. [23]. They demonstrated that the method of tissue disruption used herein resulted in consistently higher CFU counts, highlighting another problem in tissue burden studies in IA. They also showed progressive increase in fungal load by qPCR in comparison with CFU during experimental infection and recommended the use of a qPCR assay, showing it to be less variable than the GM assay, a similar conclusion as found by Singh et al. [24].

In the study of Vallor et al., fungal burden and therapeutic efficacy of voriconazole were assessed using survival, quantitative culture, GM quantification, and quantitative PCR in a guinea pig model of IPA using an aerosol challenge of A. fumigatus spores [25]. Quantitative PCR, when used for tissue burden measurement, was positive earlier, correlated with the fungal aerosol delivery, and the burden continued to increase throughout the course of the study as was expected on the basis of histopathological examination and the rise in levels determined by GM. The tissue fungal burden assessed by qPCR consistently increased throughout infection, even after the other two diagnostic markers (CFU counts and GM results) appeared to have reached a plateau by day 5.

Similar to previous studies GM assessment of lung tissue burdens showed increased burdens after day 5, which correlated with the extent of infection similar to the results of previously described [20,21,25,26]. Administration of the drug produced statistically significant decreases in pulmonary fungal burden, as detected by CFU counting, qPCR, and GM. In daily assessment of the progression of fungal infection in serum, GM detection demonstrated a statistically significant reduction in the fungal load on days 6 and 7 postchallenge in voriconazole treated animals, compared to time matched controls.
Similar findings were reported in an inhalational rat model of invasive pulmonary aspergillosis, using a real-time nucleic acid sequence-based amplification (NASBA) method [27]. Fungal load in bronchoalveolar lavage (BAL) fluid, lung tissues, and whole blood was compared by CFU, quantitative PCR (18S rDNA) and real-time NASBA (28S rRNA). As expected, both NASBA and qPCR showed a progressive increase in lung tissue burdens, while the CFU counts were stable over time. The fungal burdens in BAL fluid were more variable and not indicative of a progressive infection. The data of this study are in line with results of van Vianen and van de Sande [28,29] in a model of aerogenic A. fumigatus infection in neutropenic rats.

Recently, Arendrup et al. evaluated therapeutic efficacy of posaconazole and anidulafungin singly or in combination in an immunosuppressed haematogenous IA mouse model of infection and compared survival, fungal CFU, and detection of DNA load in kidney by quantitative PCR was used for determination of fungal burden in kidney [30]. Anidulafungin alone or in combination with posaconazole significantly improved survival, significantly reduced kidney CFU by days 4 and 8 and copy number by days 4, 8 and 11 (Figure 2).

Surprisingly, in contrast to the studies reported above, in experimental IA in the p47 phox -/- mouse model of chronic granulomatous disease (CGD), lung fungal burden assessed by qPCR did not differ among treatment groups despite significant differences in survival between treatment groups [31]. A treatment effect was demonstrated in a high-inoculum hyper acute model of pulmonary aspergillosis in which survival and fungal burden were assessed 4 days after fungal challenge [32,33]. It was concluded that, the lack of utility of qPCR in modeling a treatment effect may reflect factors specific to the CGD mouse model as well as variables related to the inoculum and the route of fungal challenge.
Figure 2. Relationships between qPCR and effect of various antifungal therapeutic regimens during therapy in a murine model of infection. From Ref. [30].
Issues related to performance of quantitative PCR

Whereas the performance of qPCR is promising there are a number of issues, primarily technical that still need careful attention as well as standardization and this may have a significant impact on the qPCR results and interpretation thereof. Although specific primers are available and there is sufficient genomic information for developing *A. fumigatus* PCR assays (e.g. end point vs. quantitative) [34], DNA extraction techniques, specific primer design and tissue extraction can be further optimized, as well as the choice and volume of animal specimens (e.g. blood, kidney or respiratory secretions), with fraction of blood; serum vs. whole blood or homogenization of tissues [35,36,37].

The majority of PCR assays target multicopy genes, in particular the ribosomal DNA (rDNA) genes (18S, 28S, and 5.8S) and internal transcribed spacer (ITS) regions (ITS1 and ITS2) particularly for *A. fumigatus*, in order to maximize sensitivity and specificity (Table 1). This complex contains both conserved and variable sequences and there is a large volume of data deposited in public databases for a wide range of genera and species. The mitochondrial genes encoding some of the tRNA genes and cytochrome b have also been used as primer targets [38,39,40]. On the other hand, PCR-based assays have been reported for use in quantifying *A. fumigatus* in experimental infection using FKS1, which is a single-copy gene [25]. Whereas multicopy genes are good targets for improving sensitivity of the assay, it may not be ideal for standardization of a qPCR, and a balance needs to be found between a higher sensitivity, specificity and dynamic range.

When using tissues, utilizing the multicopy rRNA operon for PCR amplification significantly improves the available target. The targeting of the rRNA operon is common practice and designing primers to be pan-fungal allows the assay to be modified for additional fungi by using specific probes. However, cross reactivity with non-target fungi or animal host DNA may arise and this is a significant problem in evaluating treatment outcome [41].

In comparison, 28S region is multicopy (>100 copies in the fungal genome), universal fungal primers are available, and it contains highly variable regions for species identification. Also DNA sequence analysis is available for species identification [42]. With a focus on this target, quantitative PCR assays can detect DNA from a fraction of a single organism and minimize the probability of false negatives, which is an important aspect to define the lower limit of quantification in exposure-response studies.

To facilitate and maximize fungal DNA extraction and subsequent qPCR, primary tissue homogenates should be subjected to a secondary homogenization step to aid in the release and extraction of cell nuclei from all of the conidial and hyphal forms present [18]. This will decrease inter-experimental error.
Conventional techniques used to homogenize tissues may not completely disperse the fungus, leading to inconsistent results. Discrepancies can be circumvented by employing standardized procedures for homogenizing organs, as with a mechanical homogenizer for fixed times [37].

Numerous strategies have been adopted for DNA extraction from different sources including whole blood, serum, BAL, biopsy tissues and cerebrospinal fluids after homogenization. DNA extraction process is critical to the success of most PCR amplification systems, since efficiency of the A. fumigatus PCR is limited by the extraction procedure [37]. Considerable differences in DNA extraction protocols and performance are one aspect of molecular assays that hinders the comparison of studies. A standardized protocol for DNA extraction of A. fumigatus from whole blood specimens has been recently published by the EAPCRI (The European A. fumigatus PCR initiative), to improve the analytical sensitivity. They recommend the use of EDTA blood specimens, a red and white cell lysis step, and bead-beating to lyse the fungal element and elution volumes of less than 100 µl [36,37].

**Timepoints of measurement: dynamics of the assays**

In the study of Bowman et al, both GM and DNA are present as viable as well as nonviable fungal masses that have not yet been cleared by the host. This strong correlation exists because both methods allow the detection of an increasing fungal burden during the course of infection [21].

Similarly, other investigators demonstrated that fungal burdens assessed by qPCR and GM increased significantly during the progression of infection so that those assessed by semi quantitative culture, after an initial drop, remained relatively stable. The tissue fungal burden assessed by qPCR consistently increased throughout infection, even after the other two diagnostic markers (CFU counts and GM results) appeared to reach a plateau by day 5 [23,25,26].

In addition, qPCR and GM quantification appeared to correlate most closely with the rise in the fungal burden and the extent of infection. The relatively high GM concentration and CE counts at day 21 in the treatment groups indicate that a substantial fungal burden is still present in the infected lung tissue in animals that are clinically cured. Whether this fungal burden represents viable A. fumigatus organisms is not known [28].

From the above, it is clear that the dynamics of the various markers is different and conclusions with respect to efficacy of drugs therefore also depend on the time points used...
to determine the effects of the drugs. This will directly reflect on the interpretation of the qPCR results in the different models of IA, such as inhalation models and systemic models of infection.

Conclusive remarks

Real-time qPCR assay is a reliable and promising technique to detect *A. fumigatus* DNA in vivo when performed after an infection is firmly established and the organisms have germinated. The best correlations were observed between survival and non-culture qPCR methods, at all time-points of infection.

This technique is also promising to differentiate susceptible and azole-resistant *A. fumigatus* isolates with known polymorphisms in their CYP 51 gene to distinguish the differential effect of treatment for parent strains and resistant daughter strains.

Conserved multi copy region 28SrDNA of *Aspergillus* spp. seems to be the best choice for PCR amplification that contains highly variable regions for species identification.

Although technical consensus on DNA extraction is currently achieved by EAPCRI, a major problematic issue is that the use of quantitative PCR in animal models or human IA is still lacking in standardization, and results of investigators are difficult to compare with each other.

Further studies are still required to integrate this technique as primary outcome in efficacy and PK/PD studies of antifungals in experimental models of IA.
References


Pharmacodynamics of Anidulafungin against Clinical *Aspergillus fumigatus* Isolates in a Nonneutropenic Murine Model of Disseminated Aspergillosis

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Abstract

Azole resistance is an emerging increasing problem in *Aspergillus fumigatus* that results in treatment failure. Alternative treatments may improve the therapeutic outcome in patients with azole-resistant invasive aspergillosis (IA). Little is known about the *in vivo* efficacy of the echinocandin anidulafungin (AFG) in IA. The in vivo efficacy of 2.5, 5, 10, and 20 mg/kg of body weight AFG was assessed against two clinical *Aspergillus fumigatus* isolates with identical AFG minimum effective concentrations (MECs; 0.03 mg/liter) in a murine model of IA: a wild-type voriconazole (VCZ)-susceptible (VCZs) *A. fumigatus* isolate (AZN8196) and a VCZ-resistant (VCZr) *A. fumigatus* isolate (V52-35) harboring the TR34/L98H resistance mechanism (substitution at codon L98 in combination with a 34-bp tandem repeat in the promoter region of the *CYP51A* gene). The pharmacokinetics of AFG were also assessed for each dose. Increasing doses increased survival for both isolates in a manner dependent on the AFG dose level ($R^2 = 0.99$ and 0.95, respectively) up to a maximum of 72.7% and 45.45% for the VCZs and VCZr isolates, respectively. The area under the concentration-time curve (AUC) correlated significantly with the dose in a linear fashion over the entire dosing range ($R^2 = 0.86$). The Hill equation with a variable slope fitted the relationship between the 24-h AUC/MEC ratio and 14-day survival well ($R^2 = 0.87; P < 0.05$). The 50% effective AUC/MEC for total AFG was 126.5 (95% confidence interval, 79.09 to 202.03). AFG treatment improved the survival of mice in a dose-dependent manner; however, a maximal response was not achieved with either isolate even in those treated with the highest AFG dose.

Keywords

Azole-resistance, Pharmacokinetics, Pharmacodynamics, Anidulafungin, *Aspergillus fumigatus*
Introduction

Aspergillus fumigatus may cause life-threatening infections in both immunocompetent and immunocompromised patients (1–3). Voriconazole (VCZ) is considered the first choice of therapy for invasive aspergillosis (IA) (4, 5). However, the rate of azole resistance is increasing in A. fumigatus, which significantly complicates the management of IA, as azole resistance is associated with therapeutic failure and a mortality rate of up to 88% (6–13). Primary invasive infections due to resistant isolates involving the lung (13, 14), bone (15) and brain (14, 16) have been reported, as have respiratory isolates in patients with allergic bronchopulmonary aspergillosis (7).

Seventy-nine percent of isolates with the TR34/L98H mutation are VCZ resistant (VCZ'), and this mutation is the most prevalent resistance mechanism in clinical isolates (11). All patients with pulmonary aspergillosis due to TR34/L98H mutant isolates who received VCZ monotherapy died by the 12th week of therapy (11). Therefore, it is important to explore alternative treatment regimens, as alternative treatments may improve the therapeutic outcome in patients with azole-resistant IA.

Anidulafungin (AFG) belongs to the echinocandins but has a unique site of action different from that of azoles and polyenes, as it targets cell wall synthesis, and has fungistatic activity against Aspergillus spp., in addition to an excellent safety profile (17 - 19). Little is known about the in vivo efficacy of the echinocandin AFG in IA.

Here we investigated the pharmacokinetic(PK)-pharmacodynamic(PD) properties of AFG in a nonneutropenic murine model of IA. For this purpose, we used two clinical isolates with different profiles of susceptibility to voriconazole: a VCZ-susceptible (VCZ') A. fumigatus isolate and a VCZ' A. fumigatus isolate harboring a TR34/L98H mutation in the cyp51A gene.

Materials and Methods

Fungal isolates

Two clinical A. fumigatus isolates obtained from patients with proven IA were used in the experiments: a VCZ² isolate without mutations in the cyp51A gene (AZN 8196) and a VCZ' isolate (V52-35) harboring the TR34/L98H resistance mechanism. Strain identifications and the cyp51A gene substitutions were confirmed by sequence-based analysis as described previously (9). The isolates had been stored in 10% glycerol broth at -80°C and were revived by subculturing on Sabouraud dextrose agar (SDA) supplemented with 0.02%
chloramphenicol for 5 to 7 days at 35 to 37°C. The *in vitro* antifungal susceptibility test was performed on the basis of EUCAST guidelines, using a broth microdilution format (20).

<table>
<thead>
<tr>
<th>A. <em>fumigatus</em> isolate</th>
<th>Cyp51A substitution</th>
<th>MIC (mg/liter)</th>
<th>MEC (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amphotericin B</td>
<td>Posaconazole</td>
</tr>
<tr>
<td>AZN 8196</td>
<td>None</td>
<td>0.5</td>
<td>0.031</td>
</tr>
<tr>
<td>V 52-35</td>
<td>TR34/L98H</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Table 1.** Origin, *in vitro* susceptibilities, and underlyingazole resistance mechanisms of VCZ<sup>S</sup> and VCZ<sup>T</sup> A. *fumigatus* isolates

<sup>a</sup> The A. *fumigatus* isolates were from patients with proven invasive aspergillosis.

<sup>b</sup> Susceptible.

<sup>c</sup> Resistant.

**Infection model**

A total of 170 outbred female CD-1 mice (age, 4 to 5 weeks; weight, 20 to 25 g; Charles River, the Netherlands) were randomized into groups of 17 mice for AFG monotherapy. Animals were infected using the procedure described before (21, 22). Before performing the experiment, the isolates were cultured once on SDA for 7 days at 35 to 37°C and subcultured twice on 15-cm Takashio slants for 5 days at 35 to 37°C. The conidia were harvested in 20 ml of sterile phosphate-buffered saline (PBS) plus 0.1% Tween 80 (Boom B.V. Meppel, the Netherlands). The conidial suspension was filtered through sterile gauze folded four times to remove any hyphae, and the number of conidia was counted in a hemocytometer. After the inoculum was adjusted to the required concentration, the conidial suspension was stored overnight at 4°C.
The 90% lethal dose (LD₉₀) was separately determined for each isolate. Mice were infected via injection into the lateral tail vein of an inoculum corresponding to the LD₉₀ of each isolate. The LD₉₀s of VCZ² and VCZ' (TR34/L98H mutant) A. fumigatus isolates used in the current study were 2.4x10⁷ and 2.5x10⁷ conidia, respectively. Postinfection viability counts of the injected inocula were determined to ensure that the correct inoculum had been injected.

The animals were housed under standard conditions, with drink and feed supplied *ad libitum*. The animal studies were conducted in accordance with the recommendations of the European Community (Directive 86/609/EEC, 24 November 1986), and all animal procedures were approved by the Animal Welfare Committee of Radboud University (RU-DEC 2010-187). The infected mice were examined at least three times daily. These clinical inspections were carried out in order to ensure that there were no cases of desiccation, torticollis, staggering, high weight loss (a decrease of 15% within 48 h or 20% within 24 h), or body temperature drop to below 33°C. Mice demonstrating these signs of disease were humanely terminated.

On day 15 postinfection, all remaining surviving mice were humanely euthanized under isoflurane anesthesia, and blood and internal organs were collected. The survival (in number of days postinfection) was recorded for each mouse in each group and was the outcome effect measure used to assess the therapeutic efficacy of AFG monotherapy (23).

**Antifungal compound and treatment regimens**

Treatment groups consisted of AFG (Pfizer, Capelle a/d IJssel, the Netherlands) monotherapy at 2.5, 5, 10, 20, and 40 mg/kg of body weight/day. Intraperitoneal therapy was begun at 24 h postinfection for 7 consecutive days and was given once daily with standard daily dosing, in addition to a single loading dose of AFG. The control group received single doses of saline.

**Pharmacokinetic analysis of AFG in mice**

A total of 144 outbred female CD-1 mice (age, 4 to 5 weeks; weight, 20 to 22 g; Charles River, the Netherlands) were used for separate PK experiments. On day 0, mice were infected with the wild-type *A. fumigatus* isolate through the lateral tail vein, and after 24 h, treatment was initiated, as described above, at dosages of 5, 10, 20, and 40 mg/kg AFG. At day 2 of treatment (day 3 after infection), blood samples were drawn through
an orbital vein or by heart puncture and placed into lithium-heparin-containing tubes at 12 predefined time points: immediately before administration of drugs and subsequently at 0, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 48, and 72 h post-dose. Blood samples were cooled and centrifuged for approximately 10 min at 1,000 x g within 30 min of collection. Plasma was aspirated, transferred into two 2-ml plastic tubes, and stored at -80 °C.

**Analytical assay of anidulafungin**

Anidulafungin samples were measured by ultraperformance liquid chromatography (UPLC) with fluorescence detection. Samples were pretreated using a protein precipitation procedure (acetonitrile-methanol [50/50] and formic acid [0.1%]). A seven point calibration curve with three quality control samples was used. All measurements were done in duplicate. The dynamic range of the assay was 0.008 to 8.4 mg/liter, and the accuracy range (n = 15), which was dependent on the concentration, was 94.2% to 103.5%. The intraday precision varied between 0.9% and 1.8%, and the interday precision was between 0.5% and 1.6%. Validation in mouse plasma was over the dynamic range of 0.008 mg/liter to 5.9 mg/liter. The intraday precision varied between 101.0% and 104.8%. Three freeze-thaw cycles did not impact the stability of anidulafungin. Geometric mean concentrations of AFG in plasma from three mice were separately calculated per time point. Maximum concentrations in plasma (Cmax) were directly observed from the data. Pharmacokinetic parameters were derived using non-compartmental analysis with WinNonLin, version 5.2, software (Pharsight, Inc.). The area under the plasma concentration-time curve (AUC) from time zero to 24 h post-infusion (AUC0-24) was determined by use of the log-linear trapezoidal rule. The elimination rate constant was determined by linear regression of the terminal points of the log-linear plasma concentration-time curve. The terminal half-life was defined as ln 2 divided by the elimination rate constant. Clearance (CL) was calculated as dose/AUC0-24.

**Statistical analysis**

All data analyses were performed by using GraphPad Prism, version 5.0, software for Windows (GraphPad Software, San Diego, CA). A regression analysis was conducted to determine the linearity between dose and AUC. Mortality data were analyzed by the log rank test. The survival data were plotted against the dose/minimum effective concentration (MEC), and the Hill equation with a variable slope was fitted to the data both for each individual isolate and for pooled survival data. The goodness of fit was checked by use of the R² value and visual inspection. Statistical significance was defined as a P value of <0.05 (two-tailed). Dose/MEC
and AUC$_0$–$24$/MEC ratios were calculated by dividing the dose (in milligrams per kilogram of body weight) or AUC by the MEC. Dose/MEC and AUC$_0$–$24$/MEC ratio data were log$_{10}$ transformed to approximate a normal distribution prior to statistical analysis.

**Results**

**In vitro susceptibility**

The characteristics and in vitro susceptibility of the two selected *A. fumigatus* isolates are shown in Table 1. Both isolates grew well after 48 h of incubation at 35°C to 37°C. VCZ showed reduced in vitro activity against the TR$_{34}$/L98H mutant isolate, with a VCZ MIC of 4 mg/liter for the TR$_{34}$/L98H mutant isolate compared to one of 0.25 mg/liter for the wild-type isolate. There was no difference in AFG activity, and both isolates had identical MECs.

**Pharmacokinetics of AFG**

A total of 144 mice (3 mice per time point, 12 time points, 4 different dosages) were analyzed. All 144 mice were alive at the time of sample collection. The observed plasma concentration-versus-time profiles of AFG are shown in Fig. 1. The corresponding pharmacokinetic parameters are tabulated in Table 2. The AUC normalized to a dose of 2.5 mg/kg resulted in ratios of 18.06, 18.6, 14.1, 16.3, and 20.1 for dosages of 2.5, 5, 10, 20, and 40 mg/kg, respectively. The AUC correlated significantly with the dose in a linear fashion over the entire dosing range ($R^2$ = 0.86).
Figure 1. Plasma concentrations of anidulafungin following intraperitoneal administration of 5, 10, 20, and 40 mg/kg to immunocompetent infected mice. Each symbol corresponds to the geometric mean and standard error of the mean plasma levels for three mice.
Table 2. Pharmacokinetic parameters of anidulafungin after intraperitoneal administration of various doses of AFG

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>AUC_{0-24} (mg · h/liter)</th>
<th>Dose-normalized AUC [(mg · h)/(liter · kg)]</th>
<th>T\textsubscript{max} (h)</th>
<th>C\textsubscript{max} (mg/liter)</th>
<th>C\textsubscript{min} (mg/liter)</th>
<th>CL\textsubscript{ss}/F [liter/(h · kg)]</th>
<th>t\textsubscript{1/2} (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>46.5b</td>
<td>18.6\textsuperscript{b}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>93</td>
<td>18.6</td>
<td>8</td>
<td>7.9</td>
<td>0.82</td>
<td>0.05</td>
<td>17.34</td>
</tr>
<tr>
<td>10</td>
<td>141.4</td>
<td>14.1</td>
<td>2</td>
<td>10.7</td>
<td>3.3</td>
<td>0.07</td>
<td>17.38</td>
</tr>
<tr>
<td>20</td>
<td>326.3</td>
<td>16.3</td>
<td>0.5</td>
<td>22.2</td>
<td>6.4</td>
<td>0.06</td>
<td>22.78</td>
</tr>
<tr>
<td>40</td>
<td>802.7</td>
<td>20.1</td>
<td>4</td>
<td>49.5</td>
<td>20.7</td>
<td>0.05</td>
<td>15.33</td>
</tr>
</tbody>
</table>

\textsuperscript{a} AFG doses of 2.5 to 40 mg/kg were used. Intraperitoneal therapy was begun at 24 h postinfection with standard daily dosing of AFG, in addition to a single loading dose of AFG.

\textsuperscript{b} Simulated analysis of pharmacokinetic assay with concentrations ranging from 5 to 40 mg/kg.

T\textsubscript{max}, time to C\textsubscript{max}

C\textsubscript{min}, minimum concentration in plasma

CL\textsubscript{ss}/F, apparent steady-state clearance

t\textsubscript{1/2}, half-life.
Efficacy of AFG monotherapy

(i) Survival curves

The survival curves for all control groups receiving saline intraperitoneally showed a mortality of 90 or 100% and a median survival time of 3.5 to 4 days (Fig. 2). For both isolates, a dose-response relationship with increasing survival with increasing dose was observed. The maximum dose of AFG resulted in 72.7% survival in mice infected with the VCZ s isolate, whereas it resulted in 45.45% survival in mice infected with the VCZ r isolate. Of note, the response was lower in those infected with the VCZ r isolate than the VCZ s isolate for each dose (Fig. 2).

(ii) Dose-response analysis

The dose-response curves for the dosing regimen and control groups of AFG monotherapy are shown in Fig. 3. AFG treatment improved the survival of the mice in a dose-dependent manner. The dose-response curve for mice infected with the VCZ r isolate was shifted to the right compared to that for mice infected with the VCZ s isolate, indicating that higher doses of AFG were required to achieve similar efficacy. In mice receiving AFG monotherapy, a maximal response could not be achieved with either isolate, even in those treated with the highest AFG dose.

(iii) Exposure-response analysis

The AUC for each dose, determined from PK experiments (Table 2), was used to calculate the AUC0–24/MEC ratio for each isolate, as shown in Fig. 4. The exposure-response relationship had a sigmoidal shape. Increased AFG exposure was required to obtain maximum efficacy in mice infected with the VCZ r isolate compared to those infected with the VCZ s isolate. The Hill equation with a variable slope fitted the relationship between the 24-h AUC/MEC ratio and 14-day survival well ($r^2 = 0.87$), as statistically significant pharmacodynamic indices (PDIs) for single-agent regimens ($P < 0.05$). The 50% effective AUC for AFG was 126.5 (95% confidence interval, 79.09 to 202.03). We also determined the relationship between the in vivo efficacy and other PDIs, such as the cumulative percentage of a 24-h period that the drug concentration exceeded the MIC under steady-state PK conditions and the peak level (Cmax)/MEC (data not shown). However, AUC0–24/MEC appeared to be the most important pharmacodynamic index correlating with efficacy.
Figure 2. Efficacy of anidulafungin monotherapy against voriconazole-susceptible (MIC, 0.25 mg/liter) and voriconazole-resistant (MIC, 4 mg/liter) *A. fumigatus* isolates. Both isolates had the same AFG MEC (0.03 mg/liter). Control groups received saline. For all groups, *n* = 11.
Figure 3. Anidulafungin dose-survival relationships for voriconazole-susceptible and voriconazole-resistant *A. fumigatus* isolates. The curves indicate fits with the Hill equation for each isolate.

Figure 4. Percent of survival as a function of the anidulafungin AUC$_{0-24}$/MEC ratio for voriconazole-susceptible and voriconazole-resistant *A. fumigatus* isolates. Increased voriconazole and anidulafungin exposure was required to obtain maximum efficacy in mice infected by the voriconazole-resistant isolate. The curve is the model fit with the Hill equation for each datum.
Discussion

Our animal model indicated that AFG monotherapy is moderately effective against isolates with a VCZ MIC within the susceptible range and in groups of mice infected with the resistant TR34/ L98H mutant isolate, which had a VCZ MIC of 4 mg/liter. Although increasing doses increased survival in a dose-dependent manner, a maximal response was not achieved with either isolate, even in those treated with the highest AFG dose (20 mg/kg of body weight). Apparently, this explains why AFG is not effective as single-drug therapy against Aspergillus infections, whereas VCZ is (24), confirming that AFG is a less potent drug for the treatment of IA (21, 22). A higher dose (40 mg/kg) of AFG was also studied for some groups in order to achieve higher efficacy; however, a dose limiting toxicity was defined, and thus, we were not able to explore the effect of higher doses.

Of note, AFG appeared to be slightly less effective against the VCZ' isolate than the VCZ isoate, despite identical MECs, which raises a possible concern regarding the efficacy of anidulafungin monotherapy forazole-resistant IA. Although this difference could be due to differences in the virulence of the two isolates, we have no indications that this is the case, as we have used this isolate in our previous animal models, and the LD90 inocula were almost identical ($2.4 \times 10^7$ versus $2.5 \times 10^7$ conidia). We also investigated the fitness of both isolates using a growth kinetic system (25) but found no differences in germination times or growth rates (results not shown). An alternative possibility might be that changes in ergosterol biosynthesis through mutations in the $cyp51A$ gene might have indirect effects on fungal cell wall synthesis. These changes might not be reflected in in vitro susceptibility, as the MEC may not be sufficiently sensitive to detect subtle differences in echinocandin drug activity. Further research into this phenomenon is needed through, for instance, determination of the levels of the glucan synthase target enzyme in azole-resistant A. fumigatus isolates. A range of resistance mechanisms should be investigated, as the effect on the cell wall might differ depending on the underlying mutations.

The exposure-response relationship of AFG indicated that improvement of survival for both VCZ and VCZ' isolates was dependent on the dose, and since the dose-AUC relationship was linear for the doses studies, this was also the case for the AUC3-24/ MEC ratio. The latter has relevance for predicting therapeutic efficacy (26).

In the present study, the AUC of total AFG was relatively high (326 mg · h/liter for the 20-mg/kg dose) and the MECs were quiet low (0.03 mg/liter), so that AFG treatment alone does not result in 100% survival. The major factor here is that AFG is highly protein bound, with protein binding estimated to be 99% (27) or possibly more. The AUC for the free, unbound fraction of AFG ($f_{AUC}$)/ MEC is therefore about 100 or even lower.
The exact amount of free drug is not well-known, however, since protein binding at these high values is difficult to measure (27).

For echinocandins such as AFG, the 24-h fAUC/MIC ratio is considered the PK/PD index determining therapeutic efficacy, as indicated previously (26, 28). In humans, the AUC after a standard dose is slightly over 100 mg · h/liter (28, 29). Andes et al. found that a fAUCₐ₄/MEC value of 100 was required to result in a static effect in a Candida infection model (27). This value is somewhat higher than the value found for other echinocandins, indicating that 99% protein binding may be an underestimation. van de Sande et al. reported only 18% survival of rats with IA after administration of AFG at human-equivalent doses. In this study, the steady-state fAUC₀₋₄ for AFG was calculated to be 120.3 µg·h/ml (29). Our results for AFG monotherapy at those values are in line with those reports. We also used higher doses, however, which resulted in increased survival of mice, although it did not reach 100%.

A possible limitation of the model used to explore the PK/PD relationships is that the effects were observed in nonneutropenic animals and the route of infection was dissemination rather than inhalation. However, IA in the nonneutropenic host is observed with increasing frequency, although other host factors might be impaired in such patients, in particular, those in an intensive care unit (3). The effects observed could therefore be an underestimation of the exposure required. On the other hand, studies with posaconazole and voriconazole in neutropenic (30) and nonneutropenic (21, 22) models have shown that the exposure-response relationships are of the same order of magnitude; in fact, slightly lower exposures were required in the neutropenic model.

With respect to the discussion presented above, the AUC₀₋₄/MEC appeared to be the most important pharmacodynamic index, which can be used to predict the outcome of AFG mono-therapy. However, compared to the results of our previous study describing the pharmacodynamics of voriconazole monotherapy (24), the results of the present study indicate that AFG is less potent for the treatment of IA. Therefore, instead of using AFG monotherapy for IA, other treatment modalities including this agent in combination therapy can be useful approaches in the clinical setting to improve the therapeutic outcomes of patients with underlying IA.

AFG belongs to the echinocandins, has a unique site of action, as it targets cell wall synthesis, and has fungistatic activity against Aspergillus spp. (18). The echinocandin AFG offers a particularly interesting option for combination antifungal therapy because of its mechanism of action, which is completely different from that of azoles and polyenes, and such combinations should be explored (17, 19).
In addition, clinical studies have suggested that combinations of echinocandins with other antifungals are safe and may improve the response in patients with IA (17). However, preclinical studies and the results of a multi-center trial investigating such combinations will provide more data to judge this strategy.
References


Acknowledgements

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4.1. In Vitro Interaction of Voriconazole and Anidulafungin against Triazole-Resistant Aspergillus fumigatus

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2 Nijmegen Institute for Infection, Inflammation and Immunity (N4i), Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands
3 Clinical Microbiology Laboratory, Attikon University Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece

Abstract

Voriconazole is the recommended drug of first choice to treat infections caused by *Aspergillus fumigatus*. The efficacy of voriconazole might be hampered by the emergence of azole resistance. However, the combination of voriconazole with anidulafungin could improve therapeutic outcomes in azole-resistant invasive aspergillosis (IA). The *in vitro* interaction between voriconazole and anidulafungin was determined against voriconazole-susceptible and voriconazole-resistant (substitutions in the cyp51A gene, including single point [M220I and G54W] and tandem repeat [34-bp tandem repeat in the promoter region of the cyp51A gene in combination with substitutions at codon L98 and 46-bp tandem repeat in the promoter region of the cyp51A gene in combination with mutation at codons Y121 and T289] mutations) clinical *A. fumigatus* isolates using a checkerboard microdilution method with spectrophotometric analysis and a viability-based XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide) assay within 2 h of exposure after 24 and 48 h of incubation at 35°C to 37°C. Fractional inhibitory concentration (FIC) indexes (FICis) were determined using different MIC endpoints and Bliss independence analysis performed based on the response surface calculation of the no-drug interaction. Significant synergistic interactions obtained based on measuring the FIC index were dependent on the MIC endpoint, in which FICs were inversely related to voriconazole and anidulafungin MICs and were influenced by the CYP51A genotype. A statistically significant difference was observed between FIC indexes of isolates harboring tandem repeat mutations and wild-type controls (P = 0.006 by one-way analysis of variance [ANOVA]), indicating that synergy is decreased in azole-resistant strains. Our results indicated that a combination of voriconazole and anidulafungin might be effective against infections caused by both azole-susceptible and azole-resistant *A. fumigatus* isolates, but the combination could possibly be less effective in voriconazole-resistant strains with high MICs. Studies in vivo and *in vitro-in vivo* correlation investigations are required to validate the potential synergy of voriconazole and anidulafungin.

Keywords

Introduction

Voriconazole (VCZ) is an extended-spectrum triazole which affects the integrity of the fungal cell membrane by inhibiting ergosterol biosynthesis. Voriconazole is the recommended first-choice therapy for infections caused by *Aspergillus* species (1, 2). However, acquired resistance to azoles was recently described for *Aspergillus fumigatus*, which may hamper the efficacy of voriconazole (3).

To date, a wide range of mutations in *A. fumigatus* have been described to confer azole resistance (3), which commonly involves changes in the *cyp51A* gene, the target for azole antifungals (4, 5). The emergence of azole resistance has been documented with increasing reports of azole-resistant clinical *A. fumigatus* isolates in multiple European countries, Asia, and the United States (5–11). There is increasing evidence that azole resistance is associated with treatment failure (4, 11, 12), and in a recent Dutch survey, azole-resistant invasive aspergillosis (IA) carried a mortality rate of 88% (11). These clinical observations are supported by preclinical studies in animal models of IA (5, 11, 13–19), where the MIC was shown to have a major impact on the efficacy of voriconazole and posaconazole (15, 20). Evidence is accumulating that azole resistance may develop in our environment with the consequence that in up to two-thirds of patients with azole-resistant *Aspergillus* disease, there was no history of previous azole exposure (11). Therefore, there is an urgent need for new approaches to manage azole-resistant *Aspergillus* diseases.

Although combination therapy is presently not recommended for the primary therapy of IA, it may be an effective alternative approach for treatment of patients with azole-resistant *Aspergillus* disease (21, 22). Several studies have shown the potential of combining an echinocandin with voriconazole to improve outcomes in IA (23–33), but in a recent prospective randomized study, the combination of voriconazole and anidulafungin (AFG) was found not to be more effective than voriconazole monotherapy (34).

Anidulafungin is a cyclic lipopeptide antifungal agent of the echinocandins with *in vitro* and *in vivo* activity against *Aspergillus* spp. (35), which acts via inhibition of 1, 3-β-D-glucan synthesis present only in fungal cell walls (36). However, the drug is not clinically licensed for the treatment of IA. Despite the failure to show a benefit of voriconazole and anidulafungin therapy in IA, this combination might be an option for patients with azole-resistant IA disease.

In this study, we investigated the *in vitro* antifungal activity of voriconazole either alone or in combination with anidulafungin against a collection of 25 clinical *A. fumigatus* isolates, including voriconazole-resistant isolates with various substitutions in the *cyp51A* gene and voriconazole-susceptible isolates, to determine the interaction between these two agents.
CHAPTER 4.1

Materials and Methods

Fungal isolates

A collection of 25 clinical *A. fumigatus* isolates was used in this study. Clinical isolates harbored various substitutions in the *cyp51A* gene, including isolates with single point (M220I and G54W) and tandem repeat (TR$_{34}$/L98H and TR$_{46}$/Y121F/T289A) mutations, and voriconazole-susceptible clinical isolates without mutations in the *cyp51A* gene were used as wild-type controls (Table 1.). All isolates were obtained from the fungus culture collection of the Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands. The *cyp51A* gene substitutions and morphological strain identification were confirmed by sequence-based analysis, as described previously (5). The isolates had been stored in 10% glycerol broth at -80°C and were revived by subculturing on Sabouraud dextrose agar (SDA) supplemented with 0.02% chloramphenicol for 5 to 7 days at 35°C to 37°C. All isolates were subcultured again on SDA for 5 to 7 days at 35°C to 37°C before preparation of the inoculum. *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used for quality control in all experiments.

Preparation of inoculum

Conidial suspensions were harvested after isolates were subcultured on SDA at 35°C to 37°C 2X 5 to 7 days and were suspended in normal saline containing 0.025% Tween 20. *Aspergillus* inocula were then prepared spectrophotometrically and further diluted in normal saline in order to obtain a final inoculum concentration of 2X10$^5$ to 5X10$^5$ CFU/ml (37)

Antifungal agents

Voriconazole and anidulafungin (Pfizer, Capelle aan den Ijssel, the Netherlands) were obtained as standard pure powders, and serial dilutions were prepared according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (37).
<table>
<thead>
<tr>
<th>Aspergillus fumigatus (Cyp51A substitution)</th>
<th>N</th>
<th>MIC&lt;sub&gt;VRC&lt;/sub&gt; mg/L</th>
<th>MIC&lt;sub&gt;APG&lt;/sub&gt; mg/L</th>
<th>FIC index</th>
<th>C&lt;sub&gt;VRC&lt;/sub&gt; mg/L</th>
<th>C&lt;sub&gt;APG&lt;/sub&gt; mg/L</th>
<th>Bliss Independence</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Mutation</td>
<td>7</td>
<td>0.58 (0.25-2)</td>
<td>≥1 (≥1-1)</td>
<td>0.46 (0.16-1.01)</td>
<td>0.25 (0.13-0.50)</td>
<td>0.01 (0.01-0.25)</td>
<td>193.3 (30.39-352.6)</td>
</tr>
<tr>
<td>Single Point Mutation (M 220 I and G 54 W)</td>
<td>5</td>
<td>1.49 (0.13-4)</td>
<td>≥1 (≥1-2)</td>
<td>0.38 (0.05-1.03)</td>
<td>0.48 (0.13-2.00)</td>
<td>0.04 (0.02-0.06)</td>
<td>90.16 (-207.6 - 409.6)</td>
</tr>
<tr>
<td>Tandem Repeat Mutation (TR&lt;sub&gt;Ru&lt;/sub&gt;L98H and TR&lt;sub&gt;Ru&lt;/sub&gt;Y121F/T289A)</td>
<td>13</td>
<td>8.94 (2-32)</td>
<td>≥1 (≥1-2)</td>
<td>0.33 (0.04-1.01)</td>
<td>2.18 (0.02-8.00)</td>
<td>0.04 (0.01-0.25)</td>
<td>95.74 (-335.5 - 692.8)</td>
</tr>
<tr>
<td>No Mutation</td>
<td>7</td>
<td>0.40 (0.25-1)</td>
<td>0.68 (0.03-2)</td>
<td>0.08 (0.02-0.54)</td>
<td>0.01 (0.01-0.13)</td>
<td>0.02 (0.02-0.06)</td>
<td>193.3 (30.39-352.6)</td>
</tr>
<tr>
<td>Single Point Mutation (M 220 I and G 54 W)</td>
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<td>1.16 (0.13-4)</td>
<td>0.61 (0.06-1)</td>
<td>0.10 (0.02-0.56)</td>
<td>0.02 (0.02-0.25)</td>
<td>0.04 (0.02-0.13)</td>
<td>90.16 (-207.6 - 409.6)</td>
</tr>
<tr>
<td>Tandem Repeat Mutation (TR&lt;sub&gt;Ru&lt;/sub&gt;L98H and TR&lt;sub&gt;Ru&lt;/sub&gt;Y121F/T289A)</td>
<td>13</td>
<td>10.22 (2-32)</td>
<td>0.09 (0.02-1)</td>
<td>0.19 (0.01-1.03)</td>
<td>0.04 (0.01-4.00)</td>
<td>0.01 (0.01-0.13)</td>
<td>95.74 (-335.5 - 692.8)</td>
</tr>
</tbody>
</table>

Table 1. FIC indices based on 10% and 25% growth endpoints and Bliss independence results for VRC-susceptible and VRC-resistant *A. fumigatus* isolates

a The MICs of voriconazole and anidulafungin alone (MIC<sub>VRC</sub> and MIC<sub>APG</sub>, respectively) and the concentrations of voriconazole (C<sub>VRC</sub>) and anidulafungin (C<sub>APG</sub>) in combination are presented. Data are presented as geometric means and ranges.

b Bliss interaction.
Susceptibility and drug interaction testing

Antifungal susceptibility MICs and minimum effective concentrations [MECs]) and drug interaction testing were performed by using the EUCAST broth microdilution checkerboard (two dimensional, 8 by 12) method (37), utilizing XTT dye (2, 3-bis (2- methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (38–40). XTT (Sigma-Aldrich, St. Louis, MO) was dissolved in normal saline at concentrations of 0.5 mg/ml. Menadione (Sigma-Aldrich, St. Louis, MO) was initially dissolved in absolute ethanol at a concentration of 10 mg/ml and subsequently added to the above-mentioned XTT solutions at concentrations of 6.25 lLM for each solution. The final concentrations of the antifungal agents ranged from 0.016 to 16 mg/liter for voriconazole and 0.008 to 0.5 mg/liter for anidulafungin. Aliquots of 50 µl of each drug at a concentration four times the targeted final concentration were dispensed into the wells of flat-bottom 96-well microtiter plates (Costar; Corning, NY). Trays were maintained for a period of less than 1 month at −70°C until the day of testing. After the microtitration trays were defrosted, 100 µl of the inoculum was added to each well, corresponding to a final concentration of 2 X 10^5 to 5 X 10^5 CFU/ml from each isolate. The microtiter plates were incubated at 35°C to 37°C for 48 h. Subsequently, 50 µl of the above-mentioned XTT-menadione solutions was added to each well, as previously described (40, 41). The microtitration plates were further incubated at 35°C to 37°C for 2 h in order to allow conversion of XTT to its formazan derivative. XTT conversion was measured as optical density (OD) with a microtitration plate spectrophotometric reader (Anthos htIII; Anthos Labtec Instruments, Salzburg, Austria) at 450 nm/630 nm. For each well, XTT conversion was calculated after subtraction of the background OD, which was the OD of a simultaneously incubated well with 200 µl of medium and 50µl of XTT-menadione solution but no inoculum. Percentages of fungal growth were calculated for each well by dividing the XTT conversion of each well by the XTT conversion of the drug-free growth control well. All experiments were performed in three independent replicates, and the breakpoints reported previously by Verweij et al. were used for classifying voriconazole-susceptible and voriconazole-resistant isolates (3).

MIC and MEC determination

The MIC of voriconazole was defined as the lowest concentration that completely inhibited growth compared with that of the drug-free well, as assessed by visual inspection. The MEC of anidulafungin was defined as the lowest concentration in which abnormal, short, and branched hyphal clusters were observed, in contrast to the long, unbranched hyphal elements that were seen in the growth control well (37). Because the voriconazole MIC corresponds to the lowest drug concentration corresponding to <10% growth and the MEC corresponds to
the lowest concentration corresponding to <50% growth with the XTT assays, for the voriconazole-anidulafungin combination, both 10% and 50% growth endpoints in addition to the 25% growth endpoint were considered MIC endpoints.

**Definitions for drug interaction modeling**

In order to assess the nature of *in vitro* interactions between voriconazole and anidulafungin, the data obtained as described above were analyzed using two different models. These models were nonparametric approaches of the following two no (zero)-interaction theories: the Loewe additivity (LA) and the Bliss independence (BI) theories (42-45). The fractional inhibitory concentration (FIC) index is defined as $\sum \text{FIC} = \frac{C_A}{\text{MIC}_A} + \frac{C_B}{\text{MIC}_B}$, where $\text{MIC}_A$ and $\text{MIC}_B$ are the MICs of the drugs A and B when acting alone and $C_A$ and $C_B$ are concentrations of the drugs A and B at the iso-effective combinations, respectively (42).

To determine synergistic and antagonistic interactions among all $\sum \text{FIC}$s calculated for each isolate and replicate, the FIC index was determined as the $\sum \text{FIC}_{\text{min}}$ (the lowest $\sum \text{FIC}$) or the $\sum \text{FIC}_{\text{max}}$ (the highest $\sum \text{FIC}$) (42). The 10%, 25%, and 50% endpoints of fungal growth were used to assess pharmacodynamic interactions at different concentrations. In order to determine the nature of the interaction between voriconazole and anidulafungin, previously described cutoff values were used (46), in which an interaction was defined as synergistic if the FIC index was <1, additive if the FIC index was >1 to <1.25, and antagonistic if the FIC index was >1.25. These cutoff values were derived from experiments that investigated the voriconazole-echinocandin interaction (46). Furthermore, we compared our analysis with the commonly used FICI range of 0.5 to 4 that is generally recommended to define drug-drug interactions in combination studies of antifungal agents (21, 47, 48).

The BI was described by the equation $l_{\text{ind}} = I_A + I_B - I_A \times I_B$, where $l_{\text{ind}}$ is the predicted percentage of inhibition of an non-interactive theoretical combination, calculated based on the experimental percentages of inhibition ($I_A$ and $I_B$) of each drug acting alone, respectively (43). In the three-dimensional plots, peaks above and below the zero plane indicate synergistic and antagonistic combinations, respectively, whereas the zero plane itself indicates no statistically significant interactions. The average sum of the three replicates of all Bliss interactions was used as a measure of the pharmacodynamic interactions for each strain.
Data analysis

All data analyses were performed by using the software package GraphPad Prism, version 5.0, for Windows (GraphPad Software, San Diego, CA). The FICs among the different genotype groups were compared by analysis of variance (ANOVA) followed by a posttest for linear trends. The correlation between the mean FIC indexes and voriconazole and anidulafungin MIC endpoints was determined by Spearman’s correlation coefficient (r); a P value of 0.05 was considered significant (two tailed).

Results

The MIC and MEC characteristics of the 25 clinical *A. fumigatus* isolates used for the current study are shown in Table 1. The mean MICs of voriconazole (and ranges) based on 10% and 25% growth endpoints were 0.58 (0.25 to 2) mg/liter and 0.40 (0.25 to 1) mg/liter, respectively, for the voriconazole-susceptible (VCZ-S) isolates, whereas higher MICs were observed for isolates harboring single point mutations, 1.49 (0.13 to 4) mg/liter and 1.16 mg/liter (0.13 to 4), respectively, and tandem repeat mutations, 8.94 (2 to 32) mg/liter and 10.22 (2 to 32) mg/liter, respectively. Anidulafungin MIC endpoints based on the 50% growth endpoint were off scale for most of the isolates, and therefore, this growth endpoint was excluded from the analysis.

The mean values of FIC indexes based on 10% and 25% growth endpoints as well as BI response surface analysis results for different groups of *A. fumigatus* isolates with regard to substitutions in the cyp51A gene are also shown in Table 1, whereas Fig. 1 shows the distribution of FICs at each growth endpoint. None of the data sets analyzed had $\sum$FIC$_{\text{max}}$s higher than 1.25, indicating that antagonism was not observed. Therefore, the FIC index corresponded to the $\sum$FIC$_{\text{min}}$. The lowest FIC index values found for isolates without a mutation in the cyp51A gene ranged between 0.16 and 1.01 based on the 10% growth endpoint and between 0.02 and 0.54 with the 25% growth endpoint, followed by isolates harboring single point and tandem repeat mutations, respectively.

For isolates with the tandem repeat resistance mechanism (TR$_{34}$/ L98H and TR$_{46}$/Y121F/T289A), the FIC index values averaged 0.33 (range, 0.04 to 1.01) based on the 10% growth endpoint and 0.19 (range, 0.01 to 1.03) with the 25% growth endpoint. When analyzing interactions, considering the 10% and 25% growth endpoints, significant synergy ($P < 0.05$) was found for all isolates, with mean FICI$_{\text{min}}$s of 0.42 and 0.12, respectively (Fig. 1).
However, the wide distribution observed for mean FIC values of each growth endpoint indicated that for some strains, there appeared to be no synergism.

<table>
<thead>
<tr>
<th></th>
<th>10%Growth</th>
<th>25%Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>0.1250</td>
<td>0.02267</td>
</tr>
<tr>
<td>Maximum</td>
<td>0.8790</td>
<td>0.7750</td>
</tr>
<tr>
<td>Mean</td>
<td>0.4437</td>
<td>0.2941</td>
</tr>
</tbody>
</table>

**Figure 1.** Graphical distribution of mean and standard error of the mean of FIC indexes determined at 10% and 25% growth endpoints for 25 A. fumigatus isolates. None of the data sets analyzed had $\Sigma FIC_{\text{max}}$ higher than 1.25, indicating that antagonism was not observed.
As shown in Fig. 2, the mean FICI based on the 25% growth endpoint did not differ significantly among voriconazole-susceptible isolates and those with single point mutations (M220I and G54W). However, a statistically significant difference was observed between isolates harboring tandem repeat mutations (TR\textsubscript{34}/L98H and TR\textsubscript{46}/Y121F/T289A) and wild-type controls ($P = 0.006$ by one-way analysis of variance [ANOVA]). Therefore, the dependence of the FIC index on resistance mechanisms indicates that synergistic interactions may be lost for the isolates with higher MICs of voriconazole. The consequence of this observation is that in isolates where voriconazole has no \textit{in vitro} activity (MIC>8 mg/liter), the efficacy of the combination relies solely on anidulafungin.

**Figure 2.** Mean and standard errors of the mean of FICs with respect to \textit{cyp51} substitutions for 25 \textit{A. fumigatus} isolates, indicating that the FIC indexes are dependent on the type of mutation. The vertical bars indicate that the mean FIC indexes did not differ significantly among VCZ-S isolates and those with M220I and G54W mutations; a statistically significant difference was observed between isolates harboring TR\textsubscript{34}/L98H and TR\textsubscript{46}/Y121F/T289A mutations and wild-type controls ($P < 0.05$).
Figure 3. Interaction surfaces obtained from response surface analysis of the Bliss independence no-interaction model for the in vitro combination of VCZ plus AFG against a VCZ-susceptible A. fumigatus isolate (MIC of VCZ, 0.25 mg/liter; MEC of AFG, 0.03 mg/liter) and a VCZ-resistant A. fumigatus isolate (MIC of VCZ, 4 mg/liter; MEC of AFG, 0.03 mg/liter). The x and y axes represent the efficacies of VCZ and AFG, respectively. The z axis is the percent dE. The zero plane represents Bliss-independent interactions, whereas the volumes above the zero plane represent statistically significantly synergistic (positive dE) interactions. The magnitude of interactions is directly related to dE. The different tones in three-dimensional plots represent different percentile bands of synergy. (a) Synergistic interaction. The mean dE ± standard error of the mean and sum dE were 3.23% ± 1.09% and 271%, respectively, after 48 h. (b) Antagonistic interaction. The mean dE ± standard error of the
mean and sum \( dE \) were \(-2.47\% \pm 0.40\%\) and \(-208\%\), respectively, after 48 h.

Furthermore, the results of FICi analysis are supported by response surface analysis using the BI no-interaction model for all isolates where the synergistic interactions in wild-type isolates were higher than those in the other two groups harboring CYP51A gene mutations for which some antagonistic interactions were observed. Bliss antagonism reflects additive/indifferent interactions by Loewe additivity. Thus, the presence of antagonistic interactions correlates with the reduction of Loewe synergistic interactions at the 25% growth endpoint. The selected interaction surface plots indicating synergy and antagonism for a voriconazole-susceptible \( A. \ fumigatus \) isolate (MIC of voriconazole, 0.25 mg/liter; MEC of anidulafungin, 0.03 mg/liter) and a voriconazole-resistant \( A. \ fumigatus \) isolate (MIC of voriconazole, 4 mg/liter; MEC of anidulafungin, 0.03 mg/liter) are shown in Fig. 3.

In comparison, Fig. 4 shows the interpretation of FIC indices, using two different cutoff values, in which the commonly used FIC index range of 0.5 to 4 indicated synergism for 38.1% and indifference for 61.9% of isolates, while the use of recently reported cutoff values (46) indicated synergism for 75.0% and additivity for 25.0% of isolates. Antagonism was not observed with either definition of the interaction.

**Figure 4.** Interpretation of voriconazole and anidulafungin interactions for 25 \( A. \ fumigatus \) isolates utilizing two different definitions: the cutoff values proposed previously by Meletiadis et al. (46) (synergistic if the FIC index was <1, additive if the FIC index was >1 to <1.25, and antagonistic if the FIC index was >1.25) and the commonly used FIC index range of 0.5 to 4 proposed previously by Greco et al. (47) and which is generally recommended to define drug interactions in most combination studies of antifungal agents (synergistic if the FIC index was <0.5, indifferent if the FIC index was >0.5 to <4, and antagonistic if the FIC index was >4).
Discussion

A number of studies have reported data on the efficacy of combination therapy against A. fumigatus. Most studies investigating combinations of azoles and echinocandins have shown a synergistic or additive interaction against Aspergillus spp. (24, 27, 29, 30, 33). Antagonism was not reported. The combination of voriconazole and an echinocandin in advanced invasive pulmonary aspergillosis in transiently neutropenic rats improved the therapeutic outcome (49).

Notably, synergy was documented by the majority of studies when susceptibility testing endpoints were defined as a substantial inhibition of growth. For example, in a previous study by Shalitet et al., caspofungin and itraconazole were studied alone and in combination against 31 clinical Aspergillus isolates (33). MICs and MECs were recorded, and synergy was calculated by using both endpoints. Synergy or synergy to additivity was found for 30 of 31 isolates by using MIC endpoints. With MEC endpoints, no synergy was found, and indifference was detected for 26 of 31 strains. In a previous study by Philip et al., significant synergy was noticed with regard to combinations of voriconazole and anidulafungin for 18/26 isolates, depending on the drug concentration and interaction definitions (32). Voriconazole in combination with anidulafungin has been shown to be efficient in treating infections caused by A. fumigatus in an immunosuppressed guinea pig model of IA (25, 49). We recently also found a synergistic interaction between voriconazole and anidulafungin in a model of disseminated IA when mice were infected with a voriconazole-susceptible isolate (50).

Although retrospective clinical studies indicated a benefit of combining an echinocandin, i.e., caspofungin, with voriconazole (28), a recent randomized prospective trial of voriconazole and anidulafungin showed no superiority to voriconazole mono-therapy (34). This apparent discrepancy between this prospective clinical trial and retrospective trials and preclinical research may be due to methodological issues related to the prospective clinical trial (27). However, preclinical studies involved only wild-type isolates, and it can be assumed that the vast majority of patients enrolled in clinical studies would have suffered from invasive aspergillosis due to wild-type isolates.

In azole-resistant disease, combination therapy has potential benefit, as the reduced efficacy of the azole might be overcome by the concomitant administration of an echinocandin. In our murine model, we found that the interaction between voriconazole and anidulafungin was indifferent in mice infected with an A. fumigatus isolate with a voriconazole MIC of 4 mg/liter (50), which indicated that the drug interaction varied according to the susceptibility of the isolate to voriconazole. As only one azole-resistant isolate was investigated in the animal model, we used an in vitro interaction model to investigate this relationship in more detail using a larger collection of isolates and a wide
range of voriconazole MICs. Furthermore, fitting an interaction model to the whole response surface and estimation have the additional advantage that confidence intervals of the interaction are obtained (44).

We found that synergistic drug interactions obtained for the FIC indexes were dependent on the MIC endpoints. Significant variations were observed in the FIC distributions using MIC endpoints. However, for some strains, there appeared to be no synergism (FIC > 1), which was dependent on the MIC of voriconazole. This variation in FIC index results could be explained largely by the CYP51A gene mutation and the associated voriconazole phenotype of the strain. In addition to the analysis with the nonparametric fractional inhibitory concentration model (FIC index), similar results were found when the data were analyzed using the response surface approaches of the Bliss independence (BI) no-interaction theory.

The statistically significant difference between isolates harboring tandem repeat mutations and wild-type controls ($P = 0.006$ by ANOVA) is in keeping with the observation in our in vivo model (50). FICs were inversely related to voriconazole and anidulafungin MICs and influences by CYP51A genotype.

The interpretation of data from in vitro interaction studies depends on the definition used for FIC calculation (21, 22, 46–48, 51–55), which can vary depending on the cutoff values used (Fig.4). In our study, we used cutoff values to indicate that the interactions were synergistic if the FIC index was <1, additive if the FIC index was >1 to <1.25, and antagonistic if the FIC index was >1.25 (46), since an additivity range of 0.5 to 2 is more symmetrical than a range of 0.5 to 4. Furthermore, the cutoffs of 1 and 1.25 were previously investigated for drug interactions of voriconazole and anidulafungin against A. fumigatus and validated by an in vivo model (46). Interpreting our data by this definition indicated synergism for 75% of isolates and additivity for 25% of isolates. In comparison, the application of the generally used FIC index range of 0.5 to 4 (21, 47, 48) indicated synergism for 61.9% of isolates and indifference for 38.1% of isolates.

We used XTT for a more precise quantification of hyphal growth. It has been shown that the assessment of metabolic activity provides useful quantitative endpoints for in vitro studies of both azoles and echinocandins against Aspergillus spp. (38, 40, 41, 43).

The significant relationship between FICI and CYP51A genotype raises concern regarding if the combination of voriconazole and anidulafungin can be used in the management of azole-resistant disease. In the Netherlands, the TR$_{34}$/L98H mutation is highly prevalent (5, 11), and more recently, a TR$_{46}$/Y121F/T289A mutation was found in A. fumigatus isolates recovered from patients from multiple Dutch hospitals (56). This new resistance mechanism has characteristics similar to those of TR$_{34}$/L98H, indicating that it may also originate from the environment. These two resistance mechanisms correspond to the highest voriconazole MICs
IN VITRO COMBINATION OF VORICONAZOLE AND ANIDULAFUNGIN

(8 and \(>16\) mg/liter), and our results indicate that we can expect the least benefit from combination therapy with voriconazole and anidulafungin in patients infected by \(A.\ fumigatus\) strains harboring these resistance mechanisms. As the targets of azoles and echinocandins are unrelated, a lack of voriconazole activity may indicate that the efficacy of combination therapy relies solely on anidulafungin.

Evidence to support treatment choices for azole-resistant \(Aspergillus\) disease is scarce at present. Although the \textit{in vitro} activity of echinocandins and amphotericin B appears unaffected in azole-resistant isolates, \textit{in vivo} efficacy studies are lacking. Clearly, more research is warranted to explore treatment options in azole-resistant disease. Our results indicate that azole and echinocandin combination therapy should be used with great caution in patients with azole-resistant \(Aspergillus\) diseases.
References


Acknowledgements

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4.2. Efficacy and pharmacodynamics of voriconazole combined with anidulafungin in azole-resistant invasive aspergillosis

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Abstract

Azole resistance is an emerging problem in the treatment of *Aspergillus fumigatus* infections. Combination therapy may be an alternative approach to improve therapeutic outcome in azole-resistant invasive aspergillosis (IA). The in vivo efficacy of voriconazole and anidulafungin was investigated in a non-neutropenic murine model of IA using voriconazole-susceptible and voriconazole-resistant *A. fumigatus* clinical isolates. Treatment groups consisted of voriconazole monotherapy, anidulafungin monotherapy and voriconazole + anidulafungin at 2.5, 5, 10 and 20 mg/kg body weight/day for 7 consecutive days. In vitro and in vivo drug interactions were analysed by non-parametric Bliss independence and non-linear regression analysis. Synergistic interaction between voriconazole and anidulafungin against the voriconazole-susceptible isolate (AZN 8196) was observed in vitro and in vivo. However, among animals infected with the voriconazole-resistant isolate (V 52-35), 100% survival was observed only in groups receiving the highest doses (20 mg/kg voriconazole + 20 mg/kg anidulafungin). For this isolate, additivity, but not synergy, was observed in vivo. Combination of voriconazole and anidulafungin was synergistic in voriconazole-susceptible IA, but additive in voriconazole-resistant IA. There is a clear benefit of combining voriconazole and anidulafungin, but the reduced effect of combination therapy in azole-resistant IA raises some concern.

Keywords

Azoles, Echinocandins, Combination therapy, Synergy, Additivity
Introduction

Invasive aspergillosis (IA) is an increasingly common infection in immunocompromised patients (1-3). Voriconazole is considered the first-choice therapy for invasive infections caused by Aspergillus species, based on the results of randomized clinical trials (4-5). However, the emergence of acquired azole resistance has been reported in clinical Aspergillus fumigatus isolates, (6) in different continents (7-11).

There is increasing evidence that azole resistance is associated with azole treatment failure (8,12,13), and in a recent Dutch survey azole-resistant IA was associated with a 12 week mortality rate of 88% (8). These clinical observations are supported by animal models of IA, in which the MIC has been shown to have major implications for the efficacy of voriconazole and posaconazole (14, 15). Alternative treatment regimens need to be explored in order to improve the outcome of patients with azole-resistant IA.

Alternative options to treat infections caused by azole-resistant A. fumigatus include a lipid formulation of amphotericin B and combination therapy (5). Combination therapy can potentially increase the spectrum of efficacy, reduce toxicity, stabilize pharmacokinetic (PK)/pharmacodynamic (PD) characteristics and possibly prevent the emergence of resistance (16, 17).

In one clinical study, the combination of voriconazole and caspofungin was shown to produce a better response than voriconazole monotherapy in patients with IA, but in that study a historical control group was used (16). However, these patients were probably infected with azole-susceptible Aspergillus isolates, although in vitro susceptibility test results were not reported.

As in vitro and in vivo interaction studies suggest that the combination of an azole and an echinocandin may be synergistic (18, 21), this combination might be useful as a strategy in patients with documented azole-resistant IA or as primary therapy in those centres with a high prevalence of azole resistance. However, there are no in vivo data that confirm the observed synergistic interaction in azole-resistant IA.

We report the efficacy of combination therapy with voriconazole and anidulafungin in an established animal model of disseminated IA. Although anidulafungin is currently not clinically licensed for the treatment of IA, we investigated the voriconazole + anidulafungin combination as it is currently being evaluated in a large Phase III clinical trial (22, 23). The efficacy and interaction between voriconazole and anidulafungin were evaluated using voriconazole-susceptible and voriconazole-resistant A. fumigatus isolates.
Methods

Organisms
Two clinical A. fumigatus isolates obtained from patients with proven IA were used in the experiments: a voriconazole-susceptible isolate without mutations in the cyp51A gene (AZN 8196) and a voriconazole-resistant isolate (V 52-35) harbouring the TR34/L98H resistance mechanism. Strain identifications and cyp51A gene substitutions were confirmed by sequence-based analysis as described previously (7). The isolates had been stored in 10% glycerol broth at -80°C and were revived by subculturing on Sabouraud dextrose agar (SDA) supplemented with 0.02% chloramphenicol for 5 – 7 days at 35-37°C.

In vitro antifungal susceptibility testing
The in vitro antifungal susceptibility test for voriconazole and anidulafungin (Pfizer, Capelle aan den IJssel, The Netherlands) was performed in triplicate based on EUCAST guidelines (24). The interaction testing of voriconazole and anidulafungin was performed by using a broth microdilution chequerboard (two-dimensional 8×12) method, utilizing XTT dye, as previously described (25, 26).

Mouse infection model
Outbred CD-1 (Charles River, The Netherlands) female mice, 4–5 weeks old and weighing 20 – 25 g, were used in all experiments. Animals were infected using the procedure described previously by injection of an inoculum corresponding to the LD90 of each isolate into the lateral tail vein (14, 27). The LD90 of the voriconazole-susceptible and voriconazole-resistant isolates used was 2.4×10^7 and 2.5×10^7 conidia, respectively. Post-infection viability counts of the injected inocula were determined to ensure that the correct inoculum had been injected.

The animals were housed under standard conditions with drink and feed supplied ad libitum and were examined at least three times daily. The animal studies were conducted in accordance with the recommendations of the European Community (Directive 86/609/EEC, 24 November 1986), and all animal procedures were approved by the Animal Welfare Committee of Radboud University (RU-DEC 2010-187).

For the efficacy study, 882 animals were randomized into groups of 11 mice. Treatment groups consisted of voriconazole monotherapy and anidulafungin monotherapy at 2.5, 5, 10 and 20 mg/kg once daily and combinations of these regimens.
All data for efficacy of anidulafungin monotherapy were from a previous study (28). Briefly, intraperitoneal therapy was begun 24 h post-infection and comprised standard once-daily dosing of voriconazole and anidulafungin for 7 consecutive days. In addition, a single loading dose of the same amount of anidulafungin was injected in order to keep its PK parameters at a steady-state level. The control groups received a single dose or multiple doses of saline as a control for monotherapy or combination therapy, respectively. On day 15 post-infection, surviving mice were humanely euthanized under isoflurane anaesthesia, and blood and internal organs were collected. The survival time in days post-infection was recorded (29).

A total of 144 mice were used for separate PK experiments of voriconazole monotherapy. Treatment was initiated with intraperitoneal dosages of 5, 10, 20 and 40 mg/kg voriconazole 24 h after infection with the voriconazole-susceptible isolate. On day 2 of treatment (day 3 after infection), blood samples were drawn through the orbital vein or heart puncture into lithium – heparin-containing tubes at six predefined time points (immediately before administration of drugs and subsequently at 0.5, 1, 2, 4 and 8 h post-dose), three mice per timepoint. Blood samples were centrifuged for ×10 min at 1000 g within 30 min of collection. Plasma was aspirated, transferred in two 2 mL plastic tubes and stored immediately at –80°C.

**Analytical assay of voriconazole and anidulafungin**

Voriconazole concentrations were measured by a validated (for human and mouse matrices) HPLC method with fluorescence detection (Thermo Scientific, Breda, The Netherlands). The dynamic range of the assay was 0.05 to 10 mg/L and it had an accuracy range (n = 15), depending on the concentration, of 96.7% – 101.4%. Geometric mean concentrations of voriconazole in plasma from three mice were calculated separately for each timepoint. Plasma Cmax values were directly observed from the data. PK parameters were derived using non-compartmental analysis with WinNonLin, version 5.2 (Pharsight, Inc., Mountain View, CA, USA). The AUC from time 0 to 24 h post-infusion (AUC0–24) was determined by use of the log-linear trapezoidal rule. The elimination rate constant was determined by linear regression of the terminal points of the log-linear plasma concentration – time curve. The terminal half-life was defined as ln2 divided by the elimination rate constant. CL was calculated as dose/AUC0–24. The procedure and PK parameters for anidulafungin monotherapy are described in a previous study (28).
Exposure–response and statistical analysis

All data analyses were performed by using GraphPad Prism, version 5.0, for Windows (GraphPad Software, San Diego, CA, USA).

A regression analysis was conducted to determine linearity between dose and AUC. Mortality data were analysed by the log-rank test. The survival data were plotted against dose/MIC and the Hill equation with a variable slope fitted to the data, both for each individual isolate and for pooled survival data. The curve was then fitted with minimum and maximum survival constrained at ≥ 0% and ≤ 100%, respectively. The goodness of fit was checked by the R² and visual inspection. Statistical significance was defined as a P value of < 0.05 (two-tailed). Dose/MIC and AUC/MIC ratio data were transformed to log₁₀ values to approximate a normal distribution prior to statistical analysis.

In order to assess the nature of in vitro interactions between voriconazole and anidulafungin, the results of the checkerboard experiments were analysed using two non-parametric no-interaction models: fractional inhibitory concentration indexes (FICIs) based on Loewe additivity theory, and a Bliss independence-based drug-interaction model based on the response surface approach developed by Prichard et al. (30).

The effects of combinations of voriconazole and anidulafungin in vivo were analysed by response surface analysis of the Bliss independence-based no-interaction model using survival as the end-point (29). The expected effect was determined using the model of Prichard et al. (30). Observed versus expected percentage survival for various dosing regimens of combinations was also plotted for both isolates as described previously (31).
Results

In vitro susceptibility

The characteristics and in vitro susceptibilities of the two selected A. fumigatus isolates are shown in Table 1. Both isolates grew well after 48 h of incubation at 35 – 37°C. Voriconazole showed reduced in vitro activity against the TR34/L98H isolate, with an MIC of 4 mg/L (MIC of 0.25 mg/L for the wild-type isolate). There was no difference in anidulafungin activity.

In vitro drug interaction experiments

The FICIs obtained for each isolate at 48 h are shown in Table 1. Voriconazole and anidulafungin appeared to act synergistically against both the voriconazole-susceptible isolate and the voriconazole-resistant isolate, with an FICI of 0.35 and 0.43, respectively. Bliss independence-based response surface analysis showed statistically significant synergistic interactions with a sum DE of 271.04% and a mean of DE 3.23%+SEM 1.10% for the voriconazole-susceptible isolate and a sum DE of 27.43% and a mean DE of 0.33%+SEM 10.27% for the voriconazole-resistant isolate (Table 1).

<table>
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<th>Origin</th>
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<td>Proven invasive aspergillosis</td>
<td>None</td>
<td>0.25 (Susceptible)</td>
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<td>TR34/L98H</td>
<td>4 (Resistant)</td>
<td>0.031</td>
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Table 1. Origin, in vitro susceptibilities, underlying azole resistance mechanisms and in vitro interaction of voriconazole + anidulafungin of voriconazole-susceptible and voriconazole-resistant A. fumigatus isolates

MEC, minimum effective concentration.

aDifference between observed versus expected percentage of fungal growth.
PK of voriconazole and anidulafungin

The PK parameters of voriconazole and anidulafungin are shown in Table 2. In the case of voriconazole, the dose-normalized AUC increased and CL decreased with increasing dosages, confirming the non-linear PK of voriconazole. For anidulafungin, the AUC correlated significantly with the dose in a linear fashion over the entire dosing range ($R^2 = 0.86$) (28).

<table>
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<td>2.5</td>
<td>46.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>93</td>
<td>2.6</td>
<td>18.6</td>
<td>0.51</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>141.4</td>
<td>12.9</td>
<td>14.1</td>
<td>1.3</td>
<td>2</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>326.3</td>
<td>58.1</td>
<td>16.3</td>
<td>2.9</td>
<td>0.5</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>802.7</td>
<td>192.8</td>
<td>20.1</td>
<td>4.8</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2. PK parameters of voriconazole and anidulafungin following single- and multiple-dose intraperitoneal administration of 2.5–40 mg/kg AFG, anidulafungin; VRC, voriconazole. Intraperitoneal therapy was begun 24 h post-infection with standard daily dosing of voriconazole and anidulafungin in addition to a single loading dose of anidulafungin. All PK parameters for anidulafungin monotherapy are reproduced from a previous study (28).

<sup>a</sup> Simulated analysis of PK assay ranging from 5 to 40 mg/kg.
Efficacy of voriconazole and anidulafungin monotherapy

For the voriconazole-susceptible isolate as well as the voriconazole-resistant isolate, a dose–response relationship was observed for both drugs. Voriconazole and anidulafungin treatment improved the survival of the mice in a dose-dependent manner (Table 3), although, for each dose, the response was lower in those infected with the voriconazole-resistant isolate than in those infected with the voriconazole-susceptible isolate. The maximum dose of voriconazole resulted in 100% survival in mice infected with the voriconazole-susceptible isolate compared with 72.2% in mice infected with the voriconazole-resistant isolate, indicating that higher doses of voriconazole were required to achieve similar efficacy. In mice receiving anidulafungin monotherapy, the survival rate was 72.7% and 45.4% for 20 mg/kg, respectively, and a maximal response could not be achieved in mice infected with either isolate, even in those treated with the highest anidulafungin dose (28).

The AUC for each dose (Table 2) was used to determine the AUC$_{0-24}$/MIC ratio for each isolate. Increased voriconazole exposure was required to obtain maximum efficacy in mice infected with the voriconazole-resistant isolate compared with those infected with the voriconazole-susceptible isolate. The 50% effective AUC$_{0-24}$/MIC for voriconazole was 3.71 (95% CI = 1.19 – 11.59) compared with 126.5 (95% CI = 79.09 –202.03) for anidulafungin. The Hill equation with a variable slope fitted well the relationship between 24 h AUC/MIC ratio and 14 day survival ($R^2$= 0.80 voriconazole and $R^2$=0.70 anidulafungin), as statistically significant PD indices for single-agent regimens ($P\leq0.05$).

Efficacy of voriconazole and anidulafungin combination therapy

Figure 1 shows selected survival curves for mice infected with voriconazole-susceptible and voriconazole-resistant isolates and treated with the highest dose regimens of voriconazole and anidulafungin monotherapy (10 and 20 mg/kg voriconazole) or with voriconazole + anidulafungin combination therapy. Survival of 100% was observed in the groups of mice infected by the voriconazole-susceptible isolate and treated with 20 mg/kg voriconazole or with 10 mg/kg voriconazole when combined with anidulafungin (10 and 20 mg/kg). In contrast, in the groups infected by the voriconazole-resistant isolate, 100% survival was not achieved in groups receiving monotherapy, but only in one treatment group, that receiving 20 mg/kg voriconazole + 20 mg/kg anidulafungin.
Figure 1. Efficacy of 10 and 20 mg/kg voriconazole and anidulafungin monotherapy versus voriconazole + anidulafungin combination therapy against (a) voriconazole-susceptible and (b) voriconazole-resistant *A. fumigatus* isolates. Survival is increased following combination therapy compared with single-drug therapy. AFG, anidulafungin; VRC, voriconazole.
Table 3 shows the survival rates of voriconazole and anidulafungin monotherapy versus voriconazole + anidulafungin combination therapy incorporating the full range of dose regimens for each isolate.

Interestingly, combination therapy with voriconazole + anidulafungin was found to significantly improve the efficacy of antifungal therapy compared with that obtained with each drug alone. To determine possible synergism between voriconazole and anidulafungin, the efficacy was analysed based on Bliss expected effect ($E_{\text{expected}}$) and the experimentally observed effect ($E_{\text{observed}}$) was calculated to assess antifungal efficacy of combination therapy. Significant Bliss independence-based synergy was found in vivo between voriconazole and anidulafungin, with observed effects being 119.0% and 35.5% higher than would be expected if the drugs were acting independently against voriconazole-susceptible and voriconazole-resistant *A. fumigatus* infection, respectively (Figure 2).

Figure 3 shows the relationship between observed versus expected percentage of survival for all voriconazole + anidulafungin combinations.

Based on the AUC$_{0-24}$/MIC–response relationships, there appeared to be an excellent linear relationship between observed and expected AUC$_{0-24}$/MICs of the combinations. For the voriconazole-susceptible isolate, the slope was significantly different from 1 and the intercept significantly different from 0, indicating synergism. In contrast, the slope was not significantly different from 1 for the voriconazole-resistant isolate, and the intercept was not significantly different from 0, indicating additivity.
### Table 3. Observed in vivo efficacy of voriconazole + anidulafungin combination therapy against infection caused by the voriconazole-susceptible (MIC 0.25 mg/L) and voriconazole-resistant (MIC 4 mg/L) *A. fumigatus* isolates

AFG, anidulafungin; VRC, voriconazole.

Results are presented as observed percentage of survival.

<table>
<thead>
<tr>
<th>Dose mg/kg</th>
<th>Voriconazole-susceptible <em>A. fumigatus</em></th>
<th>Voriconazole-resistant <em>A. fumigatus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5 VCZ&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>18.2</td>
</tr>
<tr>
<td>2.5 AFG</td>
<td>18.2</td>
<td>54.5</td>
</tr>
<tr>
<td>5 AFG</td>
<td>27.3</td>
<td>72.7</td>
</tr>
<tr>
<td>10 AFG</td>
<td>45.4</td>
<td>81.8</td>
</tr>
<tr>
<td>20 AFG</td>
<td>72.7</td>
<td>90.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Voriconazole concentration in mg/L.

<sup>b</sup> Anidulafungin concentration in mg/L.
**Table 1.**

<table>
<thead>
<tr>
<th>A. fumigatus</th>
<th>SUM ΔE (%)</th>
<th>Mean ΔE (%)</th>
<th>± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voriconazole-susceptible (MIC 0.25 mg/L)</td>
<td>119.0</td>
<td>7.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Voriconazole-resistant  (MIC  4  mg/L)</td>
<td>35.5</td>
<td>2.2</td>
<td>2</td>
</tr>
</tbody>
</table>

**Figure 2.**
Figure 2. Interaction surfaces obtained from response surface analysis of Bliss independence no-interaction model for in vivo combination of voriconazole and anidulafungin against (a) voriconazole-susceptible (voriconazole MIC = 0.25 mg/L and anidulafungin MEC = 0.03 mg/L) and (b) voriconazole-resistant (voriconazole MIC = 4 mg/L and anidulafungin MEC = 0.03 mg/L) A. fumigatus isolates.

The x-axis and y-axis represent the efficacy of voriconazole and anidulafungin, respectively. The z-axis is DE in %. The 0-plane represents Bliss independent interactions, whereas the volumes above the 0-plane represent statistically significantly synergistic (positive DE) interactions.

The magnitude of interactions is directly related to DE. The different tones in three-dimensional plots represent different percentile bands of synergy. AFG, anidulafungin; VRC, voriconazole; MEC, minimum effective concentration.
**Figure 3.** Observed versus predicted (using non-parametric Bliss independence no-interaction model) percentage of survival for various intraperitoneal dosing regimens (2.5, 5, 10 and 20 mg/kg) of voriconazole + anidulafungin combinations. The slope significantly deviated from 0 for both voriconazole-susceptible (bottom) and voriconazole-resistant (below) A. fumigatus isolates (non-0 slope, with P value <0.0001). The PD indices (AUC₀−24/MIC) of single agents were used to predict the efficacies of the combination therapy on the basis of a linear regression analysis to determine the presence of synergism. By plotting predicted versus observed effect, any deviation of slope from 1 and intercept from 0 indicates interaction in vivo.
Discussion

In the present study we investigated whether the combination of voriconazole and anidulafungin could be used as a treatment option in patients with azole-resistant IA using an experimental model of infection. We observed that the combination of voriconazole and anidulafungin was synergistic in voriconazole-susceptible IA, but additive in voriconazole-resistant IA. Voriconazole monotherapy was effective against the voriconazole-susceptible isolate, whereas efficacy of voriconazole was significantly reduced in mice infected with the TR34/L98H isolate, with an MIC of 4 mg/L. For the latter, maximum survival was not reached with the highest dose.

The exposure–response relationships indicate that increased survival of both voriconazole-susceptible and voriconazole-resistant isolates was dependent on the dose, but a much better relationship existed with the total AUC0-24/MIC ratio, in line with previous results (14, 27). Those studies, as well as others (32), have indicated that achieving a serum free-drug AUC/MIC ratio of greater than 25 is the value of the PD index linked to successful treatment. Since the MIC for the voriconazole-resistant isolate is 4 mg/L and the AUC0–24 is 58.1 h.mg/L for the 20 mg/kg voriconazole dose (and the unbound fraction even lower, 17.0 h.mg/L), this explains why a maximum effect could not be reached for the resistant isolate.

Anidulafungin monotherapy was less effective and, although increasing doses increased survival, maximum survival was not achieved for infections with either isolate, which explains why anidulafungin is not an effective echinocandin for single-drug therapy against aspergillus infections.

A possible limitation of the experimental design used in the current study is that the effects were observed in non-neutropenic animals and the route of infection was dissemination rather than inhalation. However, IA in the non-neutropenic host is observed with increasing frequency, although other host factors might be impaired in such patients, in particular those in intensive care units (1). The effects observed could therefore be an underestimate of the exposure required. On the other hand, studies with posaconazole and voriconazole in a neutropenic model (15), and non-neutropenic model (14, 27), have shown that the exposure–response relationships are of the same order of magnitude; in fact, slightly lower exposures were required in the neutropenic model.

There are conflicting reports on the efficacy of combination therapy with voriconazole+ anidulafungin. van de Sande et al. (34), using human-equivalent doses of both drugs, found no additional benefit of adding anidulafungin to treatment with voriconazole for a voriconazole-susceptible isolate.
In our study there was a clear benefit of adding anidulafungin, and the combination was found to be synergistic rather than additive. In other studies, the combination was also found to significantly enhance the efficacy of antifungal therapy compared with either drug alone as measured by increased survival, reduction in residual fungal burden (log cfu/g), reduced galactomannan antigenaemia and decreased pulmonary injury (determined by lung weights, pulmonary infarct scores and CT scan image score), indicating a synergistic action in vivo (19).

Our results are also comparable to in vitro or animal studies comparing other combinations of echinocandins and triazoles. Indeed, synergistic or additive effects of echinocandins combined with an azole antifungal have been observed in some in vitro and experimental animal models (16,17, 19, 20, 34-45). In such studies, synergistic combinations were obtained when voriconazole was combined with caspofungin in a guinea pig model of IA (40, 46), or in combination with micafungin in the study of Lewis and Kontoyiannis (39).

In vivo synergistic interaction between ravuconazole and micafungin in experimental invasive pulmonary aspergillosis led to significant reductions in mortality, residual fungal burden and serum galactomannan antigenaemia, compared with either agent alone (46). Cuenca-Estrella et al. (35), reported in vitro synergistic interactions between itraconazole and caspofungin, similar to the study of Shalit et al. (36). In vivo synergy between voriconazole and caspofungin has been demonstrated by Kirkpatrick et al. (41), in an experimental model of IA in guinea pigs.

Although we did find that the combination had a beneficial effect on infection with the voriconazole-resistant isolate, and 100% survival could be reached using the highest doses of both drugs, the effect of the combination on the voriconazole-resistant isolate was somewhat different from the effect on the voriconazole-susceptible isolate, and appears to be additive rather than synergistic. This was confirmed by applying the methodology developed previously for antibacterial drugs (31).

We found a good correlation between the expected effects of the combination, based on the relationship between \( \text{AUC}_{0-24}/\text{MIC} \) and mortality during single drug therapy, and the observed effects. Thus, for the voriconazole-resistant isolate, the effect of the combination could be predicted by adding the predicted effects based on the \( \text{AUC}_{0-24}/\text{MIC} \) relationships found for monotherapy based on the Prichard model, whereas clear synergy was observed for the voriconazole-susceptible isolate. Translating these results to treatment for infections in humans, it can be concluded that the addition of anidulafungin to voriconazole has merit, in particular for infections caused by voriconazole susceptible isolates.
Although most guidelines indicate voriconazole as an agent of choice, the cure rate in patients treated with voriconazole alone is not optimal (4-6), and addition of anidulafungin, especially in patients with advanced or severe disease, may be of benefit. The question that remains is whether the additive effect of anidulafungin in patients with voriconazole resistant isolate infections is enough to overcome resistance and up to which MIC of voriconazole application of anidulafungin is meaningful.

The loss of synergistic drug interaction between voriconazole and anidulafungin in mice infected with an *A. fumigatus* isolate for which voriconazole has an MIC of 4 mg/L raises concern regarding the use of this combination in azole-resistant IA. This indicates that the drug interaction varies according to the susceptibility of the isolate to voriconazole. It seems that the azole target is lost in isolates for which the MIC is high and the loss of voriconazole efficacy cannot be overcome by adding anidulafungin.

The MIC of voriconazole for the TR34/L98H isolate we used in our experiments was 4 mg/L, which is above the resistance breakpoint of > 2 mg/L. In isolates for which the MIC of voriconazole is higher, i.e. 8, 16 or >16 mg/L, possibly even less drug interaction can be expected. As the drug interaction between echinocandins and triazoles is most likely due to simultaneous independent mechanisms of action, the consequence could be that in those isolates for which the MIC of voriconazole is 16 mg/L the efficacy of the voriconazole + anidulafungin combination relies solely on the efficacy of anidulafungin, which is suboptimal.

Clearly, it is difficult to extrapolate our observations to general statements regarding the use of voriconazole + anidulafungin in the management of azole-resistant IA as we have investigated only one voriconazole-resistant isolate with one resistance mechanism. More isolates should be investigated in order to understand in more depth the potential of voriconazole + anidulafungin combination therapy in azole-resistant aspergillus disease.

In conclusion, there is a clear benefit of combining voriconazole and anidulafungin, but the reduced effect of combination therapy in azole-resistant IA raises some concern.
References


Acknowledgements

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Parts of these results were presented at the Fifty-first Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, USA, 2011 (Abstract A2-584).
Pharmacodynamics and Dose-Response Relationships of Liposomal Amphotericin B against Different Azole-Resistant *Aspergillus fumigatus* Isolates in a Murine Model of Disseminated Aspergillosis

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Abstract

The management of invasive aspergillosis (IA) has become more complicated due to the emergence of acquired azole resistance in Aspergillus fumigatus, which is associated with treatment failure and a mortality rate of 88%. Treatment with liposomal amphotericin B (L-AmB) may be a useful alternative to improve therapeutic outcome in azole-resistant IA. Four clinical A. fumigatus isolates obtained from patients with proven IA were studied in a nonneutropenic murine model of infection: a wild-type isolate without mutations in the cyp51A gene and three azole-resistant isolates harboring a single mutation at codon 220 (M220I) and tandem repeat mutations (a 34bp tandem repeat mutation in the promoter region of the cyp51A gene in combination with substitutions at codon L98 [TR34/L98H] and a 46-bp tandem repeat mutation in the promoter region of the cyp51A gene in combination with mutation at codons Y121 and T289 [TR46/Y121F/T289A]), respectively. Female CD-1 mice were infected intravenously 24 h prior to the start of therapy. Groups of 11 mice were treated at days 1, 2, and 5 postchallenge with increasing 4-fold doses of L-AmB ranging from 0.004 to 16 mg/kg/day and observed for 14 days. Survival for all 4 isolates at day 14 was significantly better than that of controls. A dose-response relationship was observed independent of the azole resistance mechanism. The Hill-type model with a variable slope fitted the relationship between the dose and 14-day survival well for all isolates, with $R^2$ values of 0.95 (wild-type), 0.97 (M220I), 0.85 (TR34/L98H), and 0.94 (TR46/Y121F/T289A), respectively. Multiple logistic regression analysis confirmed that there was no significant difference between groups. The results of these experiments indicate that L-AmB was able to prolong survival in vivo in disseminated IA independent of the presence of an azole resistance mechanism in a dose-dependent manner, and therefore, they support a role for L-AmB in the treatment of azole-resistant IA.

Keywords

Liposomal Amphotericin B, Azole-resistant, Invasive aspergillosis
Introduction

Voriconazole is the recommended first-choice therapy for invasive infections caused by Aspergillus species (1, 2). However, the management of invasive aspergillosis (IA) has become more complicated due to the emergence of acquired azole resistance in Aspergillus fumigatus (3), and azole resistance has been reported on different continents (4–8). There is increasing evidence that azole resistance is associated with treatment failure (5, 9, 10), and a mortality rate of 88% has been reported (5). These clinical observations are also supported by animal models of IA, where the MIC was shown to have major implications for the efficacy of voriconazole and posaconazole (11, 12). Therefore, it is important to explore alternative treatment regimens. Lipid formulations of the amphotericin B and echinocandin antifungals or combination therapy may be important alternative options, in patients with azole-resistant Aspergillus diseases.

We previously investigated the pharmacodynamics of anidulafungin monotherapy and the combination of voriconazole and anidulafungin (13–15). Although anidulafungin treatment improved the survival of mice in a dose-dependent manner, a maximal response was not achieved when mice were infected with an azole-susceptible or azole-resistant isolate, even in those treated with the highest anidulafungin dose. The results of combination therapy suggested that voriconazole and anidulafungin have a synergistic interaction in mice infected with a voriconazole-susceptible isolate. However, the synergistic interaction was lost in the azole-resistant isolate (voriconazole MIC, 4 mg/liter), as only an additive interaction was observed (13). A relation between the voriconazole MIC and the fractional inhibitory concentration (FIC) index was observed in vitro, which indicated that further increase of the voriconazole MIC was associated with less favorable drug interaction (14). In infection due to isolates which are highly resistant to voriconazole, the efficacy of the combination might rely only on that of anidulafungin, which is suboptimal. In clinical practice, this is a major drawback, as isolates that are highly resistant to voriconazole are increasingly common (4), and in culture-negative patients we will be unable to determine voriconazole susceptibility.

Treatment with a liposomal amphotericin B (L-AmB) may be a useful alternative to improve therapeutic outcome in azole-resistant IA, as the in vitro activity of AmB appears not to be affected in azole-resistant isolates (J. W. M. Van der Linden, S. M. Camps, G. A. Kampinga, J. P. A. Arends, Y. J. Debets-Ossenkopp, P. J. A. Haas, B. J. A. Rijnders, E. J. Kuijper, F. H. van Tiel, J. Varga, A. Karawajczyk, J. Zoll, W. Melchers, and P. E. Verweij, submitted for publication). L-AmB has been developed to reduce toxicity and enhance the safety profile and efficacy of AmB (16–20). L-AmB significantly reduced dose-limiting toxicities by allowing administration of higher doses of the drug and improving the pharmacokinetic and pharmacodynamic properties (21, 22).
There are currently no data on the in vivo efficacy of L-AmB inazole-resistant IA. Here we investigated the pharmacodynamics and dose-response relationships of L-AmB against wild-type and three clinical azole-resistant A. fumigatus isolates harboring different resistance mechanisms in an immunocompetent murine model of disseminated aspergillosis.

Materials and Methods

Fungal isolates

Four clinical A. fumigatus isolates obtained from patients with proven IA (classified according to EORTC/MSG [European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group] consensus definitions) (23) were used in the experiments: a wild-type isolate without mutations in the cyp51A gene (isolate AZN 8196) and three azole-resistant isolates harboring an M220I (isolate V28-37), a TR34/L98H (isolate V52-35), and a TR46/Y121F/T289A (isolate V54-10) resistance mechanism. Strain identification and the cyp51A gene substitutions were confirmed by sequence-based analysis as described previously (4). The isolates had been stored in 10% glycerol broth at −80°C and were revived by subculturing on Sabouraud dextrose agar (SAD) supplemented with 0.02% chloramphenicol for 5 to 7 days at 35 to 37°C. The in vitro antifungal susceptibilities to AmB, itraconazole, voriconazole, and posaconazole were determined based on the EUCAST guidelines, using a broth microdilution format (24).

Infection model

A total of 604 outbred CD-1 (Charles River, the Netherlands) female mice, 4 to 5 weeks old, weighing 20 to 25 g, were randomized into groups of 11 mice for L-AmB monotherapy. Animals were infected using the procedure described before (11, 13, 15, 25). Before performing the experiment, the isolates were cultured once on SAD for 5 days at 35 to 37°C and subcultured twice on 15-cm Takashio slants for 5 days at 35 to 37°C. The conidia were harvested in 20 ml of sterile phosphate-buffered saline (PBS) plus 0.1% Tween 80 (Boom B.V., Meppel, the Netherlands). The conidial suspension was filtered through sterile gauze folded four times to remove any hyphae, and the conidia were counted in a hemocytometer. After the inoculum was adjusted to the required concentration, the conidial suspension was stored overnight at 4°C. The 90% lethal dose (LD90) was determined for each isolate separately.
Mice were infected via injection of an inoculum corresponding to LD90 of each isolate into lateral tail vein. The LD90 was 2.4 $\times$ 10^7 (wild-type control), 5 $\times$ 10^7 (M220I), 2.5 $\times$ 10^7 (TR90/L98H), and 3.5 $\times$ 10^7 (TR60/Y121F/T289A) conidia, respectively. Postinfection viability counts of the injected inocula were determined to ensure that the correct inoculum had been injected. The animals were housed under standard conditions, with food and water supplied ad libitum. The animal studies were conducted in accordance with the recommendations of the European Community (directive 86/609/EEC, 24 November 1986), and all animal procedures were approved by the Animal Welfare Committee of Radboud University (RU-DEC2012-050). The infected mice were examined at least three times daily. These clinical inspections were carried out in order to ensure that there were no cases of desiccation, torticollis, staggering, extreme weight loss (a decrease of 15% within 48 h or 20% within 24 h), or body temperature drop to below 33°C. Mice demonstrating these signs of disease were humanely terminated. On day 15 postinfection, all remaining surviving mice were humanely euthanized under isoflurane anesthesia, and blood and internal organs were collected. The survival (in days postinfection) was recorded for each mouse in each group and was considered an outcome effect measure to assess the therapeutic efficacy of L-AmB monotherapy (26).

**Antifungal compound and treatment regimens**

The commercial formulation of L-AmB (Ambisome) was obtained from manufacturer (Gilead Sciences, Amsterdam, the Netherlands). Drug solutions were prepared on the day of study following instructions of the manufacturer, diluted with a standard 5% glucose solution to obtain the desired concentration. Mice were treated intravenously at days 1, 2, and 5 postchallenge with increasing 4-fold doses of L-AmB ranging from 0.004 to 16 mg/kg once daily and observed for 14 days. Control mice were infected but received only 5% glucose.

**Statistical analysis**

All data analyses were performed by using GraphPad Prism, version 5.0, for Windows (GraphPad Software, San Diego, CA). Mortality data were analyzed by the log rank test. The relationship between the in vivo efficacy (survival) and dose was determined by non-linear regression analysis and the Hill equation with a variable slope fitted to the data, with the maximum effect (maximum survival) constrained at <100%. The goodness of fit was checked by the $R^2$ and visual inspection. Statistical significance was defined as a P value of <0.05 (two-tailed). For comparison, an F test was performed to define whether the best-fit values (log 50% effective concentration [EC50]) differed between the four groups.
Results

**In vitro susceptibility**

The characteristics and in vitro susceptibilities of the four *A. fumigatus* isolates are shown in Table 1. All isolates grew well after 48 h of incubation at 35 to 37°C. The isolates harboring a resistance mechanism showed variable susceptibility profiles against the three azoles. In comparison to a MIC of 0.25 mg/liter for the wild-type isolate, voriconazole showed similar activity against the isolate harboring the M220I resistance mechanism (MIC, 0.5 mg/liter) but reduced in vitro activity against TR34/L98H and TR46/Y121F/T289A isolates, with MICs of 4 and 16 mg/liter, respectively. There was no difference in the AmB activity between the isolates.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th><em>Aspergillus</em> disease</th>
<th>Prior azole exposure</th>
<th>Cyp51A substitution</th>
<th>MIC or MEC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AMB</td>
</tr>
<tr>
<td>AZN 8196</td>
<td>Proven IA</td>
<td>No</td>
<td>None</td>
<td>0.5</td>
</tr>
<tr>
<td>V 28-77</td>
<td>Proven IA</td>
<td>Yes</td>
<td>M220I</td>
<td>0.5</td>
</tr>
<tr>
<td>V 52-35</td>
<td>Proven IA</td>
<td>No</td>
<td>TR₃₄/L98H</td>
<td>0.5</td>
</tr>
<tr>
<td>V 94-10</td>
<td>Proven IA</td>
<td>No</td>
<td>TR₄₆/Y121F/T289A</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Table 1.** Characteristics of *A. fumigatus* isolates. All isolates were associated with proven IA, according to EORTC/MSG consensus definitions (23).

MEC, minimum effective concentration; IA, invasive aspergillosis; AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; AFG, anidulafungin.
Efficacy of L-AmB monotherapy

(i) Survival curves

Figure 1 shows the survival curves of L-AmB-treated mice by dose. The survival curves for all control groups receiving intravascular 5% glucose, showed a mortality of 90 or 100%. Survival for all four azole-resistant isolates at day 14 was significantly better than that of controls. The maximum effect (100% survival) was reached at a dose of 16 mg/kg for the wild-type, M220I, and TR34/L98H isolates and at a dose of 4 mg/kg for the TR46/Y121F/T289A mutant.

Figure 1. Efficacy of L-AmB against 4 A. fumigatus isolates. Survival curves are depicted by strain. Animals were treated intravenously at days 1, 2, and 5 postchallenge with increasing 4-fold doses of L-AmB ranging from 0.004 to 16 mg/kg/day and observed for 14 days. Placebo groups received 5% glucose. For all groups, \( n = 11 \).
(ii) Dose-response analysis

The dose-response curves for dosing regimens and control groups of L-AmB monotherapy are shown in Fig. 2. L-AmB treatment improved the survival of the mice in a dose-dependent manner. A dose-response relationship was observed that depended on the L-AmB dose level but was independent of the azole-resistance mechanisms. The Hill-type model with a variable slope fitted the relationship between the dose and 14-day survival well, with $R^2$ values of 0.95 (wild type), 0.97 (M220I), 0.85 (TR$_{34}$/L98H) and 0.94 (TR$_{46}$/Y121F/T289A), respectively. The 50% effective dose (ED$_{50}$) was 0.29 mg/kg (95% confidence interval [CI], 0.05 to 1.65 mg/kg) for the wild type, 0.20 (95% CI, 0.05 to 0.74 mg/kg) for M220I, 0.59 (95% CI, 0.02 to 14.80 mg/kg) for TR$_{34}$/L98H, and 0.078 (95% CI, 0.008 to 0.69 mg/kg) for TR$_{46}$/Y121F/T289A isolate.

**Figure 2.** Fourteen-day survival as a function of L-AmB dose against 4 A. fumigatus isolates. Shown are data for the wild type (AZN81-96; voriconazole [VRC] MIC, 0.25 mg/liter) and for the M220I (isolate V28-77; VRC MIC, 0.5 mg/liter), TR$_{34}$/L98H (V52-35; VRC MIC, 4 mg/liter), and TR$_{46}$/Y121F/T289A (V94-10; VRC MIC, 16 mg/liter) mutants. L-AmB treatment improved the survival of the infected mice in a dose-dependent manner for all four isolates. The curves indicate fits with the Hill equation for each isolate.
(iii) Comparative efficacy of L-AmB against the four isolates

In order to compare the efficacy of L-AmB in treating infection caused by the different isolates, the best-fit values for the curves were defined based on the EC50, EC80, and EC90 of L-AmB and compared to each other (Table 2). The efficacy of L-AmB was not different between the isolates with an azole resistance mechanism and wild-type controls \((P > 0.05)\), and no difference in efficacy was found when different azole resistance mechanisms were compared \((P > 0.05)\). The null hypothesis was not rejected in an \(F\) test \((P = 0.92, \quad F = 0.2241, \quad \text{DFn} = 4, \quad \text{DFd} = 54)\), indicating that log EC50 did not significantly differ between the four groups (Table 2). In addition, multiple logistic regression analysis confirmed that there was no significant difference between groups (results not shown).

<table>
<thead>
<tr>
<th>Efficacy of L-AmB</th>
<th>(A.\ fumigatus\ isolates)</th>
<th>(\text{P value})</th>
<th>(F) test ((\text{DFn}, \text{DFd}))</th>
<th>Difference between efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Log EC}_{50})</td>
<td>Wild Type</td>
<td>M 220 I Mutant</td>
<td>(\text{TR}_{34})/L98H Mutant</td>
<td>(\text{TR}_{46}/\text{Y121F/T289A}) Mutant</td>
</tr>
<tr>
<td>(\text{Log EC}_{80})</td>
<td>0.71 (-0.036 to 1.46)</td>
<td>0.47 (-0.09 to 1.03)</td>
<td>1.24 (-0.21 to 2.70)</td>
<td>0.10 (-0.73 to 0.94)</td>
</tr>
<tr>
<td>(\text{Log EC}_{90})</td>
<td>1.44 (0.34 to 2.53)</td>
<td>1.15 (0.34 to 1.96)</td>
<td>2.10 (-0.12 to 4.32)</td>
<td>0.81 (-0.37 to 2.00)</td>
</tr>
</tbody>
</table>

**Table.** Efficacy of L-AmB against four \(A.\ fumigatus\) isolates. No differences were significant. DFn, degrees of freedom numerator; DFd, degrees of freedom denominator.
Discussion

In the present study, we investigated whether L-AmB could be used as an alternative treatment option in patients with azole- resistant IA, using an experimental model of infection. We observed that L-AmB was able to prolong survival in vivo in disseminated IA due to azole-resistant A. fumigatus isolates independent of the azole resistance phenotype. Despite a wide variation in susceptibility to the primary treatment option, voriconazole (range, 0.5 to 16 mg/liter), a maximal response was achieved with each isolate. Our model indicated that L-AmB exhibited a dose-dependent effect on survival, with increasing doses corresponding with increased survival.

Both in vitro and in vivo AmB and lipid AmB formulations generally display concentration-dependent fungicidal activity that begins to plateau once concentrations exceed the MIC of the infecting pathogen by 4- to 10-fold (27, 28). The concentration-dependent killing and post antifungal effects of AmB had already been shown in several in vitro and in vivo studies of efficacy against Candida and Aspergillus species, when the concentration of AmB was increased to a level multiple times higher than the MIC (27, 29–35). We previously compared dose-response relationships of three AmB formulations in our nonneutropenic murine model of IA using the same wild-type isolate that was used as the control strain in the present study (20). Our previous work indicated that L-AmB exhibited a dose-dependent effect on survival and that the maximum effect in terms of survival was higher for L-AmB than for conventional AmB and AmB lipid complex (79 to 100%, P <0.05). The maximum effect was reached at a dose of 16 mg/kg of L-AmB, and the ED50 was 0.06 mg/kg (95% CI, 0.03 to 0.127) (20).

These results are similar to the present results, as the maximum effect (100% survival) was reached at a dose of 4 or 16 mg/kg for all isolates. Our observations are also in keeping with previously published experimental studies of aspergillosis (18, 19, 36–40). Leenders et al. reported that in a pulmonary aspergillosis infection in rats, L-AmB monotherapy at 5 and 10 mg/kg was effective in pre-venting dissemination from the lungs to the kidneys, liver, and spleen (36). Survival improved from 57% to 86% as the dose of L-AmB increased from 5 to 15 mg/kg. In another study, Takemoto et al. reported that both 3 and 10 mg/kg of L-AmB were efficacious, although 10 mg/kg was the most protective in disseminated murine aspergillosis (19).

The pharmacodynamic target C\text{max}/MIC (maximum concentration-to-MIC ratio) has been shown to be the best predictive parameter for AmB treatment efficacy (28, 41). In one study, the pharmacokinetics and pharmacodynamics of L-AmB were investigated in a small cohort of pediatric oncology patients with Aspergillus infections (42). In this study, the maximal efficacy associated with a higher probability of treatment response was observed when the C\text{max}/MIC of L-AmB was greater than 40, taking into account the individual patient pharmacokinetic
data, the MIC of the infecting organism, and clinical outcomes. In a similar fashion, when pharmacodynamics of AmB in a neutropenic mouse model of disseminated candidiasis were evaluated, increased killing was observed when the concentration of drug exceeded the MIC 2- to 10-fold (27, 33). However, Wiederhold et al. evaluated the pharmacodynamic characteristics of AmB in a murine model of invasive pulmonary aspergillosis (28) in which the maximal efficacy against this Aspergillus strain was observed at $C_{\text{max}}$/MIC values near 2.

In the present study, the maximum survival was reached at a dose of 16 mg/kg for the wild-type, M220I, and TR34/L98H isolates and at a dose of 4 mg/kg for the TR46/Y121F/T289A isolate. For most adults, standard L-AmB doses of 3 to 5 mg/kg should surpass the maximum concentration-to-MIC ratio of 40 unless the pathogen has an AmB MIC of 2 mg/liter or greater (43). In addition, in a recent clinical study that examined the benefits of increasing the dosage to 10 mg/kg of L-AmB daily in patients with proven or probable aspergillosis, the effectiveness of 3 mg/kg of L-AmB per day as first-line therapy for invasive aspergillosis was demonstrated (44).

We used survival as the endpoint. Although the disadvantage remains that a relatively large number of animals is needed to determine dose-response relationships, at present survival studies are still considered the most reliable effect measure to assess the efficacy of antifungals in experimental models of IA (26). However, efforts are underway and new techniques such as qPCR will probably replace survival in the near future, once these models have been sufficiently validated (26, 45, 46).

A possible limitation of the experimental design used in our study is that the effects were observed in nonneutropenic animals and the route of infection was intravenous, which is not the natural route of infection. The advantage of the model is that administration of immunosuppressive drugs, with potential implications for the results, and bacterial sepsis do not occur. We believe that our model performs well, giving exposure-response relationships similar to those in other models of infection. Studies with posaconazole and voriconazole in neutropenic (12) and nonneutropenic (11, 25) models have shown that the exposure-response relationships are on the same order of magnitude; in fact, slightly lower exposures were required in the neutropenic model.

Given that the clinical experience with the treatment of azole-resistant IA is still limited, it remains difficult to develop a treat-ment algorithm. Our study showed that the efficacy of L-AmB against isolates harboring different azole resistance mechanisms was similar to that against the wild-type control, indicating that L-AmB might have a role in the management of azole-resistant *A. fumigatus* disease.

In patients receiving azole therapy or prophylaxis, it seems appropriate to change to L-AmB when azole resistance in *A. fumigatus* is suspected or documented. In cases with
central nervous system involvement, the optimum therapy is unknown. L-AmB may have a lower efficacy than voriconazole (47), and therefore combination therapy might be more successful. Although the echinocandins are known to penetrate the cerebrospinal fluid poorly, the combination with L-AmB needs to be investigated in experimental models. Another potentially effective combination is L-AmB plus flucytosine, a combination that has been shown to be effective in central nervous system infections due to *Cryptococcus neoformans* (48). The efficacy of flucytosine against *A. fumigatus* infection has long been controversial, but we previously showed efficacy of flucytosine against *A. fumigatus* infection in our animal model (49) and a good correlation with *in vitro* activity when the MIC was determined at pH 5 instead of pH 7 (50). Clearly, more research is needed to allow recommendations to be made. In the absence of clinical trials that could provide clinical evidence for treatment choices inazole-resistant *Aspergillus* diseases, we believe that our findings support a role for L-AmB.
References


Acknowledgements

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6.1. The role of azoles in the management of azole-resistant aspergillosis: From the bench to the bedside

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Abstract

Azole resistance is an emerging problem in Aspergillus fumigatus and is associated with a high probability of treatment failure. An azole resistance mechanism typically decreases the activity of multiple azole compounds, depending on the mutation. As alternative treatment options are limited and in some isolates the minimum inhibitory concentration (MIC) increases by only a few two-fold dilutions steps, we investigated if voriconazole and posaconazole have a role in treatingazole-resistant Aspergillus disease. The relation between resistance genotype and phenotype, pharmacokinetic and pharmacodynamic properties, and (pre)clinical treatment efficacy were reviewed. The results were used to estimate the exposure needed to achieve the pharmacodynamic target for each MIC. For posaconazole adequate exposure can be achieved only for wild type isolates as dose escalation does not allow PD target attainment. However, the new intravenous formulation might result in sufficient exposure to treat isolates with a MIC of 0.5 mg/L. For voriconazole our analysis indicated that the exposure needed to treat infection due to isolates with a MIC of 2 mg/L is feasible and maybe isolates with a MIC of 4 mg/L. However, extreme caution and strict monitoring of drug levels would be required, as the probability of toxicity will also increase.

Keywords

Aspergillus fumigatus; Invasive aspergillosis; Voriconazole; Posaconazole; Azole-resistance; Management
Introduction

Azole resistance is an emerging problem in species of the genus *Aspergillus* (1, 2). The polyphasic approach to the taxonomic classification of aspergilli has resulted in the recognition of new species (3). These new or sibling species are difficult to identify using conventional methods, often requiring molecular techniques (4, 5). Recent epidemiologic research indicates that sibling species of *Aspergillus* may cause invasive aspergillosis in susceptible hosts (6-11). Many of these species show a susceptibility profile that differs from the conventional species, usually with reduced activity of specific antifungal agents (4).

In addition to intrinsic resistance within the aspergillus family (12), there are increasing reports of acquired resistance to azoles (1). The majority of reports concern *Aspergillus fumigatus* (13), although azole resistance has been reported sporadically in other species as well, such as *A. flavus* (14) and *A. terreus* (15).

In *A. fumigatus* two routes of resistance selection have been reported; Azole resistance has been reported in patients with chronic cavitating aspergillus diseases that receive long-term azole therapy (16). In these patients the initial infection is caused by an azole-susceptible isolate, but through therapy azole-resistant isolates may be cultured. A second route of resistance selection is believed to occur through exposure of *A. fumigatus* to azole compounds in the environment (2, 17-19). Azoles are commonly used for crop protection or material preservation. Some of the fungicides were found to have a molecule structure very similar to that of the medical triazoles (18, 19). The fungus is believed to develop mutations that confer resistance to fungicides, but due to the molecule similarity with the medical triazoles, the latter become inactive as well.

A wide range of mutations in *A. fumigatus* have been described conferring azole-resistance commonly involving modifications in the *cyp51A*-gene, the target of antifungal azoles. Cyp51A mutations in *A. fumigatus* commonly affect the activity of all mold-active antifungal azoles. Specific mutations correspond with various phenotypes characterized by complete loss of activity of a specificazole, and with decreased activity of others (20).

If a role for the azoles remains in the management of azole-resistant aspergillosis (21), optimizing drug exposure appears critical to increase the probability of treatment success. In this context, understanding of the pharmacokinetics (PK) and pharmacodynamics (PD) and more importantly defining the pharmacodynamic target of theazole compounds is crucial to increase the probability of a favorable clinical response (22).

Reduced susceptibility of the fungus for azoles has significant impact on the ability to achieve the PD-target, and sometimes targets can only be achieved at the cost of increased probability of toxicity. Many variables, such as the underlying azole resistance mechanism and
PK/PD properties of the antifungal agent, are important to determine if treatment with an azole remains feasible (23). Furthermore, in the absence of extensive clinical experience with the treatment of azole-resistant aspergillosis, data obtained through in vitro susceptibility testing and experimental models of infection are needed to design treatment strategies.

We reviewed our current understanding of azole resistance and the potential role of voriconazole and posaconazole in order to guide clinicians to manage patients with azole-resistant aspergillus disease. The results of in vitro and preclinical studies were extrapolated to humans to provide evidence that may support the use of voriconazole and posaconazole in isolates with attenuated azole susceptibility.

### Triazole antifungals: Mode of action and label indication for invasive aspergillosis

The antifungal triazoles are synthetic compounds that have >1 triazole ring attached to an isobutyl core (e.g., voriconazole, ravuconazole, and isavuconazole) or to an asymmetric carbon atom with a lipophilic complex mixed functional aromatic chain (e.g., itraconazole and posaconazole) (24). Triazoles inhibit the synthesis of ergosterol from lanosterol in the fungal cell membrane (24, 25); the target is the cytochrome (CYP)-dependent 14-a-demethylase (CYP51 or Erg11p), which catalyses this reaction. Thereby, ergosterol is depleted and methyl-sterols accumulate within the cell membrane and lead to either inhibition of fungal cell growth or death, depending on the species and antifungal compound involved. Triazoles are generally fungistatic, although itraconazole, voriconazole, posaconazole and isavuconazole have been shown to be fungicidal against Aspergillus spp. (25-27). The various azoles have different affinities for the CYP-dependent 14-a-demethylase, which in return results in various antifungal activities (28); and therefore various susceptibilities to Aspergillus spp.

Four triazole compounds (fluconazole, itraconazole, voriconazole, and posaconazole) have been clinically licensed and are currently in wide use for the prevention and treatment of invasive fungal infections (29, 30).

Fluconazole has a lack of efficacy against moulds such as Aspergillus spp., therefore targeted prophylaxis or treatment against aspergillosis cannot be covered by this agent. Itraconazole is commonly used for the treatment of chronic and allergic conditions (29, 30).
Voriconazole has broad in vitro activity against *Aspergillus* spp., is recommended first choice treatment of invasive aspergillosis with a label indication in adults and children aged 2 and above (30). In addition, voriconazole is the drug of choice for treatment of central nervous system aspergillosis (31).

Posaconazole is licensed only for patients aged 18 years or older (29); for prophylaxis in patients receiving remission-induction chemotherapy for acute myelogenous leukemia (AML) or myelodysplastic syndromes (MDS) expected to result in prolonged neutropenia and who are at high risk of developing invasive fungal infections; for prophylaxis of invasive fungal infections in hematopoietic stem cell transplant (HSCT) recipients who are undergoing high-dose immunosuppressive therapy for graft versus host disease and who are at high risk of developing invasive fungal infections; and for salvage therapy of invasive aspergillosis in patients with disease that is refractory to amphotericin B or itraconazole or in patients who are intolerant of these medicinal products (21, 32-34).

**Phenotypic detection of azole resistance and clinical breakpoints for *Aspergillus* spp**

In recent years major advances have been made in the detection of azole resistance in *Aspergillus* spp. Both the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial susceptibility Testing-subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST) have developed and standardized phenotypic methods that enable the reliable and reproducible determination of the minimal inhibitory concentration (MIC) for conidia-forming moulds or *Aspergillus* spp. (35, 36).

When a collection of fungal strains is tested, typically a Gaussian distribution of MICs is found referred to as the wild type population. The right side of the distribution, i.e. growth of isolates that is inhibited only by a higher concentration of the drug or any isolates / populations to the right side of the wild type distribution might contain isolates that possess a resistance mechanism. These isolates are considered non-wild type (37). Testing of large collections of fungi enables the determination of an epidemiological cut-off, which is the concentration of drug that inhibits 95% of the fungal species.

Notably, a clinical breakpoint is needed to obtain a clinically meaningful interpretation of the MIC of individual isolates. A standardized approach is followed, which incorporates standard dosing recommendations and formulations of antifungal agents, the PK/PD characteristics, information from experimental models of infection and results from clinical trials. All this information is analyzed and leads to the clinical breakpoint, i.e. the classification
of the isolate as susceptible to the drug or resistant. There are currently three sets of breakpoints and epidemiological cut-off values available; The first was published in 2009 by Verweij et al. based on clinical experience and the available knowledge at that time (13). Since then breakpoints have been published by the CLSI (38) and the EUCAST-AFT (39, 40). The breakpoints are shown in table 1.

<table>
<thead>
<tr>
<th>Minimum inhibitory concentration (MIC) mg/L</th>
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<tbody>
<tr>
<td><strong>EUCAST</strong></td>
</tr>
<tr>
<td>Azole antifungal</td>
</tr>
<tr>
<td>Itraconazole</td>
</tr>
<tr>
<td>Voriconazole</td>
</tr>
<tr>
<td>Posaconazole</td>
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<tr>
<td><strong>Verweij et al.</strong></td>
</tr>
<tr>
<td>Itraconazole</td>
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<td>Voriconazole</td>
</tr>
<tr>
<td>Posaconazole</td>
</tr>
<tr>
<td><strong>CLSI-ECVs</strong></td>
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<tr>
<td><strong>Non-Wild-Type</strong></td>
</tr>
<tr>
<td>Itraconazole</td>
</tr>
<tr>
<td>Voriconazole</td>
</tr>
<tr>
<td>Posaconazole</td>
</tr>
</tbody>
</table>

**Table 1.** Epidemiological cut-off values, proposed EUCAST breakpoints (MIC, mg/L) for the *A. fumigatus* and clinically licensed active azoles ( adopted from Hope et al. 2013, Arendrup et al. 2012 and Verweij et al. 2009) and CLSI breakpoints adopted from Espinel-Ingroff et al. 2010.
Genotypic detection of azole resistance mechanisms in *Aspergillus* spp

In addition to the phenotypic methods, significant insight has been obtained regarding the underlying genetic mechanisms that confer an azole resistant phenotype. In *A. fumigatus* two distinct but closely related cyp51 genes were found (cyp51A and cyp51B) that share 63% sequence identity and encode for two different cyp51 proteins (41, 42). Azole binding studies showed that fluconazole has the weakest binding with cyp51A and cyp51B proteins, which is in keeping with the lack of activity against *A. fumigatus*. Furthermore cyp51B showed more tight bindings with azoles compared to cyp51A and is generally more susceptible to azole compounds compared to cyp51A (41). Therefore, it has been postulated that the cyp51A gene encodes for the major 14-alpha-demethylase enzyme activity required for growth, and the cyp51B gene encodes for alternative functions for particular growth conditions or even being functionally redundant (41). This provides a possible explanation to why mutations in azole-resistant *A. fumigatus* isolates are predominantly detected in the cyp51A gene and only rarely in the cyp51B gene (41, 43). Also in cyp51B no mutations have yet been proven to be correlated to azole resistance (44).

Three different studies adapted the X-ray crystallography of *Mycobacterium tuberculosis* protein to develop an *A. fumigatus* cyp51A 3-D protein model (45-47). All models show that two ligand entry channels can be identified in the cyp51A protein. The ligand access channels immersed in the endoplasmic reticulum (ER) membrane would allow highly lipophilic sterol substrates as well as azole compounds to dock into the channels and restrict access of other metabolites (41). The azole compounds can bind to the active heme molecule located in the center of the cyp51A protein and thereby inhibiting its enzyme function.

Different single nucleotide polymorphisms (SNPs) in the cyp51A-gene are related to resistance against one or more azole compounds found in clinical induced azole resistant *A. fumigatus* isolates (Table 2). Although several SNPs have been reported, codons 54, 98 and 220 are the most frequently characterized hot spots. According to protein homology modeling, these codons are located in the opening of one of the ligand access channels, which is thought to interfere with the entry of azole compounds into the hydrophobic access channel (48).

In addition to single point mutations, a combination of genetic changes has been described in azole-resistant *A. fumigatus* isolates. The duplication of a set of sequences in the promoter region significantly increases the expression of cyp51A which for one part provides an explanation for the decrease in azole susceptibility.
Importantly, recombinant experiments showed that only when both mutations were introduced the multi azole resistance (MAR) phenotype was observed (20, 49). Up until now, three mechanisms have been described: a 34 base pair tandem repeat (TR) combined with an L98H substitution in the cyp51A-gene (TR\textsubscript{34}/L98H) (2, 49-51), a 53 bp TR without substitutions in the cyp51A-gene (TR\textsubscript{53}) (52, 53), and recently a 46 bp TR with two substitutions in the cyp51A-gene (TR\textsubscript{46}/Y121F/T289A) (54, 55). Unlike the point mutations, the resistance mechanisms with a TR appear not to have a predictable phenotype for all azole compounds. Isolates with TR\textsubscript{53} are associated with a pan-azole-resistant phenotype and was reported to have caused aspergillus osteomyelitis in a pediatric patient in 2006 (53). Isolates harboring the TR\textsubscript{34}/L98H resistance mechanism are all highly resistant to itraconazole and have a MIC of 0.5 mg/l for posaconazole, but the activity of voriconazole varies, ranging from 1 to >16 mg/L. Likewise in isolates with the TR\textsubscript{46}/Y121F/T289A, voriconazole is inactive but the activity of itraconazole may vary ranging from 0.5 to 16 mg/L. Notably, this type of resistance mechanisms has been found in isolates that are associated with the environmental route of resistance selection. A TR in the promoter region has been described in several azole-resistant plant pathogenic fungi, which adds to the evidence that selection of this type of resistance mechanism occurs in the environment (17).

Although the azole target cyp51A is a hotspot for mutations that confer phenotypic resistance, there is an increasing number of resistant isolates with a wild type cyp51A sequences, indicating the presence of another, yet unknown resistance mechanism. Recently, Camps et al. reported a novel resistance mechanism, consisting of a mutation in the CCAAT binding transcription factor complex subunit HapE (56). The substitution was found in P88L within the exonic region of HapE gene causing the resistance phenotype. Unlike cyp51A-mediated resistance mechanisms, HapE was associated with a fitness cost (57). In addition, the increased mRNA expression of the Aft1 transposon (AfuMDR1 and AfuMDR4 transporters) was demonstrated in pan-azole resistant A.\textit{fumigatus} isolates, which could contribute to azole resistance or simply represent a stress response (58).

Nevertheless, as is the case for \textit{A. fumigatus}, azole resistance in other species of \textit{Aspergillus} such as \textit{A. flavus} (14), and \textit{A. terreus} (15), may be caused by alterations and over-expression of the azole target 14a-demethylase. This indicates that acquired azole resistance is a clinical challenge that is not restricted to \textit{A. fumigatus}. The voriconazole-resistant strain of \textit{A. flavus} was isolated from the surgical lung specimen of an invasive aspergillosis patient with no response to voriconazole therapy (14). Through sequencing and gene replacement studies the T788G mis-sense mutation in the cyp51C gene was identified as responsible for voriconazole resistance in \textit{A. flavus}. In another study by Arendrup et al., azole resistance in \textit{A. terreus} isolates was explored (15). The itraconazole MIC was elevated (1–2 mg/L), and voriconazole and posaconazole MICs were 0.5–4 and 0.06–0.5 mg/L, respectively. Sequencing
of the cyp51A-gene suggested a potential role of the M217I alteration in itraconazole resistance in *A. terreus*. This codon corresponds to M220 in *A. fumigatus*.

<table>
<thead>
<tr>
<th>Cyp51A substitution</th>
<th>Maximum number of isolates</th>
<th>MIC (mg/l)</th>
<th>Reference</th>
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<tr>
<td></td>
<td></td>
<td>ITC</td>
<td>VRC</td>
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<tr>
<td>G54E</td>
<td>6</td>
<td>&gt;8</td>
<td>0.25-0.5</td>
</tr>
<tr>
<td>G54R</td>
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<td>G13BC</td>
<td>10</td>
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<td>4 to &gt;8</td>
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<td>P216I</td>
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<td>1</td>
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<tr>
<td>TR&lt;sub&gt;5&lt;/sub&gt;/Y121F/T289A</td>
<td>29</td>
<td>0.5 to &gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>TR&lt;sub&gt;5&lt;/sub&gt;</td>
<td>1</td>
<td>&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>F219I</td>
<td>6</td>
<td>&gt;16</td>
<td>1, 4, 8</td>
</tr>
<tr>
<td>P216I</td>
<td>1</td>
<td>&gt;16</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. The minimum inhibitory concentrations (MICs) of clinical *A. fumigatus* isolates with various cyp51A conferring azole-resistance phenotype.
Clinical implications of azole resistance in *A. fumigatus*

There are currently no randomized controlled trials showing that azole resistance is associated with an increased probability of treatment failure compared to infection with wild type isolates. Case series have been published including both patients with azole-resistant chronic aspergillus diseases and azole-resistant invasive aspergillosis that show the recovery of an azole-resistant isolate is associated with a high probability of azole treatment failure (2, 16, 53, 59-64).

In the study of Howard et al. a wide range of mutations was found in azole-resistant *A. fumigatus* isolates that were cultured from clinical samples in patients with primarily non-invasive aspergillus infections (16). A total of 14 patients were investigated; 2 patients had invasive disease; 9 had chronic diseases with ≥ 1 aspergillomas; 2 had allergic bronchopulmonary aspergillosis; and one had aspergillus bronchitis. Thirteen of 14 evaluated patients had prior itraconazole exposure. Eight patients failed azole therapy (progressed), and 5 failed to improve (clinically stable disease). In patient 14 the infection also failed to treatment with voriconazole for 18 months, in which the corresponding isolates had MICs of >8 mg/L for both itraconazole and voriconazole.

Two case series of patients with azole-resistant invasive aspergillosis were reported from the Netherlands (54, 65). In one study, eight patients with proven or probable, culture-positive invasive aspergillosis due to *A. fumigatus* harboring the TR34/L98H resistance mechanism were described. All five patients with invasive pulmonary aspergillosis failed to voriconazole therapy and had died at 12 weeks post diagnosis, while two of three patients presenting with disseminated aspergillosis died, despite treatment with multiple drugs with anti-aspergillus activity (65). Overall, seven of eight (88%) patients had died at 12 weeks after diagnosis. Although all TR34/L98H isolates were highly resistant of itraconazole, approximately 80% were found to also be voriconazole resistant.

In the second study the emergence of a voriconazole highly resistance mechanism was described, associated with the TR46/Y121F/T289A resistance mechanism (54). Among the 15 patients identified with a TR46/Y121F/T289A isolate, 8 were diagnosed with azole-resistant invasive aspergillosis. Three of these patients were classified as having probable disease and 4 as proven invasive aspergillosis. One patient could not be classified according to the EORTC/MSG consensus definitions (66). This patient showed bone destruction of the skull on computed tomography scan and *A. fumigatus* was recovered repeatedly from the ear, without any other explanation. All patients with invasive aspergillosis due to TR46/Y121F/T289A were azole-naive, except 1 patient with probable and 1 patient with proven invasive aspergillosis. At 12 weeks after recovery of the TR46/Y121F/T289A isolate, 4 of 8 patients with invasive aspergillosis had died and 2 patients had a persisting infection. All
patients who died had received primary therapy with voriconazole. In 4 patients, primary therapy was initiated with liposomal amphotericin B. In 3 of these patients, invasive aspergillosis was diagnosed, and all patients were alive at 12 weeks. In addition, a number of single cases have been described harboring TR34/L98H (2, 16, 51, 53, 59-64) or TR46/Y121F/T289A resistance mechanisms (55, 67). In all cases, patients with infection due to an azole-resistant isolate failed to azole therapy.

Although the current clinical experience suggests that azole resistance is associated with treatment failure, it should be recognized that there are numerous factors that impact on treatment outcome. Patients with underlying malignancy are prone to fail to azole therapy, even if the infection is caused by an azole-susceptible isolate. Azole exposure might have been insufficient in patients failing therapy and as most patients may not be culture-positive, treatment might have been initiated relatively late in the course of the infection. Therefore, azole-resistant infection might occur predominantly in patients in poor clinical condition, compared to wild type isolates. In the absence of robust clinical evidence, experimental models of aspergillus infection can help us to understand the implications of MIC elevation on treatment efficacy.

### Efficacy of voriconazole and posaconazole in experimental models of azole resistant aspergillosis

Several experimental models have been used to explore PK and PD properties of voriconazole and posaconazole in the setting of azole-resistant aspergillosis. These models are summarized in Table 3 (68-72). Using a non-neutropenic murine model of invasive aspergillosis, voriconazole and posaconazole (response measured as survival) showed a clear exposure-dependent relation with response for both voriconazole-susceptible and voriconazole-resistant strains. For each dose the response was lower in mice infected with the azole-resistant isolate compared to that of mice infected with the azole-susceptible isolate. As for voriconazole and posaconazole the dose correlates with exposure, a higher exposure of theazole was required to achieve similar efficacy when harboring azole-resistant strains.
Table 3. Pharmacodynamic index (PDI) of voriconazole and posaconazole correlated with measures of efficacy in *in vitro* and preclinical models of invasive aspergillosis.
Jeans et al. developed an *in vitro* dynamic model of the human alveolus invasive pulmonary aspergillosis to study the impact of MIC on exposure-response relationships of voriconazole, against wild-type and azole-resistant *A. fumigatus* (70). The antifungal effect of voriconazole was assessed by measuring levels of galactomannan. Galactomannan concentrations began to increase approximately 16–24 hours post-inoculation, and a maximum was reached approximately after 36 hours. The rate of increase and the maximum galactomannan concentrations were comparable. The isolates with higher MICs required higher area under the concentration time curves (AUCs) to achieve similar suppression of galactomannan compared to the wild-type controls.

Howard et al. also used this *in vitro* model to study the impact of MIC on PK/PD relationships of posaconazole (72). The results were validated using an inhalational murine model of invasive pulmonary aspergillosis. Similarly, the administration of posaconazole caused a dose-dependent decline in serum galactomannan concentrations with near-maximal suppression following 20 mg/kg/day. The posaconazole MICs affected the exposure-response relationships, those strains with a higher MIC had higher 50% effective pharmacodynamic index (EI50).

In another study, Lepak et al. investigated the pharmacodynamic target of posaconazole in an immunocompromised murine model of invasive pulmonary aspergillosis against Cyp51A wild-type isolates and isolates carrying Cyp51A mutations conferring azole resistance (71). Efficacy was assessed by quantitative PCR (qPCR) of lung homogenate and survival. Mortality mirrored qPCR results, with the greatest improvement in survival noted at the same dosing regimens that produced fungistatic or fungicidal activity. The results demonstrated that more posaconazole, on a mg/kg basis, was required for efficacy against organisms with reduced *in vitro* susceptibility.

In conclusion, all models show a clear exposure-response relationship. In most models, the exposures that are required for efficacy are in a similar range and therefore underscore the value of these models. However, it should be realized that the models are designed and optimized to find these relationships, and the pharmacodynamic targets that are derived from the models may therefore over- or underestimate the ‘true’ target. For instance, the EC50 in survival studies is a reproducible measure for efficacy, but not necessarily coincides with the same endpoint in humans.

Importantly, the pharmacodynamic endpoint in experimental models of invasive aspergillosis that best predicts the outcome of patients with pulmonary infections is not well known and still suffers lack of standardization. Using the abovementioned studies the EI50, EI80 and EI90 over 7 or 14 days survival for voriconazole and posaconazole can be estimated (Table 3).
In addition, in vitro studies and in vivo models differ concerning the route of infection, the efficacy parameter, the presence or absence neutropenia, level of protein binding and other variables.

Both in vitro and in vivo studies have indicated that the ratio of the area under the concentration time curve (AUC) at 24 hours to the minimum inhibitory concentration (MIC) is the main PK/PD parameter that best predicts voriconazole and posaconazole efficacy in invasive aspergillosis.

Therefore, for purpose of further discussion, we decided to use the effective exposure index at 50% (EI\textsubscript{50}) AUC\textsubscript{0-24}/MIC as the value most predictive of treatment, which is generally considered in the relationships between the PK/PD evaluation of antimicrobial agents (73). However, one should consider that higher values such as EI\textsubscript{80} or EI\textsubscript{90} are more reliable when translating to the patient setting. Importantly, this higher value was similar to EI\textsubscript{50} in voriconazole studies, and not consistent/achievable in all posaconazole experimental studies analyzed in our review. Moreover, the range of PD-target predicting therapeutic success using either in vitro or in vivo model was in the same range in abovementioned voriconazole and posaconazole studies (Table 4 and 5).

**Bridging experimental results to humans: is there a role for voriconazole and posaconazole in azole-resistant invasive aspergillosis?**

Based on the estimates of the PD-targets for voriconazole and posaconazole we can now determine if there remains a role for voriconazole or posaconazole in the management of azole-resistant disease. The integration of all above information is given in Table 4 for voriconazole and Table 5 for posaconazole. The underlying resistance mechanisms are provided for each MIC-value. Based on the estimates of the PD-target, the exposure can be calculated that is needed to achieve the PD-target for each MIC. The exposure corresponds with plasma levels, which are typically higher than those needed for treating infection due to wild-type isolates. The feasibility of achieving higher exposure depends on characteristics of the drug related to absorption and clearance, but is limited by toxicity.
For voriconazole, a total drug AUC/MIC ratio of 21.96 was associated with 50% probability of success (E<sub>50</sub>) to suppress galactomannan concentrations in a dynamic <i>in vitro</i> model of the human alveolus (70). Using an immunocompetent murine model of invasive aspergillosis, we observed that achieving a serum total AUC<sub>0-24</sub>/MIC ratio of 17.61 was the PD-target linked to half-maximum antifungal effect predicting therapeutic success (Table 3) (69).

Recently, Pascual et al. performed a population pharmacokinetic analysis (NONMEM) on 505 plasma concentration measurements involving 55 patients with invasive mycoses who received recommended voriconazole doses in order to describe factors influencing the pharmacokinetic variability, to assess associations between plasma concentrations and efficacy or neurotoxicity/hepatotoxicity, and to define intravenous and oral doses required for achieving drug exposure with the most appropriate efficacy/toxicity profile (74). A logistic multivariate regression analysis revealed the therapeutic target with a clinically appropriate efficacy-safety profile, close to that recently reported by others (75). An independent association between voriconazole trough concentrations and probability of response or neurotoxicity was identified for a therapeutic range of 1.5 mg/L (>85% probability of response) to 4.5 mg/L (<15% probability of neurotoxicity). Population-based simulations with the recommended 200 mg oral or 300 mg intravenous twice-daily regimens predicted probabilities of 49% and 87%, respectively, for achievement of 1.5 mg/L and of 8% and 37%, respectively, for achievement of 4.5 mg/L. With 300–400 mg twice-daily oral doses and 200–300 mg twice-daily intravenous doses, the predicted probabilities of achieving the lower target concentration were 68%–78% for the oral regimen and 70%–87% for the intravenous regimen, and the predicted probabilities of achieving the upper target concentration were 19%–29% for the oral regimen and 18–37% for the intravenous regimen (74). Apparently, patients achieving higher concentrations of voriconazole may show higher exposure and a better response to therapy, but they are at higher risk for toxicity. In contrast, patients achieving lower concentrations may have reduced therapeutic response but subsequently a lower risk for adverse events.

Whereas the Pascual study is based on trough levels as a measure of exposure (74), because it is much easier to determine than the AUC, all preclinical models are AUC based (69, 70, 76). However, voriconazole trough levels correlate well with AUC as determined in several studies. Estimates of total AUC<sub>0-24</sub> in patients showed that standard dose on the basis of 200 mg twice daily oral voriconazole results in a total AUC value of 18 to 23 mg.h/L (77). Population PK modeling of voriconazole in adults (78) and other PK studies (figure 1) (79), revealed that the trough concentration are well correlated with the AUC, and a drug level of 1 and 5.3 mg/L corresponded with a total AUC<sub>0-24</sub> of 43 and 175 mg.h/L, respectively.
<table>
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<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
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<td>F219I</td>
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<td>F219I</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>G54A/R; W</td>
<td>G54F; R; V; W</td>
<td>G54E; V</td>
<td>G54V</td>
<td>-</td>
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<td>G448S</td>
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</tr>
</tbody>
</table>

Pharmacodynamic target (total AUC_{24h}/MIC) predicting therapeutic success [adapted from preclinical study of Mavridou 2010 and Jeans 2012]

Calculated exposure (total AUC_{24h}) needed to be achieved [calculation made by us] 1.10-1.372, 2.26-2.74, 4.40-5.49, 8.8-10.98, 17.62-21.96, 35.22-43.92, 70.44-87.84, 140.88-175.68, 281.76-351.36

Calculated trough concentration (C_{trough}) needed to be achieved [adapted from clinical data of Bruggemann et al. 2010] 0.05, 0.05, 0.06-0.09, 0.11-0.19, 0.22-0.39, 0.81-1.03, 2.15-2.65, 4.25-5.30, 8.70-10.60

EUCAST interpretation

| S | S | S | S | S | - | R | R | R |

Proposed interpretation [adapted from Verweij et al. 2009]

| S | S | S | S | S | I | R | R | R |

Probability of achieving trough concentration (C_{trough}) with 400 mg/kg twice daily [adapted from Passaual et al. 2012]

<table>
<thead>
<tr>
<th>Oral</th>
<th>&gt; 95%</th>
<th>35-67%</th>
<th>36-59%</th>
<th>56%</th>
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<tr>
<td>IV</td>
<td>&gt; 97%</td>
<td>56-86%</td>
<td>48-50%</td>
<td>44%</td>
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</table>

Probability of reaching the exposure

| IV/oral |  |

**Table 4.** Probability of attaining therapeutic success of voriconazole against azole-susceptible and azole-resistant *Aspergillus* diseases according to MIC values (top row), PK/PD target and exposures.

**Green,** exposure using standard dose; **orange:** exposure attainable using increased dose; **red,** required exposure not attainable.
<table>
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<td>G448S</td>
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<td>TR1/299A</td>
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<td>TR130C</td>
<td>Y413C</td>
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</table>

Pharmacodynamic target (total $\text{AUC}_{0\rightarrow\infty}$) predicting therapeutic success 
[adapted from preclinical study of Howard 2011, Mavridou 2012, Lopok 2013]

$\text{AUC}_{0\rightarrow\infty}$ 167 – 170 (EUCAST)

Calculated exposure (total $\text{AUC}_{0\rightarrow\infty}$) needed to be achieved (calculation made by us)

5.22 - 5.5 - 10.43 - 11.125 - 20.87 - 22.5 - 41.75 - 44.5 - 82.5 - 89 - 167 - 170 - 234 - 256 - 660 - 712 - 1336 - 1424 - 2672 - 2848

Calculated trough concentration ($C_{\text{min}}$) needed to be achieved (adapted from clinical data of Bruggemann et al. 2010)

<0.4 <0.4 0.72 - 0.77 1.44 - 1.54 3.09 - 3.33 6.18 - 6.66 <10 >10 >10 >10 >10

EUCAST Interpretation

S S S S R R R R R

Proposed interpretation breakpoints

[S proposed from Verwoj et al. 2008]

S S S S S I R R R R R

Probability of exposure (AUC)

S <0.6 %

attainment with 800 mg a day

[adapted from Agutif et al. 2008]

96% 68% 15.3% 0.6% <0.6%

Probability of reaching the exposure [adapted from clinical data]

Oral [Courteny, 2004, Kithinji, 2009]
Fasted
Non-fasted [Courteny, 2004, Kithinji, 2009]
High fat meal

Table 5. Probability of attaining therapeutic success of posaconazole against azole-susceptible and azole-resistant *Aspergillus* diseases according to MIC values (top row), PK/PD target and exposures.

Green, exposure using standard dose; orange: exposure attainable using increased dose; red, required exposure not attainable.
The AUC levels required for efficacy as derived from the trough levels in the Pascual study correspond well with the AUC levels required for efficacy in preclinical models. Assuming no resistant strains in the Pascual study, the ECOFF can be used as the upper value of the MIC distribution and the denominator in the AUC/MIC. Since this is 1 mg/L it follows that the AUC/MIC ratio required for optimal treatment is 30, which is very close to the pharmacodynamic targets derived from preclinical models in order to achieve therapeutic success.

**Figure 1.** Linear regression analysis between exposure (AUC$_{0-24}$) of voriconazole (left) and posaconazole (right) and plasma trough concentration in human.

Therefore, it can be expected that isolates with a MIC that is classified as susceptible can be treated with voriconazole, with a probability of exposure attainment of over 90% according to Hope et al. using licensed doses of voriconazole (40, 78). For isolates with a voriconazole MIC of 2 mg/L, classified as intermediate susceptibility by Verweij et al. (13), the plasma level should exceed 1.03 mg/L which is well attainable. Voriconazole MIC of 4 mg/L is classified as resistant, and in order to achieve the PD-target a higher exposure is needed ($\geq 2.65$ mg/L). Higher exposure of voriconazole can be achieved using dose escalation, but will be associated with increased probability of toxicity. Clearly if voriconazole would be used in this setting intravenous administration would be required as well as close monitoring of plasma levels. For isolates with a MIC exceeding 4 mg/L very high plasma levels exceeding 5.30 mg/L are needed, which are in a range where toxicity can be anticipated.
Posaconazole is currently not licensed for the primary therapy of invasive aspergillosis, but may be used for salvage therapy. Similar to the other triazoles, posaconazole displays concentration-dependent with time dependence pharmacodynamic characteristics, for which a total AUC_{0-24}/MIC ratio ranging 167 to 178 was the value predictive of success associated with half-maximal efficacy. Estimates of the total AUC_{0-24} for patients infected with *A. fumigatus* with a posaconazole MIC of 0.125 mg/L receiving 800 mg/day are 13-17 mg.h/L, corresponding to the best response rate (80-82). On the other hand, optimal outcome could be achieved with posaconazole plasma concentrations of ~0.7 mg/L when administered for prophylaxis. However, for purpose of salvage therapy, Walsh et al. showed that an average concentration of 1.25 mg/L was associated with a higher probability of a clinical response for patients with invasive aspergillosis receiving posaconazole 800 mg/day (83), corresponding to an AUC of approximately 30 mg.h/L. Therefore, with fixed dosing of 800 mg/day (200 mg four times a day), drug exposures may not be high enough to cover the entire wild-type distribution, reliably in persistently neutropenic hosts with invasive aspergillosis. The patients infected with an *Aspergillus* strain with a MIC of 0.25 mg/L, will need to obtain an AUC_{0-24} of ~40–50 mg.h/L, which corresponds with trough concentrations of >1.25 mg/L, as shown in figure 1 (79, 82).

According to available data shown in table 5, the exposure needed to treat infection due to isolates that are classified as susceptible can only just be achieved with a low probability of exposure attainment in isolates with a MIC of 0.125 mg/L (13, 39). Given the current problems of increasing the exposure of the drug due to its formulation and limited absorption, there appears to be no room for posaconazole for the treatment of isolates that are not within the wild type distribution. However, a new oral tablet and intravenous formulation are under development and soon to be brought the clinical practice (84). The tablet is designed to release the entire dose of solubilized posaconazole in the small intestine, maximizing systemic absorption. In an exploratory study, this new solid oral formulation significantly increased exposure to posaconazole relative to the oral suspension in fasting healthy volunteers (85). Following single and multiple doses of posaconazole solid oral tablets (200 and 400 mg) in healthy subjects, the exposure increased in a dose-related manner. When the dose was increased in a 1:2 ratio, exposure increased in 1:1.9 and 1:1.8 ratios for days 1 and 14, respectively. On day 1, the dose-normalized posaconazole exposure (AUC_{tau}) was substantially higher than for the oral suspension under both fasted and fed conditions (85). Notably, a novel cyclodextrin formulation of posaconazole is under development for intravenous (i.v) use. In a phase 1B study, the pharmacokinetics of 2 doses of i.v. posaconazole was investigated in 55 patient volunteers (86). The higher protective blood level of posaconazole was found for the 300 mg given once daily, for which the average blood concentration at 14 days was 1.43 mg/L. The minimum effective concentration was seen in 95% of patients. Recently, Cornely et al. reported that 300 mg posaconazole i.v. was well tolerated and resulted in higher exposure compared to the oral suspension (Cornely et al., 6).
A lowest mean $C_{\text{min}}$ value of 1,297 mg/L was achieved for posaconazole i.v 300 mg vs. 751 mg/L for posaconazole oral suspension. Although our calculations indicate that a posaconazole exposure of $\geq 3.33$ mg/L would be required to treat infection due to isolates with a posaconazole MIC of 0.5 mg/L, we believe that this might be achievable using the i.v. formulation. Given that a significant proportion of isolates harboring an azole resistance mechanism exhibit a posaconazole MIC of 0.5 mg/L, this approach requires further investigation in experimental models.

Concluding remarks

The management of azole-resistant *Aspergillus* disease remains a challenge. There are currently no guidelines or recommendations that guide clinicians confronted with azole resistance. Furthermore, pre-clinical or clinical evidence that support treatment choices is scarce. Experimental models of infection indicate that liposomal-amphotericin B may be effective (87), or a combination of voriconazole or posaconazole with an echinocandin (76, 88, 89). In addition to the choice of antifungal regimen other important issues remain such as the early detection of azole resistance, especially in culture negative patients. Also treatment regimens for patients with infection in tissues that are difficult to reach, such as the brain, remain problematic.

In our current review we explored the role of azole monotherapy in the management of azole-resistant aspergillosis. We believe that only a modest role of voriconazole and posaconazole remains, if any. Clearly, the use of an azole can only be considered in patients that fail alternative regimens or are intolerant to polyene therapy. Although it appears that voriconazole can be used to treat infection due to isolates with a MIC of 2 mg/L, in isolates with a MIC of 4 mg/L the risk of toxicity is significant. In this setting of dose escalation, intravenous administration, extensive monitoring of plasma levels and close clinical and radiological follow-up is required. At current there appears to be no role for posaconazole in the treatment of isolates with a MIC outside the wild type population. We believe that this is due to the difficulty in achieving sufficient exposure. However, we anticipate that adequate drug exposure might be achieved with the i.v. formulation that might allow treatment of infections due to isolates with a MIC of 0.5 mg/L, although this possibility would need to be explored in experimental models.
References


6.2. Therapeutic drug monitoring of voriconazole and posaconazole for invasive aspergillosis

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Abstract

Voriconazole and posaconazole are extended-spectrum triazoles recommended for treatment, prophylaxis and salvage therapy of aspergillus diseases. Over the past decade many papers have emerged supporting the use of therapeutic drug monitoring (TDM) for azole antifungals. TDM is used to tailor the exposure of a specific drug to the individuals to optimize treatment response and minimize side effects. We reviewed the pharmacokinetics and pharmacodynamics (PK-PD) characteristics of voriconazole and posaconazole. We present the available evidence on target concentrations defining maximal efficacy and minimal toxicity. Finally we provide some practical recommendations how to best perform TDM in clinical practice.

Keywords

Aspergillus, Azole antifungal drugs, Pharmacodynamics, Pharmacokinetics, Therapeutic drug monitoring
Introduction

The strategy to tailor the individuals’ exposure through the assessment of a patient serum or plasma concentration and subsequently adaptation of the dosing regimen is called therapeutic drug monitoring (TDM). TDM has since long been used to optimize treatment for several drug classes, most importantly the aminoglycosides and glycopeptides, and has been shown of significant benefit to patients [1]. Over the past decade a vast amount of evidence has been published, supporting a role for TDM in the class of azole antifungal drugs.

Azole antifungals, such as voriconazole and posaconazole, are recommended drugs to manage aspergillus diseases [2, 3]. Invasive aspergillosis (IA) is a life-threatening opportunistic fungal infection in immunocompromised patients with an overall mortality ranging between 30 to 88% [4-7]. Voriconazole is most frequently used in the treatment for IA and posaconazole for prophylaxis and salvage therapy [2,3,8,9].

To select the most appropriate drug and to optimize the exposure of the drug by adapting the administered dose, understanding of the pharmacokinetics (PK) and the pharmacodynamics (PD) is crucial [7]. The aim of this manuscript is to start with a brief overview of the PK of voriconazole and posaconazole. Next we discuss the current evidence, supporting the use of TDM in the case of voriconazole and posaconazole. We will focus particularly on target concentrations that correlate with maximal response in the context of patients underlying invasive aspergillosis (IA) and on the correlation between exposure and toxicity. Finally some practical recommendations are given.

Antifungal triazoles

The antifungal triazoles are synthetic compounds that have >1 triazole ring attached to an isobutyl core (e.g., voriconazole, ravuconazole and isavuconazole) or to an asymmetric carbon atom with a lipophilic complex mixed functional aromatic chain (e.g., itraconazole and posaconazole) [10]. Triazoles inhibit the synthesis of ergosterol from lanosterol in the fungal cell membrane [10,11]. The target is the cytochrome (CYP)–dependent 14-a-demethylase (CYP51 or Erg11p), which catalyzes this reaction. Thereby, ergosterol is depleted and methyl-sterols accumulate within the cell membrane and lead to either inhibition of fungal cell growth or death, depending on the species and antifungal compound involved.

Triazoles are generally fungistatic, although itraconazole, voriconazole and posaconazole have been shown to be fungicidal against Aspergillus spp [11]. The various azoles have different affinities for the CYP-dependent 14-a-demethylase, which in return result in various
antifungal activities [12], and therefore susceptibilities. Four triazole compounds (fluconazole, itraconazole, voriconazole and posaconazole) have been approved and are currently in wide use for the prevention and treatment of invasive fungal infections (IFIs) [101,102].

**Label indication of triazoles for invasive aspergillosis**

Voriconazole has a label indication for the treatment of IA, treatment of candidemia in non-neutropenic patients, treatment of fluconazole-resistant serious invasive Candida infections (including C. krusei) and treatment of serious fungal infections caused by *Scedosporium* spp. and *Fusarium* spp [102]. Voriconazole can be used on-label in adults and pediatrics aged 2 and above.

Posaconazole is licensed for the treatment of: IA in patients with disease that is refractory to amphotericin B or itraconazole or in patients who are intolerant of these medicinal products; Fusariosis in patients with disease that is refractory to amphotericin B or in patients who are intolerant of amphotericin B; oropharyngeal candidiasis and for the treatment of less common infections such as chromoblastomycosis and mycetoma and coccidioidomycosis. In addition posaconazole has a label for the prophylaxis of invasive fungal infection in patients with graft-versus-host disease (GvHD) or after remission-induction chemotherapy for acute myeloid leukemia or myelodysplastic syndrome (AML-MDS). Posaconazole is licensed only for patients aged 18 years or older [101].
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Voriconazole</th>
<th>Posaconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formulation</strong></td>
<td>IV solution (CDx), PO capsules, PO suspension</td>
<td>PO suspension</td>
</tr>
<tr>
<td><strong>Maintenance dose for adults for antifungal treatment</strong></td>
<td>4 mg/kg BID IV; 200 mg BID PO</td>
<td>400 mg BID PO</td>
</tr>
<tr>
<td><strong>Absolute bioavailability</strong></td>
<td>+/- 90% (bioavailability decreased when taken together with a meal and (but lower in children)</td>
<td>8-47% (Variable; saturable oral absorption; dose dependent; availability increased by intake with fat-rich food and gastric acid inhibitors decrease drug absorption)</td>
</tr>
<tr>
<td><strong>Volume of distribution</strong></td>
<td>4.6 L/kg</td>
<td>5-25 L/kg</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;max&lt;/sub&gt;</strong></td>
<td>3-4.6 mg/L</td>
<td>1.5-2.2 mg/L</td>
</tr>
<tr>
<td><strong>AUC&lt;sub&gt;0-24&lt;/sub&gt;</strong></td>
<td>20.3 mg x h/L</td>
<td>8.9 mg x h/L</td>
</tr>
<tr>
<td><strong>Tissue penetration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Lung tissue</td>
<td>Good</td>
<td>Good / excellent</td>
</tr>
<tr>
<td>- Cerebrospinal fluid (CSF)</td>
<td>Good (CSF – plasma ratio 0.3 – 0.6)</td>
<td>Poor (CSF - plasma ratio 0 – 2.4)</td>
</tr>
<tr>
<td>- Vitreous humor</td>
<td>Good (around 60%)</td>
<td>Limited (around 21%)</td>
</tr>
<tr>
<td>- Urine</td>
<td>&lt;2%</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td><strong>Protein binding</strong></td>
<td>58 %</td>
<td>&gt; 98 %</td>
</tr>
<tr>
<td><strong>Half life</strong></td>
<td>6 hours (but nonlinear PK)</td>
<td>25-35 hours</td>
</tr>
<tr>
<td><strong>Time to reach steady state</strong></td>
<td>2 days</td>
<td>7-10 days</td>
</tr>
<tr>
<td><strong>Elimination</strong></td>
<td>Renal &gt; Faecal; primarily as inactive metabolites</td>
<td>Faecal &gt; Renal; extensively in unchanged form</td>
</tr>
<tr>
<td><strong>Metabolism</strong></td>
<td>Hepatic via CYP2C19, 2C9 and CYP3A4</td>
<td>Hepatic by UGT1A4</td>
</tr>
<tr>
<td><strong>CYP inhibition</strong></td>
<td>CYP3A4,2C19,&gt;2C9</td>
<td>CYP3A4</td>
</tr>
</tbody>
</table>

Table 1. Basic pharmacokinetics properties of voriconazole and posaconazole.

Current concepts in antifungal pharmacology: b.i.d: Twice daily; CDx: Suflobutylether-beta-cyclodextrin in vorinonazole for injection; CSF: Cerebrospinal fluid; CYP: Cytochrome; i.v.: Intravenous; p.o.: Per orem; PK: Pharmacokinetics; q.i.d.: Four-times a day; t.i.d.: Three-times a daily. Data taken from [60].
Pharmacokinetic & pharmacodynamic relationships

For the purpose of this review, we first explore the pharmacokinetics (PK) profile of the drug, and then integrate the information on exposure-response and exposure-toxicity.

Pharmacokinetics of voriconazole

Voriconazole, a structural congener of fluconazole, is a second-generation triazole with broad-spectrum antifungal activity, including enhanced potency against Aspergillus species and other molds that are fluconazole or itraconazole-resistant [2,14-15]. Current dosing recommendations are provided in the summary of product characteristics (SPC) [102]. The compound is available in both intravenous and oral formulations. Pharmacokinetic properties are depicted in (TABLE 1): briefly stated, when given orally to a fasting adult person, the drug has a bioavailability of > 90% yielding a maximum plasma concentration (Cmax) approximately 2 h after administration [16].

Food has a negative impact on the drug’s bioavailability, by reducing its exposure approximately 22% [17]. A meal with a high fat content will reduce the mean Cmax and area under the curve (AUC) by 34% and 24%, respectively. Thus it is recommended to administer the drug either 1 h before or after meals [102,17]. Voriconazole is extensively distributed into tissues and penetrates well into cerebrospinal fluid and into vitreous and aqueous humors with an estimated volume of distribution of around 4.6 l/kg. The steady-state plasma concentrations of voriconazole in healthy volunteers are reached at day 2 of treatment (after a loading dose) [18].

CYP2C19 is the dominant metabolic pathway with CYP2C9 and CYP3A4 being involved to a much lesser extent. Polymorphisms in CYP2C19 will render the population into ultra-rapid (2C19*17), extensive (or wild-type 2C19*1) and poor metabolizers (2C19*2 or *3). The latter will result in a prolonged clearance of voriconazole and is of particular importance in the Asian population where the prevalence of these mutations is highest at around 20-30%. The CYP3A4 route will play a more dominant role in patient with 2C19 loss of functional alleles. In addition to being a substrate to many CYP enzymes, voriconazole is also an inhibitor of CYP2C9, CYP2C19 and CYP3A4 [19]. Voriconazole displays nonlinear PK in adult patients over the therapeutic range with Cmax and AUC values that increase disproportionately as the dose increases. This non-linearity is both time- and dose dependent [18,20]. The apparent half-life of voriconazole is approximately 6 h (TABLE 1) and increases when voriconazole concentration increases. Pediatric and adolescent patients have different PK compared to adults [21], resulting in pseudo linear PK.
Pharmacokinetics of posaconazole

Posaconazole, a structural analog of itraconazole, is the most recently approved triazole antifungal that is available as an oral suspension. Posaconazole is an extended-spectrum triazole. Posaconazole has in vitro activity against Aspergillus species and many other fungi [22-24]. In vivo, posaconazole has demonstrated efficacy in several models of experimental pulmonary, cerebral and disseminated aspergillosis [25-29]. Up until today, posaconazole is only available as an oral solution but a solid oral as well as intravenous formulation are currently under development [30]. Posaconazole is slowly absorbed with a median absorption time (T\text{max}) of 5 h (TABLE 1). In healthy volunteers, posaconazole showed dose-proportional PK over the range of 50-800 mg. [31]. Dosages above 800 mg showed no further or minimal increase in plasma concentrations or total exposure due to a saturation of the gastrointestinal absorption mechanism. Posaconazole binds predominantly to albumin, and the drug protein binding is high (>98%). Posaconazole has a large mean apparent volume of distribution after oral administration (Vd/F), which is approximately 5-25 l/kg, suggesting extensive extra vascular distribution and penetration into intra-cellular spaces [32]. Steady-state PK are reached after a period of 7-10 days. The relative oral bioavailability of posaconazole varies significantly among different regimens and is significantly increased by administration in divided doses. The same total daily dose of posaconazole administered in two or four dosages results in a two to threefold increase in exposure, respectively, compared to once daily dosing [31]. Previous studies have demonstrated that food, particularly meals with high fat content, significantly increases posaconazole bioavailability. Therefore, posaconazole should be administered with food whenever possible to ensure optimal absorption [33]. Posaconazole penetrates well into the lungs, however, the drug has a limited penetration in the brain tissue that may limit its antifungal activity in IFIs of the central nervous system [31,34]. About 20-30% of posaconazole is metabolized via the UDP-glucuronosyl-transferase enyzmsystem (UGT─1A4) to an inactive metabolite, with the remainder being eliminated unchanged in the feces and to a lesser extent in urine [35]. The estimated elimination half-life in both patients and healthy volunteers is about 30-35 h. There is limited support for the dosing of posaconazole in pediatric patients and no formal dosing information for posaconazole is available. A single center study provided a dosing algorithm that was subsequently tested in chronic granulomatous disease patients and resulted in adequate exposure [36].
CHAPTER 6.2

Exposure-response relations in patients

Voriconazole concentrations associated with efficacy

There are numerous reports on the relationships between efficacy of voriconazole and its plasma concentrations. A selection of the most recent articles is outlined below.

Pascual et al., conducted a prospective, observational study in 52 patients receiving voriconazole for treatment of IFDs [37], using trough levels rather than random samples and showed a correlation between efficacy and plasma concentration. Approximately 60% of patients had a hematologic malignancy and about half were treated for IA, in which lack of therapeutic response was more common among patients with voriconazole trough concentrations <1 mg/l.

In another study investigating the role of therapeutic dose monitoring, in the treatment of fungal infections with voriconazole in 34 Japanese patients with hematologic malignancies, there was no correlation between voriconazole trough levels and response to therapy [38]. However, when patients with refractory underlying hematologic disease were removed from the analysis, cases with a concentration of >2 mg/l were associated with favorable response to voriconazole. From these results, they concluded that TDM should be executed and targeted to 2-6 mg/l to improve efficacy and to avoid side effects.

In an Australian population, the benefit of TDM was shown in 25 patients with proven or probable IFIs who experienced two or more episodes of voriconazole TDM at a tertiary referral hospital [39]. The authors performed a chart review to investigate the association between serum trough concentrations and outcomes of IFI such as IFI related mortality. Patients were more likely to die if their initial trough concentration was below 0.35 mg/l. A significant intra patient variability in voriconazole concentration was evident. Immunosuppression was a strong factor that was associated with IFD mortality. However, successful outcomes were more likely among patients with a median trough voriconazole concentration >2.2 mg/l.

Neely et al., investigated the voriconazole PK-PD and TDM in a pediatric subpopulation aged between 2 and 12 years, and notably predicted that 34% of patients receiving voriconazole at a dose of 7 mg/kg b.i.d. did not attain the target of >1.0 mg/l [40]. There was a statistically significant association between crude mortality and voriconazole 12 h trough concentrations below 1 mg/l. However, trough concentrations >1 mg/l were associated with a higher likelihood of success.
In an observational study, Troke et al., analyzed the relationship between both mean plasma voriconazole concentrations and mean concentration/MIC ratios with efficacy for 825 patients from nine Phase II and III clinical studies [41]. In this study, the patients with higher free $C_{\text{max}}$/MIC ratios had a higher probability of clinical response. A trough/MIC ratio of 2-5 was suggested as a target. The $C_{\text{avg}}$ for 72% of the patients was 0.5-5 mg/l, with the maximum response rate (74%) at 3-4 mg/l.

For patients with $C_{\text{avg}}$ <0.5 mg/l, the response rate was 57%. As a conclusion, the authors emphasized the importance of considering the MIC, when it is available, together with the plasma concentration, in predicting the therapeutic response for patients receiving voriconazole.

Dolton et al., performed a retrospective analysis investigating the relationship between voriconazole concentration and patients clinical outcome at seven hospitals in Australia [42]. Medical records were reviewed for patients who received voriconazole and had at least 1 concentration measured. 201 patients with 783 voriconazole trough concentrations were included in the analysis. Voriconazole concentrations of <1.7 mg/l were associated with a significantly greater incidence of treatment failure (19/74 patients [26%]) than concentrations of >1.7 mg/l (6/89 patients [7%]; $p < 0.01$).

Up until today, the only randomized trial investigating the use of TDM comes from Park et al. They performed a randomized, assessor-blinded, controlled, single center trial to determine whether routine TDM of voriconazole reduces drug adverse events or improves treatment response in IFIs including IA [43]. Patients were randomly assigned to TDM or non-TDM groups. In the TDM group, voriconazole dosage was adjusted (target range, 1.0-5.5 mg/l) according to the serum trough concentrations measured on the 4th day after initiation of voriconazole. The non-TDM group received a fixed, standard dosage. Both treatment response (at 12 weeks after the initiation of therapy) and voriconazole-related adverse events were monitored. The analysis included a total of 110 patients in both arms and baseline characteristics were comparable between the two groups. The primary endpoint whether routine TDM of voriconazole reduced the incidence of voriconazole-related adverse events was not met as there was no difference between the TDM group and the non-TDM group (both 42%; $p = 0.97$). Yet, the percentage of patient who discontinued voriconazole due to adverse events was significantly lower in the TDM group compared to the non-TDM group (4% vs 17%; $p = 0.02$). A complete or partial response was observed in 81% (30 of 37) in favor of patients in the TDM group compared to 57% (20 of 34) in the non-TDM group ($p = 0.04$). Although TDM of voriconazole did not decrease the overall incidence of voriconazole-related adverse events, it did significantly reduce the incidence of voriconazole discontinuation due to adverse events. And more importantly the success rate in the treatment of IFIs was more favorable in the TDM arm.
In the setting of prophylaxis there is limited evidence with regards to voriconazole breakpoints linking exposure to efficacy of prophylaxis. Mitsani and colleagues performed a prospective, observational study in 93 lung transplant recipients [44]. They found that there was fewer colonization and there were fewer IFIs at troughs of >1.5 mg/l (p = 0.01).

**Posaconazole concentrations associated with efficacy**

For posaconazole, TDM is an emerging field. TDM should certainly be considered for patients failing therapy, the treatment of infections at sanctuary sites, treatment of uncommon or in vitro less susceptible organisms, patients with mucositis or malabsorption and those unable to take drug with high-fat food [45,46]. TDM can also be used to monitor compliance in the setting of long term therapy.

In patients with IA who were refractory to or intolerant of conventional antifungal therapy, the rate of clinical response to posaconazole salvages therapy increased by increasing average plasma concentrations. Patients with an average concentration of 0.13 mg/l, 0.41 mg/l, 0.72 mg/l and 1.25 mg/l responded successfully in 24%, 53%, 53% and 74% of cases, respectively [47].

The relationship between concentrations of posaconazole in blood and efficacy were investigated in two US FDA Phase II/III prophylaxis trials in neutropenic patients undergoing allogeneic hematopoietic stem cell transplantation (acute myeloid leukemia or myelodysplastic syndrome patients) [8], and in patients under-lying GvHD [9]. All patients received posaconazole 200 mg t.i.d. for antifungal prophylaxis. The incidence of proven or probable breakthrough fungal infections was 6.5% in neutropenic patients with posaconazole average plasma concentrations below 0.7 mg/l versus 1.9% in those who attained average plasma concentrations above 0.7 mg/l [8], and 3.9% vs 0% in GvHD patients [9], respectively.

In addition, posaconazole concentrations were nearly twofold lower in the small group of patients (n = 5) who developed an IFI than in the cohort (n = 241) that did not develop infection (Cavg of 0.61 mg/l in the infected cohort and C average of 0.92 mg/l in the uninfected cohort, respectively). However, these differences were not statistically significant, possibly due to the small number of patients who developed infection (2.4%).

Jang et al., reanalyzed the exposure-response findings of the aforementioned two clinical studies [48]. According to the logistic regression results, the clinical failure rate at an average concentration <0.7 mg/l was >25% and >35% in study 1 and study 2, respectively. The exposure-response analyses thus revealed a clear relationship between a higher incidence of clinical failure, defined as the initiation of empirical therapy, and lower Cavg, consistent with
these analyses, most of the breakthrough instances of proven or probable IFIs were observed in patients who attained low C_{avg} (<0.7 mg/l). The herein proposed target concentration of 0.7 mg/l is subject to debate, [49] and need to be further evaluated.

Recently, Dolton et al., performed a retrospective chart review of 86 patients on posaconazole in whom >1 concentration had been determined [50]. Twelve patients out of 72 patients who failed prophylaxis had significantly lower concentrations compared to patients who did not fail prophylaxis (0.289 mg/l vs 0.485 mg/l; p < 0.01). Fungal breakthrough infections were significantly predicted by the median posaconazole concentration (p < 0.05).

**Concentrations associated with toxicity**

At the event of the growing proportion of heavily immunocompromised patients, there is also an increase of patients at risk for or experiencing an IFI who are receiving systemic antifungal agents. As a consequence, clinicians need to be aware of not only the more familiar dose-limiting toxicities associated with systemic antifungal agents [51], but also longer-term risks, including recurrent drug interactions, organ dysfunction, cutaneous reactions and malignancies [52].

**Voriconazole TDM target associated with toxicity**

There is a clear relationship between voriconazole exposure and the incidence of neurological side effects or neurotoxicity; however the exposure-response relationship is not as well established for many other adverse events caused by voriconazole. Particularly for hepatotoxicity there appears to be no unique cut-off point above which hepatic toxicity becomes prevailing but the odds of having elevated liver enzymes increases with the increase of exposure to voriconazole [37,42,43,53].

Tan and colleagues performed a retrospective analysis on the data on file from the manufacturer. They combined data from ten Phase II and III clinical trials. They found a positive association between mean plasma voriconazole concentration and the frequency of visual adverse events (p= 0.01). In addition they found a weaker but still significant association with increased liver function test results [53].

Likewise, Pascual and colleagues found in a prospective study a correlation between voriconazole trough levels and toxicity [37]. Patients with voriconazole plasma concentrations of >5.5 mg/l, 31% (5/16) were at higher risk for developing neurotoxicity (confusion, hallucinations, myoclonus) compared to patients with lower concentrations.
(0/36). There was no clear and distinctive correlation between trough concentration and hepatotoxicity.

In another retrospective trial of 25 allo-HSCT recipients, elevated levels of aspartate amino-transferase and alkaline phosphatase significantly correlated with elevated voriconazole trough concentrations [54]. Furthermore, there was a link between voriconazole concentrations and the probability of photopsia [53]. Cessation of therapy due to photopsia was rarely required.

There are also a number of reports of other adverse events, such as hypoglycemia and skin and pulmonary toxicity occurring in the context of voriconazole therapy, but a causal relationship with exposure has not been established up until today [55].

**Posaconazole TDM target associated with toxicity**

A few studies have investigated the exposure-toxicity relationship of posaconazole. Based on those studies, there appears to be no relationship between average posaconazole concentrations and adverse events [47,48,50]. This may be attributable to the fact that toxic concentrations are not reached due to the limited absorption.

Jang and colleagues combined the data from the two large prophylaxis trials of posaconazole (n=467 patients). Patients were stratified by quartile posaconazole concentration. Those patients with a lower posaconazole concentration had a lower incidence of treatment-related adverse events. The authors suggested a possible relationship for posaconazole safety; however, these differences did not reach statistical significance [48].

Exposure response relations of posaconazole and factors impacting PK were investigated in a multicenter study of posaconazole therapeutic drug monitoring. The authors did not observe any causal relation between posaconazole concentrations and the incidence of elevated liver function tests [50]. In another study, Moton et al., used pooled posaconazole safety data from 18 studies in healthy volunteers and two sub-sets from clinical trials with posaconazole doses ranging from 50 to 1200 mg/day, and found no relationship between adverse events and dose [56].
Factors optimizing the efficacy of voriconazole & posaconazole in invasive aspergillosis

In general, successful management strategies of IA depend on three principal aspects related to the pathogen, the host and the drug. In each aspect, a multitude of factors influence patient outcomes. However, few of these variables are under the control of the clinician. A schematic translation of such interaction is shown in (FIGURE 1). The PK (drug) factors that have impact of the target attainment of voriconazole and posaconazole have been discussed above.

Pathogen factors: the problem ofazole-resistance

Acquired azole-resistance in *A. fumigatus* is becoming an important pathogen-related factor that compromises the clinical efficacy of azole antifungals. A wide range of mutations in *A. fumigatus* have been described conferring azole-resistance commonly involves modifications in the cyp51A-gene. Cyp51A mutations in *A. fumigatus* commonly affect the activity of all mold-active antifungal azoles. Specific mutations correspond with various phenotypes characterized by complete loss of activity of a specific azole, and with decreased activity of others. If the activity of an azole is decreased, increased exposure might remain an option to treat patients successfully [57,58]. Using a non-neutropenic murine model of IA, we recently investigated the PD and PK properties of voriconazole against clinical *A. fumigatus* isolates with cyp51A mutations (MIC 2-4 mg/l) in order to determine whether the efficacy of the drug was reduced [57,58]. Voriconazole response (measured as survival) showed a clear concentration-dependent relation in both voriconazole susceptible and voriconazole resistant strains although, for each dose the response was lower in mice infected with the voriconazole-resistant isolate. As an example, at the highest dose of voriconazole investigated 100% survival was reached. In mice infected with the voriconazole-susceptible isolate compared to 72.2% survival in mice infected with the voriconazole-resistant isolate. As dose correlated with exposure, this illustrates that higher exposure of voriconazole was required to achieve similar efficacy when harboring voriconazole resistant strains. Given that, a standard dosage of voriconazole may not be adequate in azole-resistant IA, a higher drug exposure appears to be required. This could be achieved using therapeutic drug monitoring of voriconazole that may help linking to successful treatment in azole-resistance IA. Similar to voriconazole, higher posaconazole exposure was required to achieve the same therapeutic efficacy for posaconazole-resistant *A. fumigatus* isolates compared with posaconazole-susceptible strains. Recently, we used a non-neutropenic murine model of disseminated aspergillosis to investigate the comparative efficacies of various regimens of
posaconazole against clinical *A. fumigatus* cyp51A mutated isolates [59]. Both drug exposure and the susceptibility of the isolate impacted the efficacy of posaconazole treatment in *A. fumigatus*. We observed a 50% loss of efficacy if the MIC increased from 0.03-0.5 mg/l. The loss of efficacy was completely or partly compensated by increasing the posaconazole exposure by increasing the dose. Similar to voriconazole, an optimal posaconazole AUC/MIC predictive of treatment success in case of azole-resistant IA needs further elucidation in humans.

**Host & drug factors: the role of inter-patient variability**

Many covariates are known to cause significant patient-to-patient variability in the PK of voriconazole and posaconazole for treatment or prophylaxis in IA. For example, changes in oral absorption (i.e., due to mucositis), distribution (i.e., due to fluid retention) as well in metabolism and clearance (i.e., chemotherapy induced changes in liver and kidney function, drug-drug interactions, PK changes in protein binding, extracorporal elimination etc.) may be present with the consequence of higher degree of toxicity or sub-optimal effectiveness [50,60-64]. Intensive care patients will most likely have different PK compared to hematological patients [65]. This is, among many factors, attributable to the changes in hemodynamics in this population [61]. In septic shock, blood flow is directed to vital organs (i.e., brain and heart) with hypoperfusion of organs such as the liver, the gastrointestinal system and the kidney, which will result in altered clearance. Clearance of drugs will be even further influenced when extracorporeal elimination techniques are deployed [63,64]. Other challenges in this population are the increase in volume of distribution as well as shift in protein binding that may result in changes in both PK as well as PD of the drug.

In addition to the PK variability in different cohorts of patients, one should underscore the group of patients who is considered at high risk for IA; including patients underlying hematological malignancies, critically ill patients under steroids treatments, patients with allergic bronchopulmonary aspergillosis or chronic obstructive pulmonary disease who require mechanical ventilation and cystic fibrosis patients underlying lung transplantations [66].
Figure 1. The schematic triad of ‘the host’, ‘the bug (pathogen)’ and ‘the drug’ optimizing efficacy of antifungals.
Practical issues optimizing TDM

The appropriate practice of TDM is based on several disciplines, including: analytical analysis and sample selection.

Impact of analytical assays

Defining the analytical problem before embarking on development of an assay procedure is a critical step. Using TDM in routine practice implies that a validated analytical assay has to be readily available for the real-time determination of plasma or serum concentrations. In the literature reports on single or integrated assays for azoles antifungal drugs are available, but in only few hospitals have such an assay operational and instantaneously available [67-69]. A proficiency testing program is available to help further improve analytical methods [70]. Analytical assays have to be validated according to the current requirements for validation of bioanalytical assays [103]. Having an assay with a high specificity and accuracy, appears challenging. To help identify sources of errors and to further improve analytical methods, participation in an external proficiency-testing program is recommended. Results from a recent proficiency testing program show that correct analyses within the predefined range of 80 to 120% of the weighed-in concentrations were as follows: fluconazole, 79% (n = 14 analyses); itraconazole, 78% (n = 23); hydroxy-itraconazole; itraconazole, 78% (n=23); hydroxy-itraconazole, 78% (n = 18); voriconazole, 82% (n = 57) and posaconazole, 62% (n = 26). The results from this proficiency-testing program demonstrate the need for and utility of an ongoing proficiency-testing program to further improve the analytical methods for routine patient management [70].

Impact of sampling techniques

Interaction of the laboratory in sample selection and collecting technique is a key component of TDM. The sampling techniques influence outcomes from TDM specifically in drugs with a short half-life such as voriconazole. In older studies assessing the attributable value of TDM of voriconazole, the sampling has been performed in many cases randomly, which makes interpretation of the results difficult [54,71]. The ultimate sampling scheme would encompass a certain amount of sampling moments that would best predict exposure but this method is considered unfavorable for many reasons such as patient burden, required nursing time, complex logistics, etc.

In routine practice the most common sampling moment is just prior to the next dose (trough concentrations or Cmin). Using a single sample will reduce costs and more important will reduce patients burden due to frequent sampling. Since trough concentrations correlate
with exposure, this provides a measure that in clinical practice can be easily introduced. For posaconazole different aspects apply. This drug has a very long terminal half-life. The difference between peak and trough concentrations is minimal, especially when frequent dosing (i.e., t.i.d. or q.i.d.) is used [72]. Sampling at trough concentrations is therefore not necessary but may only be preferred from a practical point of view.

On the other hand, the therapeutic range for a drug is based on steady-state plasma concentrations. Concentrations drawn too soon after a dosage regimen has been started or changed may provide misleading information. In the case of voriconazole a first sample can be drawn on day 3 of therapy. But for posaconazole, steady state conditions are only reached by day 7-10. An algorithm has been suggested to be able to determine samples early after start of therapy (day 3) and targeting a lower concentration that will eventually result in a final target concentration on day 7 [48].

And lastly, the frequency of sampling remains subject to ongoing debate. A single sample is not considered TDM and will not provide the necessary input on a patient’s intra-individual pharmacokinetic variability. Multiple interventions over time are sometimes necessary to achieve target concentrations and remain within the therapeutic range. There is substantial intra-individual variation in voriconazole trough concentrations over time. Due to the high intra-subject variability an optimal sampling frequency has to be determined to timely adjust dose in order to achieve concentrations within the target range. A good starting point would be to perform TDM once or twice a week shortly after initiation of therapy. This can be reduced to once every two weeks when the patient is clinically improving or even less frequent when the patient is no longer in the hospital. Changes in clinical condition or when interacting drugs are introduced may prompt for more frequent sampling even when therapy has been given already for a longer period of time [73].
### Recommended trough concentration

<table>
<thead>
<tr>
<th></th>
<th>Voriconazole</th>
<th>Posaconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary prophylaxis</strong></td>
<td>ND, same as primary therapy</td>
<td>0.7 mg/l</td>
</tr>
<tr>
<td><strong>Secondary prophylaxis</strong></td>
<td>ND, same as primary therapy</td>
<td>ND, 0.7-1.0 mg/l</td>
</tr>
<tr>
<td><strong>Primary therapy (focus of no hepatic)</strong></td>
<td>&gt;1 mg/l and &lt;4 mg/l (&lt;6 mg/l infection: lung) if enzyme elevation)</td>
<td>ND, &gt;1.0-1.25 mg/l</td>
</tr>
<tr>
<td><strong>Primary therapy (disseminated or sanctuary sites)</strong></td>
<td>&gt;2 mg/l</td>
<td>ND, &gt;1.25 mg/l</td>
</tr>
<tr>
<td><strong>Salvage therapy</strong></td>
<td>ND, &gt;1.25 mg/l</td>
<td>ND: Not determined.</td>
</tr>
</tbody>
</table>

*Table 2.* Provisional target concentrations of voriconazole and posaconazole used for the prevention or treatment of invasive fungal infections.
Conclusion

TDM should be routinely applied for voriconazole and posaconazole treatment of infections caused by A. fumigatus. TDM will be useful to ensure adequate exposure when using a given dose in a particular patient. Based on the evidence presented in this review we propose the breakpoints for the treatment of azole susceptible IA in hematological patients that are summarized in (TABLE 2). In addition, evidence is increasing that a better understanding of the susceptibility could assist the management of patients with IA due to A. fumigatus isolates. We therefore suggest that the in vitro testing of A. fumigatus azole susceptibility should be performed systematically when considering azole therapy.

Expert commentary & five-year view

At the moment, TDM can be considered a diagnostic tool. Diagnostics may be useful to detect a change in a clinical condition such as an infection or in the case of TDM a sub- or supra-therapeutic exposure. Unfortunately, diagnostics always follow a change in clinical condition that necessitates frequent monitoring. The inherent downside of TDM is the possible delay in first time assessment as well as follow-up assessments. This may be due to pharmacokinetic aspects (i.e., steady state not reached), cost-aspects as well the availability of in-house testing facilities. A delay in the possibility to timely determinate too low or too high exposure may have clinical consequences. It has been repeatedly shown that delay in the initiation of antifungal therapy and inadequate exposures are independently associated with increased hospital mortality.

At this time point, TDM is warranted since the current dosing regimens do not fit the individual. Many antifungal drugs have been licensed at a dose that has been changed at a later stage regardless of the population examined. For instance, both the adult and pediatric dosing guideline of voriconazole has changed after market introduction. This indicates that when these drugs came on the market, no sufficient data were available to identify the optimal dose in specific cohorts of patients.

Collaborative efforts are necessary to identify as many as possible factors that contribute to changes in PK-PD to finally truly individualize upfront a patients’ dosing regimen. In vivo experimental models could be used to explicate pathogen influence. Specific patients’ populations such as children, hematology, and ICU patients need to be included in clinical trials. Host factors such as mucositis for oral absorption, obesity, renal and hepatic dysfunction and the extent of their influence need to be resolved. This research should be performed before or very short after market authorization. We can then seek for a
probabilistic model to upfront give the patient the optimal dose based on specific patient features (identified co-variates). This implies that we would switch from TDM as a concentration guided dosing tool to an approach were the drug concentration could be used as a validation set of an individualized dosing advice and thereby bringing it to earlier time point with subsequent reduction in host-risk. Also, whenever possible an attempt should be made to integrate PD parameters such as a pathogen’s MIC. This probabilistic approach, with a tailored dose followed by validation of target concentrations, will be performed very soon (i.e., 2 or 3 days) after start of therapy. Inherent to whatever new approach is chosen, we may not be able to identify all factors that cause changes in PK-PD. In other words: a residual error will always be present. And unforeseen changes in the clinical situation will prompt for new assessments of concentrations. But we strongly believe that we should put effort into bringing this diagnostic tool to an earlier time point after initiation of therapy with a tailored dose.

**From a practical point of view**

Have an adequate (in-house) technique with short turn-around time; take more than one sample; start measuring in the first few days of therapy; use trough concentrations for correct interpretation; measure again with changed clinical circumstances.

**Key issues**

- Therapeutic drug monitoring (TDM) of posaconazole and voriconazole can be recommended in the setting of prophylaxis and treatment of invasive fungal disease caused by *Aspergillus fumigatus*.

- TDM may be beneficial to warrant optimal efficacy of posaconazole and voriconazole and to reduce toxicity in the case of voriconazole.

- Controlled trials TDM versus no-TDM (specifically for posaconazole) are warranted to provide the necessary justification of the value of TDM.

- Incorporation of MIC, disease status, genomics, other covariates will allow more thorough interpretation of the results obtained.
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Websites


Chapter 7

General discussion and future prospective
General discussion and future prospectives

Azole resistance is an emerging problem in Aspergillus fumigatus. Resistance development in patients with chronic azole therapy complicates the management of aspergillus diseases in individual cases; however the emergence of azole resistance through environmental selection has been shown to be a concern for every patient at risk for aspergillus disease in endemic areas. As resistance is a relatively recent phenomenon, there is a lack of evidence and clinical experience in how this problem is best managed. The research described in this thesis is aimed to provide some experimental evidence that will help to guide physicians in the treatment of patients with azole-resistant aspergillus diseases.

1. Towards personalized management of invasive fungal infections

Several management strategies have evolved over the past decades that are aimed at the timely treatment of patients with (presumed) invasive fungal infections (IFIs). Early studies advocated the empiric treatment strategy where patients with persistent fever, despite broad-spectrum antibacterial therapy, receive a broad-spectrum antifungal agent, without having made the diagnosis of a fungal infection (1). Any fungus could cause the infection in this setting, of course depending on the local epidemiology. With the availability of biomarkers, such as galactomannan (GM), and computed tomography a diagnostic-driven approach became possible. In GM-positive patients the treating physician would know that the fungal infection was most likely caused by a member of the aspergillus family. This approach allows a more targeted use of antifungal agents and some studies indicate the diagnostic-driven strategy is as effective as the empiric strategy (2). However, biomarkers, such as GM, are at best genus-specific and do not provide information concerning the Aspergillus species or the drug-susceptibility.

Given the changes in aspergillus taxonomy and the emergence of acquired resistance, the current biomarkers may provide insufficient information to guide the treatment choice. An important focus of research should be the development of diagnostic tools that allow aspergillus speciation and the detection of resistance markers directly in clinical specimens. Direct detection of resistance markers is difficult as the Cyp-gene is a single copy gene, thus developing a diagnostic tool with sufficient sensitivity is a challenge. Furthermore, the diversity of Cyp-mediated resistance mechanisms represents another challenge. In the Netherlands surveillance studies shows that the diversity of resistance mutations is limited, i.e. mainly TR34/L98H and TR46/Y121F/T289A, and therefore a molecular tool that would allow
detection of only these two resistance mechanisms might significantly improve our ability to detect azole resistance at an early stage. In other settings where resistance mutations are more diverse the benefit of such an approach might be limited. Investigators from Manchester have reported multiple Cyp51A-mediated mutations in patient isolates, and also a substantial proportions of resistant isolates show non mutations in the Cyp51A-gene indicating that other, yet unknown, resistance mechanisms may be present (3, 4). Possibly in settings with a high diversity of resistance mechanisms a sequence-based approach might be more appropriate.

Our studies indicate that in a setting of aspergillus isolates with a greater range of MIC-values (i.e. wild type and non-wild type) the drug exposure becomes critical. Although we have shown that the role of voriconazole and posaconazole in azole-resistant aspergillosis is probably very limited, there remain situations that azole therapy will be considered. In situations that the PD-target can still be achieved, the therapeutic index will be lower and sufficient exposure more difficult to achieve. Intensive monitoring of patients, including therapeutic drug monitoring (TDM), then becomes critical. Other patient factors such as drug interactions, drug clearance and toxicity need to be considered.

The trend we anticipate is towards individualization of the management of patients with invasive aspergillosis and other aspergillus diseases. Factors such as those regarding the fungus (species, resistance mutations and virulence markers), pharmacology (drug choice and prediction of expected exposure) and host (site of infection, immune suppression and genetic susceptibility) will be used to optimize the management of the fungal infection aiming at improved outcome (Fig. 1). Given reports on increased resistance in other opportunistic fungi, such as C. glabrata (5), such an approach might be broadly applicable in the mycology field. This personalized approach is similar to that advocated in other areas of human medicine, particularly in the area of oncology that proposes the customization of healthcare, with medical decisions, practices, and/or products being tailored to the individual patient (6-8). We believe that with the availability of new molecular diagnostic techniques to detect drug resistance mechanisms in opportunistic fungi, advances in antifungal susceptibility testing, increased understanding and monitoring of antifungal drug levels and identification of host genetic risk factors, the management of invasive fungal infections will evolve towards personalized mycology (Fig. 2).
Figure 1: Key factors optimizing therapeutic approaches for antifungal therapy against invasive aspergillosis.
2. Recommendations for the management of azole-resistant aspergillosis

Current aspergillus treatment guidelines, such as IDSA (9) and ECIL (10), do not address the issue of azole resistance. In the literature only case series or individual cases of azole-resistant aspergillus diseases have been published, and therefore the best approach to manage these patients is unknown.

In the absence of clinical evidence and with limited preclinical evidence, one needs to rely on expert opinion. Therefore recently the Dutch Society for Medical Mycology organized an international expert meeting aimed to discuss how experts would manage azole resistance in different patient groups in endemic and non-endemic situations. I contributed to this expert meeting and it was evident that even among the experts opinions were divided. In the context of my thesis one important issue is the best alternative treatment option in patients with azole-resistant aspergillosis. Liposomal amphotericin B (L-AmB) is recommended as alternative treatment option for (azole-susceptible) invasive aspergillosis and our animal experiment showed that the efficacy of L-AmB in azole-resistant infections was similar to that of wild type infection (11). Nevertheless there is doubt that the efficacy of L-AmB is similar to that of voriconazole, based on post marketing studies (12-17).

In an editorial Denning et al estimated that L-AmB would be 10 to 15% less efficacious compared to voriconazole (18). If this would be true a switch of first line therapy from voriconazole to L-AmB would come with a trade off. Such a switch would then be justified only when higher levels of environmental resistance are found. However, other studies, such as the ambiload clinical trial (19), report an efficacy of L-AmB which is comparable to that of voriconazole. The problem is that we have no studies that directly compare the efficacy of voriconazole with that of L-AmB. Furthermore, comparing different clinical studies is difficult as they will differ with respect to inclusion criteria, underlying diseases and certainty of diagnosis. Also in retrospective studies that compare the efficacy of L-AmB with voriconazole, patients treated with L-AmB are usually treated before voriconazole was licensed (2002), thereby introducing other factors, such as general improved supportive care, that influence outcome (13).

As a new randomized comparative trial is unlikely to be performed, this dilemma will prove difficult to resolve. One option would be to re-analyze patients enrolled in different prospective clinical trials, as was recently published for candidemia (20). Factors that could cause potential bias, such as the year in which the patient was treated, could be matched. The feasibility of such an analysis depends amongst other factors on the willingness of the
pharmaceutical companies to share relevant data on file. An alternative might be to set up a registry of cases with azole-resistant aspergillosis, in which standardized information is collected of patients with documented azole-resistant disease. Matched control cases of azole-susceptible aspergillosis may be included as comparison.

**Figure 2:** Schematic of personalized mycology. Modified from Sadowska et al. Ther Adv Med Oncol. 2011;3(4):207-18.
3. Investigation of alternative treatment options

Although the therapeutic arsenal of antifungal drug classes for the treatment of aspergillus diseases is limited, there are compounds from other drug classes that could be re-evaluated in the setting of azole resistance. One advantage of this approach is that these drugs are already licensed and alternative treatment regimens could be implemented rapidly. Currently there are patient groups where treatment options are extremely limited, such as patients with central nervous system (CNS) aspergillosis. In CNS aspergillosis voriconazole has shown better efficacy compared to alternatives regimens, i.e. polyene-based therapy, and patients with azole-resistant CNS aspergillosis have proven extremely difficult to manage (21).

Flucytosine (5-FC) is one of the drugs that could be evaluated for treatment of azole-resistant aspergillosis, particularly in cases that involve the CNS. In the clinical setting, the combination of 5-FC and L-AmB was effective in CNS infections due to Cryptococcus neoformans, (22) but its use in the treatment of aspergillus infection has been controversial. The drug has been used in patients with invasive aspergillosis in combination with L-AmB (23). One small prospective study was published showing no benefit of AmB+5-FC combination therapy compared to AmB monotherapy (24), however only patients with proven IFIs were enrolled in this study indicating that treatment was started at a late stage of infection. 5-FC was found to be active in vitro against A. fumigatus isolates when the MIC was determined at PH 5 instead of PH 7 (25, 26). In experimental models mice infected with isolates with a low 5-FC MIC (at pH 5) could be successfully treated with 5-FC monotherapy (26, 27). As there are currently no data regarding the in vitro activity of 5-FC against azole-resistant A. fumigatus isolates, and no in vivo data to support its use, this should be investigated. Given the excellent penetration of 5-FC in CNS and eye, combination therapy with L-AmB or voriconazole might be an option in these difficult to treat infections (28).

Another drug with in vitro activity against A. fumigatus is the allylamine terbinafine. Terbinafine appeared to be as effective as amphotericin B and itraconazole in the treatment of bronchopulmonary aspergillosis in non-immunocompromised patients (29). In two cases of aspergillus endophthalmitis, terbinafine in combination with amphotericin B showed a synergistic interaction (30). The use of terbinafine in combination therapy for aspergillus infections with azoles was also promising in vitro, against both itraconazole-susceptible and -resistant A. fumigatus isolates (31, 32). In addition, the interaction between triazoles and terbinafine was synergistic in vitro and in salvage therapy of Scedosporium prolificans infection, a fungus inherently resistant to voriconazole (33-35). In this approach, the efficacy of combination therapy relies on terbinafine and its synergistic interaction with voriconazole. In our opinion, therefore, the combination of voriconazole and terbinafine may be an attractive option for azole-resistant aspergillus diseases, and requires further investigation.
Another potential strategy to overcome antifungal drug resistance is to make currently available antifungals more effective. This approach is to identify compounds that act in synergy with currently licensed antifungals.

Chemosensitizing agents possess antifungal activity, but at insufficient levels to serve as antimycotics, alone (36). Their main function is to disrupt fungal stress response, destabilize the structural integrity of cellular and vacuolar membranes or stimulate production of reactive oxygen species, augmenting oxidative stress and apoptosis. When chemosensitizers are co-applied with a commercial antifungal agent, an additive or synergistic interaction may occur, augmenting antifungal efficacy (36). This augmentation, in turn, lowers effective dosages, costs, negative side effects and, in some cases, countermands resistance. Notably, combination of such agent 2 – Adamantanamine (AC17) which is a close structural analog of amantadine, with azoles has been shown a promising lead in the search for more effective antifungal therapeutics (37). This approach might improve the efficacy of the azoles against *A.fumigatus* isolates simply allowing more drugs to enter the cell, or they may act by affecting a pathway that leads to synergy, such as inhibiting a fungal stress response.

### 4. Investigation of new targets for antifungals

Development of new antifungal drug targets for fungi has proven difficult. It is complicated to design an agent that selectively kills one eukaryote (pathogen) while not harming the larger infected eukaryote (host). Notably, decades of *Aspergillus* pathogenesis research have confirmed its multifactorial nature. There are myriad studies highlighting *A. fumigatus* virulence factors, all of which are possible antifungal drug targets. Researchers strive to elucidate the following factors: calcineurin” conserved protein phosphatase important in stress responded pmrA (38), heat shock protein 90 (Hsp90) (39), the endoplasmic reticulum transmembrane sensors(40), Basic fibroblast growth factor (BCGF) (41), and sterol regulatory element–binding protein transcription factor which mediates hypoxia adaptation to the site of infection (42). Recent data also suggest a bifunctional role for galactosaminogalactan in the pathogenesis of invasive aspergillosis, and suggest that it may serve as a useful target for antifungal therapy (43). However, clearly, to move from “virulence factor” to next critical steps as drug target requires more investigation.
5. Immunotherapy as an alternative therapeutic option

The host-fungal interactions play a critical role for all fungal pathogens. Targeting this interaction may provide novel therapies, which could be used alone or in combination with existing antifungal drugs. There is an increasing demand for novel therapeutic strategies aimed at enhancing or restoring antifungal immunity in immunocompromised patients. In this regard, modulation of specific innate immune functions and vaccination are promising immunotherapeutic strategies (44).

Colonystimulating factors (CSFs), granulocyte transfusion, and cytokines (mainly IFN – γ) are used to augment the number and the function of circulating neutrophils in neutropenic patients (45). T-cell therapy including *Aspergillus*-specific CD4+ Th1 immunity has been shown an appealing strategy to favour immunological reconstitution and early adoptive therapy. Pentraxin (PTX3), an opsonin that forms complexes on the conidial surface of *A. fumigatus*, thereby amplifying the innate immune response, is also receiving great attention as a potential therapeutic agent with anti-inflammatory properties in aspergillosis (46). Thymosin alpha1 (Tα1), a naturally occurring thymic peptide, which is approved in some countries for the treatment of a number of viral infections (47) and as an endogenous immune regulator was capable of inducing protective immunity to *A. fumigatus* (48). However, more investigation is needed to determine whether this approach is useful in either clearing *A. fumigatus* infections or prolonging survival.
References


Summary in English
Summary

In humans, *Aspergillus fumigatus* is the most common and life-threatening aerial fungal pathogen, especially among immunocompromised patients, with an overall mortality ranging between 30 to 88%. Azole antifungals, such as voriconazole and posaconazole, are recommended first choice drugs to manage aspergillus diseases. However, acquired azole-resistance in *A. fumigatus* is an emerging problem that compromises the clinical efficacy of azole antifungals. An azole-resistant phenotype in *A. fumigatus* commonly confers with modifications in the cyp51A-gene, which affect the activity of all mold-active antifungal azoles. Specific mutations correspond with various phenotypes characterized by complete loss of activity of a specific azole, and with decreased activity of others.

Although azole resistance may emerge during antifungal therapy of individual azole-treated patients, selection of resistance may also occur in the environment. The environmental route of resistance selection has the risk of global migration and indeed azole resistance in *A. fumigatus* is increasingly found in multiple European countries, Asia and the United States. Azole resistant aspergillus infection is commonly associated with treatment failure. Given the prominent role of azoles in the management of aspergillus diseases, successful management of azole-resistant aspergillus diseases in patients with chronic pulmonary aspergillosis (CPA) and invasive aspergillosis (IA) is a challenge. Therefore, it is important to explore alternative therapeutic approaches (*Chapter 1*). The research described in this thesis is aimed to provide some experimental evidence that will help to guide physicians in the treatment of patients with azole-resistant aspergillus diseases.

Experimental models of IA have been used to explore pharmacokinetic (PK) and pharmacodynamic (PD) properties of antifungal agents. Survival is considered the golden standard effect measure but has the disadvantage that a large number of animals are needed to determine the dose–response relationships and PK/PD of antifungals. In *Chapter 2* we investigated the applications and limitations of molecular techniques to assess therapeutic efficacy of antifungal agents in experimental models of invasive aspergillosis. It is expected that the use of qPCR will become the primary outcome measure for assessment of PK/PD relationships of antifungals in experimental models of IA.

Anidulafungin belongs to the echinocandins but has an unique site of action, different from that of azoles and polyenes, as it targets cell wall synthesis. *Chapter 3* describes the PK-PD properties of anidulafungin monotherapy in a non-neutropenic murine model of invasive aspergillosis. For this purpose, we used two clinical isolates with different profiles of susceptibility to voriconazole: a voriconazole-susceptible *A. fumigatus* isolate and a voriconazole-resistant *A. fumigatus* isolate harboring a TR34/L98H mutation in the cyp51A gene. Anidulafungin treatment improved the survival of mice in a dose-dependent manner;
however, a maximal response was not achieved with either isolate even in those treated with the highest anidulafungin dose.

Although combination therapy is presently not recommended for the primary therapy of invasive aspergillosis, it may be an effective alternative approach for treatment of patients with azole-resistant *Aspergillus* disease. A recent clinical trial comparing voriconazole monotherapy with voriconazole plus anidulafungin showed a trend towards a better efficacy of the combination. However, in the study the majority of patients probably had azole-susceptible invasive aspergillosis. *In vitro* interaction studies indicated that a combination of voriconazole and anidulafungin might be effective against infections caused by both azole-susceptible and azole-resistant *A. fumigatus* isolates, but the combination could possibly be less effective in voriconazole-resistant strains with high MICs (Chapter 4.1).

The in vivo efficacy of voriconazole and anidulafungin was also investigated in a non-neutropenic murine model of invasive aspergillosis using voriconazole-susceptible (voriconazole MIC of 0.5 mg/l) and voriconazole-resistant (voriconazole MIC of 4 mg/l) *A. fumigatus* clinical isolates. The combination of voriconazole and anidulafungin showed a synergistic interaction in voriconazole-susceptible invasive aspergillosis, but only an additive interaction in voriconazole-resistant invasive aspergillosis. There was a clear benefit of combining voriconazole and anidulafungin, but the reduced effect of combination therapy in azole-resistant invasive aspergillosis raises some concern (Chapter 4.2).

In Chapter 5, we investigated whether liposomal-amphotericin B (L-AmB) could be used as an alternative treatment option in patients with azole-resistant invasive aspergillosis, using an experimental model of infection. To this aim, we studied the pharmacodynamics and dose-response relationships of L-AmB against wild-type and three clinical azole-resistant *A. fumigatus* isolates harboring different resistance mechanisms in an immunocompetent murine model of disseminated aspergillosis. Our results indicated that L-AmB was able to prolong survival *in vivo* in disseminated invasive aspergillosis independent of the presence of an azole resistance mechanism in a dose-dependent manner, and therefore, supports a role for L-AmB in the treatment of azole-resistant invasive aspergillosis.

In chapter 6.1 we investigated if voriconazole and posaconazole remain to have a role in the treatment of azole-resistant aspergillus disease. *In vitro*, preclinical studies and clinical studies were reviewed and used to estimate the pharmacodynamic (PD) target. Then for each MIC the required exposure and plasma level were calculated. Our analysis showed that for posaconazole adequate exposure can be achieved only for wild type isolates and that dose escalation does not allow PD target attainment. However, the new intravenous formulation might result in sufficient exposure to treat isolates with a MIC of 0.5 mg/L. For voriconazole our analysis indicated that the exposure needed to treat infection due to isolates with a MIC of 2 mg/L is feasible and maybe isolates with a MIC of 4 mg/L. However, extreme caution and
strict monitoring of drug levels would be required, as the probability of toxicity will also increase.

In Chapter 6.2 we review the pharmacokinetics and pharmacodynamics of voriconazole and posaconazole. Given the PK/PD characteristics of these azoles monitoring of drug exposure appears necessary to ensure adequate exposure in individual patients. The increased recognition of non-wild type *A. fumigatus* isolates and other aspergillus species with different azole-susceptibility profiles, underscores the need for close and individualized patient management.
Summary in Dutch / Samenvatting in het Nederlands
Samenvatting

In de mens is *Aspergillus fumigatus* de meest voorkomende schimmel die levensbedreigende infecties kan veroorzaken met name bij immuunecompromitteerde patiënten, met een letaliteit die varieert tussen 30 en 88%. Azolen, zoals voriconazol en posaconazol, zijn aanbevolen eerste keus middelen bij aspergillusziekten. Echter, verworven azoolresistentie is een toenemend probleem die de effectiviteit van azoolgeneesmiddelen bedreigt. Een azoolresistente fenotype hangt vaak samen met genetische veranderingen in het Cyp51A-gen, waarbij de activiteit van de azolengroep wordt aangetast. Specifieke mutaties in dit gen corresponderen veelal met volledig verlies van activiteit van één bepaalde azool, en met verminderde activiteit bij de andere azolen.

Hoewel azoolresistentie kan ontstaan gedurende behandeling van individuele patiënten, kan resistentieselectie ook optreden in het milieu. Deze omgevingsroute van resistentieselectie heeft een hoog risico op mondiale verspreiding van resistentie en azoolresistentie wordt ook in toenemende mate gerapporteerd in Europese landen, Azië en de Verenigde Staten. Azoolresistentie is geassocieerd met het falen op behandeling met azolen. Gegeven de prominente rol van azolen bij de behandeling van aspergillusziekten, wordt het behandelen van azoolresistente chronische aspergillose (CPA) en acute aspergillose een uitdaging. Het is daarom van belang alternatieve behandelingsopties te exploreren (Hoofdstuk 1). Het onderzoek zoals beschreven in dit proefschrift heeft als doel wetenschappelijk bewijs op basis van experimenteel onderzoek te leveren die cliënten kan helpen bij de behandeling van patiënten met azoolresistente aspergillusziekten.

Experimentele modellen van IA zijn gebruikt bij het onderzoek naar farmacokinetische (PK) en farmacodynamische (PD) eigenschappen van antifungale middelen. Overleving wordt gezien als de gouden standaard van effectiviteit, maar heeft als nadeel dat er een groot aantal dieren nodig zijn om dosis - respons en PK/PD relaties te beschrijven. In Hoofdstuk 2 hebben we de toepassingen en beperkingen onderzocht van moleculaire technieken om de effectiviteit van antifungale middelen in diermodellen van IA te bepalen. Men verwacht dat qPCR de belangrijkste maat van effectiviteit zal worden om Pk/PD relaties van antifungale middelen in IA te beschrijven.

Anidulafungine behoor tot de klasse van de echinocandines en heeft een uniek aangrijpingspunt, verschillend van de azolen en polyenen, met als aangrijpingspunt de celwand synthese. Hoofdstuk 3 beschrijft de PK-PD eigenschappen van anidulafungine monotherapie in een niet-neutropeen muis model van IA. Voor dit onderzoek hebben we twee klinische isolaten gebruikt met verschillende gevoeligheidspatronen ten opzichte van voriconazol: een voriconazol-gevoelig *A. fumigatus* isolaat en een voriconazol-resistente *A. fumigatus* isolaat met een TR34/L98H substitutie in het Cyp51A-gen. De overleving van
muizen behandeld met anidulafungine verbeterde en was dosis afhankelijk; echter, een maximale respons werd met geen van beide isolaten bereikt, zelfs niet in de groepen die met de hoogste dosering werden behandeld.

Hoewel combinatietherapie momenteel niet wordt aanbevolen voor de behandeling van IA, zou combinatietherapie een optie kunnen zijn bij azoolresistente aspergillusziekten. In een recente klinische trial waarbij de effectiviteit van voriconazol werd vergeleken met dat van voriconazol plus anidulafungine was er een trend naar betere effectiviteit bij patiënten die met de combinatie werden behandeld. Echter we gaan ervan uit dat de meerderheid van de patiënten een infectie hadden met een azool – gevoelig isolaat. In vitro interactie onderzoek toonde aan dat de combinatie voriconazol en anidulafungine mogelijk effectief zou zijn bij zowel azool-gevoelige en azool-resistente infecties, maar dat de combinatie mogelijk minder effectief zou kunnen zijn als het isolaat volledig resistent was voor voriconazol (Hoofdstuk 4.1).

De in vivo effectiviteit van voriconazol en anidulafungine werd onderzocht in een niet-neutropeen model van IA waarbij dieren geïnfecteerd werden met klinische A. fumigatus isolaten: een voriconazol-gevoelige (voriconazol MIC van 0,5 mg/l) of een azool-resistente (voriconazol MIC van 4 mg/l). De combinatie voriconazol en anidulafungine toonde een synergistische interactie in azool-gevoelige IA, maar slechts een additieve interactie bij azool-resistente IA. Hoewel er een duidelijk toegevoegde waarde was van het combineren van voriconazol en anidulafungine, is het verlies van synergisme bij azoolresistentie zorgelijk (Hoofdstuk 4.2).

In hoofdstuk 5 hebben we onderzocht of liposomaal-amfotericine B (L-AmB) gebruikt zou kunnen worden als alternatieve behandeling voor patiënten met azoolresistente IA, gebruikmakend van ons diermodel. Hiertoe werden de farmacodynamiek en dosis-respons relaties van L-AmB onderzocht bij muizen geïnfecteerd met een wild-type isolaat en drie klinische azool-resistente A. fumigatus isolaten, met verschillende resistentie mechanismen. Het onderzoek toonde aan dat L-AmB behandeling de in vivo overleving van de muizen verlengde onafhankelijk van de aanwezigheid van een azoolresistentie mechanisme. De effectiviteit was dosis afhankelijk en het model ondersteunt het gebruik van L-AmB voor de behandeling van azoolresistente IA.

In hoofdstuk 6.1 hebben we onderzocht of er nog een rol is voor voriconazol en posaconazol bij de behandeling van azoolresistente aspergillus ziekte. Relevant in vitro, preklinische en klinische studies werden onderzocht en gebruikt om een schatting te maken van het farmacodynamisch target (PD). Vervolgens werd er voor MIC berekend wat de vereiste blootstelling zou zijn en plasma spiegel om de PD te bereiken. Deze analyse toonde dat voor posaconazol een adequate blootstelling bereikt kon worden die ons in staat stelt uitsluitend infecties met een wild type gevoeligheid te behandelen, en dat dosis escalatie niet
leidt tot een hogere blootstelling. Mogelijk dat met het beschikbaar komen van een intraveneuze formulering een voldoende blootstelling bereikt kan worden om infecties door isolaten met een MIC van 0,5 mg/l te behandelen. Voor voriconazol toonde ons onderzoek aan dat de blootstelling die nodig is om isolaten met een MIC van 2 mg/l te behandelen bereikt kan worden, en mogelijk isolaten met een MIC van 4 mg/l. Echter dit dient dan wel met grote omzichtigheid en stricte monitoring van plasma spiegels gebeuren, omdat ook het risico op toxiciteit sterk toeneemt.

In **Hoofdstuk 6.2** presenteren we literatuuronderzoek naar de farmacokinetiek en farmacodynamiek van voriconazol en posaconazol. Gegeven de PK/PD eigenschappen van deze middelen is het monitoren van spiegels noodzakelijk om verzekerd te zijn van een adequate blootstelling. De noodzaak van nauwgezette monitoring van patiënten is toegenomen door de toegenomen risico op non-wild type *A. fumigatus* en andere *Aspergillus* species met afwijkende gevoeligheidsprofielen.
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Amir
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About the author
Seyed Mojtaba Seyed Mousavi Tasieh also known as “Amir S. Seyedmousavi” was born in Khomam, a small City in Rasht County, Gilan Province, North of Iran on the 21st September 1977.


He then completed his postgraduate training and a PhD in Mycology with Cum Laude (2006) in Iran. In addition, he has been trained in several world class research and advanced educational programs and presented his teaching/lectureship expertise in many conferences, courses and workshops.

He was previously appointed as an academic lecturer (2002 to 2006) in Medical Microbiology and assistant professor (2006 to 2009) of Medical Mycology in Islamic Azad University (IAU) of Iran.

From 2009 to 2010, he did postdoctoral research in Molecular Mycology in the lab of Prof. Dr. G. Sybren de Hoog at CBS-KNAW Fungal Biodiversity Centre, The Netherlands.

In 2010, he started his second PhD and postdoctoral research presented in the current thesis in Medical Mycology and PK/PD (pharmacokinetics and pharmacodynamics) of antifungals under the supervision of Prof. Dr. Paul E. Verweij, Prof. Dr. Johan W. Mouton, and Dr. Willem Melchers at the department of Medical Microbiology, Radboudumc, Nijmegen, The Netherlands.

He is married to Haleh Rafati since 2009 and they have an adorable daughter “Rosha” who was born in 2012 in Rotterdam.
Scientific Portfolio (2010-2014)
PhD portfolio

Membership in scientific societies

2011- Present ASM (American Society for Microbiology)
2011-present ESCMID (European Society of Clinical Microbiology and Infectious Diseases)
2011-Present ISHAM (International Society for Human and Animal Mycology)
2011-Present ISAP (International Society of Anti-Infective Pharmacology)

Honors & Awards

2014 Editorial Board of Medical Mycology Journal
2013 American Society for Microbiology, ASM-ICAAC Infectious Diseases Fellows Grant Program
2013 European Society of Clinical Microbiology and Infectious Diseases, Attendance grant for postgraduate Education Course
2012 American Society for Microbiology, ASM-ICAAC Infectious Disease Fellow Travel Grant
2012 European Society of Clinical Microbiology and Infectious Diseases, Attendance grant for postgraduate Education Course
2011 American Society for Microbiology, Student and Post-Doctoral Fellow Travel Grant
2011 International Society for Human and Animal Mycology (ISHAM), Co-convener of ISHAM Veterinary Mycology Working Group

Courses & Workshops

2013 Invasive Mycoses: Consider the Source, Consider the Host. A CME event from the Mycoses Study Group Educational Committee, Denver, USA
2013 1st International Workshop on Clinical Pharmacology of Antifungal Drugs & Fungal Diseases, Berlin, Germany
2012 A Statistical Approach to PK/PD Analysis in Practice, Athens, Greece
2012 Earlier Treatment of Invasive Fungal Infections: Are We There Yet? A CME event from the Mycoses Study Group Educational Committee, San Francisco, USA
2012 Advanced PK/PD Modeling of Anti-Infective Agents with Practical Examples, San Francisco, USA
2012 Introduction to PK/PD Modeling of Anti-Infective Agents, San Francisco, USA
2012 Training the innate immunity: immunological memory in innate host defense, Nijmegen, the Netherlands
2012 Training School in the Experimental Design & Statistical Analysis of Biomedical Experiments, Nijmegen, the Netherlands
2012 Histopathology of Mycoses, 18th Congress of the International Society for Human and Animal Mycology, Berlin, Germany
2012 ESCMID Postgraduate Education Course: Antimicrobial Stewardship: Measuring, Auditing and Improving, Surrey, London, United Kingdom
2011 Zebrafish: an animal model in biomedical research, Hubrecht Institute, Utrecht, the Netherlands
2011 Clinical Relevance of Pharmacokinetics and Pharmacodynamics of Anti-Infective Agents, American Society for Microbiology, Chicago, USA
2011 Basics of Pharmacokinetics and Pharmacodynamics of Anti-Infective Agents, American Society for Microbiology, Chicago, USA
2011 The dynamics of Zygomycete research in a changing world, A Workshop on Zygomycete Biodiversity, Utrecht, the Netherlands
2010 Emerging Potential of Black Yeasts, Meeting of the ISHAM Working groups on Black Yeasts and Chromoblastomycosis, Ljubljana, Slovenia
2010 AIOS course on Clinical Mycology, RUNMC (UMC ST RADBOUD), Nijmegen, the Netherlands
2010 Course on Laboratory Animal Sciences (Article 9), Utrecht, the Netherlands
2010 Course on Medical Mycology, CBS-KNAW (FUNGAL BIODIVERSITY CENTR) Utrecht, the Netherlands

Oral presentations

2013 Fifth meeting of the ISHAM-working groups on Black Yeasts and Chromoblastomycosis, Guangzhou, China
2013 Post-ICAAC Symposium of International Society of Anti-Infective Pharmacology (ISAP), Denver, Co, USA
2013 1st International Workshop on Clinical Pharmacology of Antifungal Drugs & Fungal Diseases, Berlin, Germany
2013 1st International Workshop on Clinical Pharmacology of Antifungal Drugs & Fungal Diseases, Berlin, Germany
2013 Symposium and workshop on Medical Mycology, Nijmegen, the Netherlands
2012 Netherlands Society for Medical Mycology, Utrecht, The Netherlands
2012 Post-ICAAC Symposium of International Society of Anti-Infective Pharmacology (ISAP), San Francisco, CA
2012 18th Congress of the International Society for Human and Animal Mycology, Berlin, Germany
2012 22nd European Congress of Clinical Microbiology and Infectious Diseases, London, United Kingdom
2011 51st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, USA
2011 Joint meeting of Belgian Society of Human and Animal Mycology and Netherlands Society for Medical Mycology, Antwerp, Belgium
2011 Workshop on Zygomycete biodiversity, Utrecht, The Netherlands
2010 Workshop of the ISHAM Working group on Black Yeasts, Ljubljana, Slovenia

Poster presentations

2014 24th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain
2013 6th Trends in Medical Mycology (TIMM-6), Copenhagen, Denmark
2013 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Denver, Co, USA
2013 23rd European Congress of Clinical Microbiology and Infectious Diseases, Berlin, Germany
2012 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, USA
2012 18th Congress of the International Society for Human and Animal Mycology, Berlin, Germany
2012 22nd European Congress of Clinical Microbiology and Infectious Diseases, London, United Kingdom
2011 51st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, USA
2011 Scientific Spring Meeting NVMM & NVvM, Arnhem, the Netherlands
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روشهای درمان بیماری آسپرژیلوزیس تهاجمی ناشی از قارچ آسپرژیلوس فومیگاتوس مقاوم به دارو: از آزمایشگاه تا بالین

دکتر سید مجتبی سید موسوی

بیماری آسپرژیلوزیس مهاجم سبب مرگ و میر در ۳۰ تا ۸۸ درصد بیماران می‌باشد. عامل این بیماری قارچ آسپرژیلوس فومیگاتوس است. در حال حاضر داروهای گروه آزول شامل ووریکونازول و پوساکونازول بهترین توصیه جهت درمان بیماری‌های ناشی از این قارچ هستند. هر چند که مصرف طولانی مدت این داروها سبب بروز پدیده نوزهور مقاومت در دارویی اکتسابی در این قارچ شده است. علاوه بر این، نتایج تحقیقات نشان داده است که قارچ آسپرژیلوس فومیگاتوس می‌تواند مقاومت به این داروها را در محیط کسب کند که از جمله دلایل آن می‌توان به تأثیرات مصرف بی‌روی مشتق‌های داروهای این خانواده در کشاورزی و سایر صنایع و تغییرات ژنومی این قارچ در محیط اشاره نمود. به دلیل اینکه از یکطرف داروهای آزولی نقش بسیار مهمی در درمان بیماری آسپرژیلوسیس مزمن و مهاجم بازی می‌کنند و از طرف دیگر روند میر در بین مبتلایان به این بیماری در حال افزایش است، جستجوی روشهای درمانی جایگزین بسیار حائز اهمیت و ضروری است. در این پژوهش نامه نتایج تحقیقات به عمل آمده در طی مدت چهار سال بیان می‌گردد که در این تلاش است که از راهکارهای مورد نیاز پزشکان در درمان بیماری آسپرژیلوسیس تهاجمی ناشی از قارچ آسپرژیلوس فومیگاتوس مقاوم به دارو، از آزمایشگاه تا بالین مورد بحث و بررسی قرار گیرد.
In humans, *Aspergillus fumigatus* is the most common and life-threatening aerial fungal pathogen, especially among immunocompromised patients, with an overall mortality ranging between 30 to 88%. Azole antifungals, such as voriconazole and posaconazole, are recommended first choice drugs to manage aspergillosis diseases. However, acquired azole-resistance in *A. fumigatus* is an emerging problem that compromises the clinical efficacy of azole antifungals. Although azole-resistance may emerge during antifungal therapy of individual azole-treated patients, selection of resistance may also occur in the environment. Given the prominent role of azoles in the management of aspergillosis diseases, successful management of azole-resistant aspergillosis diseases in patients with chronic pulmonary aspergillosis and invasive aspergillosis is a challenge. Therefore, it is important to explore alternative therapeutic approaches. The research described in this thesis is aimed to provide some experimental evidence that will help to guide physicians in the treatment of patients with azole-resistant aspergillosis diseases.

**Strategies for treating aspergillosis due to azole-resistant *Aspergillus fumigatus*: From the bench to the bedside**

Seyed Mojtaba Seyed Mousavi Tasieh