

Review

Antifungal Resistance Mechanisms of *Aspergillus*

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Abstract

The incidence of invasive aspergillosis (IA), which is commonly caused by *Aspergillus fumigatus*, has increased recently in immunocompromised patients and has become the common cause of death in these patients. Antifungal resistance is one of the reasons for treatment failure. Since the first itraconazole-resistant *A. fumigatus* was reported in 1997, the reports on clinical strains of triazole-resistant *A. fumigatus* have increased, as well as studies on the resistant mechanisms. In this paper, the known molecular mechanisms of antifungal resistance in *Aspergillus*, especially in *A. fumigatus*, are reviewed.

Key words : antifungal resistance, molecular mechanisms, *Aspergillus*

Introduction

In recent decades, the incidence of invasive aspergillosis (IA) has increased in frequency in immunocompromised patients with a variety of diseases. Despite the advances in early diagnosis and antifungal therapy, the reported mortality rates of IA have ranged from 80% to 95%¹⁻³. In Japan, aspergillosis in autopsy cases increased from 40.9% to 46.0% in 1997 and 2001 in leukemia patients⁴. In China, the autopsy data from Xinan Hospital showed that 54.8% of the invasive fungal infection was caused by *Aspergillus*⁵. The most common pathogen of aspergillosis is *A. fumigatus*, followed by *A. flavus* and *A. terreus*⁶.

There are four classes of antifungal drugs with *in vitro* activity against *Aspergillus* spp.: the polyenes, including amphotericin B, lipid formulations of amphotericin B; the triazoles, such as itraconazole, voriconazole, and the recently approved posaconazole; the echinocandins, including caspofungin, micafungin and anidulafungin; and the allylamines, terbinafine^{7,8}.

Repeated exposure to antifungals, especially azoles, is a major risk factor for acquired drug resistance. Reports on drug-resistant clinical isolates of *Aspergillus* have emerged in recent years^{6,9-14}, and studies on antifungal resistance mechanisms and clinical significance are also increasing^{6,9-12,15-19}. In this paper, we review the available data on molecular mechanisms of

antifungal resistance in *Aspergillus* spp., especially in *A. fumigatus* to triazoles.

The term “resistance” should be clearly defined. Resistance refers to a relative insensitivity of a microbe to an antimicrobial drug as tested *in vitro* and compared with other isolates of the same species²⁰. Although the possibility for clinical failure of IA may be due to many factors including impaired immune function, poor bioavailability of the administered drug, and accelerated metabolism of the drug²¹, drug resistance has become increasingly important.

Antifungal susceptibility testing methods

For yeasts, standardized *in vitro* methods and interpretive MIC points have been well established. For filamentous fungi, only recently has the Clinical and Laboratory Standards Institute (CLSI) published the broth microdilution method, M38-A, for testing the antifungal susceptibility against filamentous fungi²². M38-A defines uniform testing conditions that allow reproducibility of MIC determination, at least for triazoles and amphotericin B. Breakpoints for the tested drugs, however, have not been clearly defined²² yet. The alternative susceptibility testing methods for filamentous fungi include Etest and YeastOne colorimetric assays. For amphotericin B, Etest is more accurate than the M38-A method in identifying the amphotericin B-resistant isolates from the -sensitive *Aspergillus* isolates²³. YeastOne colorimetric plates have favorable correlation with the CLSI M38-A method for amphotericin B, itraconazole and posaconazole against

Table 1. Amino acid substitutions conferring triazole resistance

Amino acid changes	Resistant drugs
M220I, M220V, M220K, M220T	Itraconazole
G54R, G54E, G54K, G54V	Itraconazole and posaconazole
L98H*	Itraconazole, posaconazole, voriconazole, ravuconazole

*Multiple triazole resistance is due to L98H combination with the presence of two copies of a 34-bp sequence in tandem in the promoter of the *CYP51A* gene.

*Aspergillus*²⁴⁾.

Triazole resistance

The first published case of itraconazole-resistant isolates of *A. fumigatus* appeared in 1997⁹⁾. Despite being uncommon, triazole resistance in *A. fumigatus* is becoming more important. A global survey of 445 *Aspergillus* isolates showed excellent antifungal activity of itraconazole against *Aspergillus* spp. (94% of MICs were $\leq 1 \mu\text{g/ml}$)²⁵⁾. In a survey of over 900 *A. fumigatus* isolates, the prevalence of itraconazole-resistant isolates was 2%²⁶⁾. In a susceptibility testing of 930 *Aspergillus* isolates in the Netherlands, there were only three which were itraconazole-resistant (0.3%), no case of voriconazole resistance, and all itraconazole-resistant isolates were recovered from patients after the period of itraconazole introduction²⁷⁾.

Although it is not common, clinical isolates of triazole-resistant *A. fumigatus* and the resistance mechanisms have been reported frequently, and the clinical significance of resistance has been noticed^{6, 9-19)}. Most triazole resistance studies in *A. fumigatus* have focused on itraconazole. Quite recently, the clinical isolates of multiple-triazole resistant *A. fumigatus* were also recognized²⁸⁻³¹⁾.

Triazoles bind to lanosterol 14 α -demethylase (Cyp51p), encoded by the *ERG11* gene (*CYP51* in *Aspergillus*), and inhibit the biosynthesis of ergosterol from lanosterol in fungi⁷⁾. These cause ergosterol depletion and toxic 14- α methylated sterols accumulation, as the result of fungal growth arrest⁷⁾.

It is well known that modification of the target enzyme is one of the most important mechanisms of azole drug resistance in *Candida albicans*³²⁾. In yeasts, such as *C. albicans* and *Saccharomyces cerevisiae*, *ERG11* gene is single-copy. Different from that of the yeasts, *Aspergillus* has two distinct genes, *CYP51A* and *CYP51B*, encoding different Cyp51p³³⁾. Many studies have shown that point mutation in the *CYP51A* confers to triazole resistance in *A. fumigatus*. This has been well reviewed elsewhere^{6, 11)}. Knockout of the *CYP51A*

in triazole resistant *A. fumigatus* leads to decreasing susceptibility of the fungus to azoles³⁴⁾. The reported point mutations in the *CYP51A* that confer to azole resistance are listed in Table 1.

We have isolated a serial strain of itraconazole-resistant *A. fumigatus* from a patient with lung aspergilloma¹⁰⁾. Different point mutations in the *CYP51A* were detected in this series of *A. fumigatus*. Before itraconazole was administered to the patient, the first isolate (AF1) was obtained from his sputum. The MIC of itraconazole against AF1 was 0.25 $\mu\text{g/ml}$. Itraconazole 200 mg daily was then started and continued for 6 months. Hyphae were still found in sputum after 6 months of itraconazole treatment; the second *A. fumigatus* (AF2) was isolated at that time. The AF2, however, was resistant to itraconazole, with an MIC of $>16 \mu\text{g/ml}$. About 2 months after discontinuation of itraconazole, *A. fumigatus* AF3, with an MIC of 0.5 $\mu\text{g/ml}$ for itraconazole, was recovered from his sputum. After reconstitution of the itraconazole therapy, three isolates called AF4, AF5 and AF6 were recovered¹⁰⁾; all three isolates were resistant to itraconazole, with an MIC of $>16 \mu\text{g/ml}$. Compared to those of the itraconazole-susceptible isolates AF1 and AF3, methionine in 220 was substituted by isoleucine (M220I) in the Cyp51p of AF2. However, glycine in 54 was substituted by arginine (G54R) in the Cyp51p of AF4, AF5 and AF6¹⁰⁾. It is clear that 'reversible resistance' to itraconazole, as well as 'changeable mutations' in *CYP51A*, occurred in this serially isolated *A. fumigatus*. In general, resistance acquired by the mechanism of mutation is genetically stable. Hence it is possible that the patient was infected by multiple substrains, and the predominant substrain was changed according to itraconazole pressure.

Overexpression of the *CYP51A* results in fluconazole resistance in *C. albicans* and *S. cerevisiae*³²⁾. Although there is no evidence that the *CYP51A* overexpression confers resistance to triazoles in *A. fumigatus*, Oshero *et al.* showed that extra copies of *A. nidulans* *CYP51A* gene in *A. fumigatus* and *A. nidulans* protoplasts result

in high level resistance to itraconazole³⁵).

Recently, a new triazole resistance mechanism in *A. fumigatus* was found³⁶. The sequences of the *CYP51A* gene showed the presence of a point mutation at t364a in 14 clinical isolates of *A. fumigatus*, which leads to the substitution of leucine 98 for histidine (L98H), together with the presence of two copies of a 34-bp sequence in tandem in the promoter of the *CYP51A* gene. In order to analyze each of the *CYP51A* modifications individually, three PCR fragments of one triazole-resistant strain that included the promoter with the tandem repeat and part of *CYP51A* with the t364a mutation or PCR fragments with only one of the modifications were used to replace the *CYP51A* gene of a triazole-susceptible *A. fumigatus* wild-type strain. They found that only transformants which had incorporated the tandem repeat in the promoter of the *CYP51A* gene combined with the L98H amino acid substitution exhibited similarly reduced patterns of susceptibility to all triazole agents and similarly increased levels of *CYP51A* expression. The tandem repeat duplication by itself is not enough to confer the triazole cross-resistant phenotype. The introduction of the L98H amino acid substitution at Cyp51A alone seems to be responsible for only a slight increase in the *A. fumigatus* MICs for triazoles. These experiments confirmed that the combination of the two alterations was responsible for the cross-triazole-resistant phenotype³⁶).

ERG3 gene encoding the C-5 sterol desaturase enzyme is involved in ergosterol biosynthesis. Mutations or inactivation of the *ERG3* gene in *C. albicans* have been associated with azole and polyene cross resistance^{37, 38}. Loss of function of the *ERG3* gene in *C. dubliniensis* has been described as the primary mechanism of generated itraconazole resistance *in vitro*³⁹. There are three different *ERG3* genes (*ERG3A*, *ERG3B* and *ERG3C*) with relations in *A. fumigatus*^{11, 40}. Unlike in yeasts, susceptibility to azoles of the *A. fumigatus* strains in which *ERG3A* and *ERG3B* are knocked out alone or in combination⁴⁰ was not altered. Our studies have shown that compared to the wild type extra copies of *ERG3B* do not confer the altered susceptibility to both triazoles and amphotericin B⁴¹; and the targeted disruption of the *ERG3C* also does not affect the susceptibility of *A. fumigatus* to azoles⁴². These results would preclude any possibility of Erg3p being involved in the resistance mechanisms against antifungal drugs, at least until *ERG3A* *ERG3B* and *ERG3C* triple mutant is analyzed.

Reduced intracellular accumulation of the drug due to increased expression of efflux pumps^{10, 43, 44} has been evidenced in itraconazole-resistant *A. fumigatus* strains.

There are two categories of drug efflux involved in antifungal resistance: ATP-binding cassettes (ABC) and the major facilitator superfamily (MFS) class^{6, 32}. Genes encoding for efflux transporters are redundant in *A. fumigatus*; there are at least 49 ABC family transporters and 278 MFS genes⁶ in *A. fumigatus* predicted by using the genome. In contrast to the extensive number of genes encoding transporters, there are only a few reports concerning the relationship between overexpression of the transporter genes and triazole resistance in *A. fumigatus*. Therefore, further insight into the genes encoding these transporters is necessary. As we know, overexpression of 5 transporter genes, *AfuMDR1*, *AfuMDR2*, *AfuMDR3*, *AfuMDR4*, and *ATRF*^{18, 45, 46}, is related to triazole resistance in *A. fumigatus*.

Tobin *et al.* isolated two genes (*AfuMDR1* and *AfuMDR2*) in *A. fumigatus* and a *AfuMDR1* encoding proteins of the ABC superfamily⁴⁶. Expression of *AfuMDR1* in *S. cerevisiae* conferred increased resistance to the antifungal agent cilofungin (LY121019), an echinocandin B analog⁴⁶. Slaven *et al.* cloned a gene, *AtrF*, from *A. fumigatus*⁴⁵. Overexpression of this gene was associated with itraconazole resistance in a clinical isolate of *A. fumigatus*⁴⁵. Nascimento *et al.* identified two novel transporter genes, *AfuMDR3* and *AfuMDR4*¹⁸: *AfuMDR3* is a transporter gene that has high similarity to MFS; *AfuMDR4*, however, is a typical member of the ABC family⁴⁵. Itraconazole-resistant *A. fumigatus* showed either constitutive high-level expression of these transporter genes or induction of expression upon exposure to itraconazole^{10, 18, 47}.

Cross-resistance among azoles

Cross-resistance among azoles is possible. An *in vitro* study of 17 itraconazole-resistant *A. fumigatus* strains showed a significant degree of cross-resistance between structurally-similar triazoles itraconazole and posaconazole, but rarely between structurally-dissimilar triazoles itraconazole and voriconazole^{48, 49}. Similarly, in another surveillance that included 400 clinical and 150 environmental *A. fumigatus* isolates, there was no cross-resistance between itraconazole and voriconazole⁵⁰. Cross-resistance was also confirmed in an animal model⁵¹. After being infected with itraconazole-resistant *A. fumigatus*, the animals were treated with itraconazole or posaconazole; those receiving itraconazole died, while those treated with posaconazole had improved survival⁵¹. However, it should be noted that multiple-azole resistant clinical isolates have been reported recently²⁸⁻³⁰. *CYP51A* gene mutation combined with the alteration of the *CYP51A* promoter as

described above can explain the multiple-azole resistance^{28, 29, 36}.

Pre-use of fluconazole increases minimal fungicidal concentrations (MFC) of other azole drugs. Ten isolates of *A. fumigatus* were cultivated for 4 passages with 8 $\mu\text{g/ml}$ or 64 $\mu\text{g/ml}$ fluconazole, then MIC/MFC was measured in itraconazole, voriconazole and amphotericin B. Itraconazole MFC increased 4-fold in 9 isolates, independent of fluconazole concentration to which it had been pre-exposed; MICs increase insignificantly. Voriconazole MFCs increased 4-fold in 8 isolates and there were no significant changes in MICs. Amphotericin B had no statistically significant increase in MIC/MFCs in all isolates⁵².

Polyene resistance

Amphotericin B binds to the plasma membrane, which is organized by ergosterol, to form a channel through which potassium ion leaks out from fungal cell, resulting in a disruption of the proton gradient⁵³. Amphotericin B can also cause oxidative damage to plasma membranes⁵⁴.

Resistance to polyenes is extremely rare in *Aspergillus* spp., except among *A. terreus* isolates⁵³. The known mechanisms of polyene-resistance mostly come from studies on *S. cerevisiae* and pathogenic yeasts, including *Cryptococcus neoformans* and *C. albicans*. As described above, mutations in the *ERG3* gene of yeasts cause deficiency of 5, 6 isomerase function to result in ergosterol depletion. This results in cross resistance between polyenes and azoles^{37, 55}. Deletion of *ERG3A*, *ERG3B* alone or both genes, however, does not affect the susceptibility of *A. fumigatus* to amphotericin B. Nor do extra copies of *A. fumigatus ERG3B* affect the susceptibility of *A. fumigatus* to amphotericin B⁴¹. Recently, Vandeputte *et al.* found a novel polyene-resistant mechanism: mutation in the *ERG6* gene leads to polyene resistance in a clinical isolate of *C. glabrata*⁵⁶. However, we have found that *A. fumigatus* transformants harboring extra copies of *A. fumigatus ERG6* have similar susceptibility to amphotericin B and the wild type strain⁵⁷. These results indicated that *ERG3B* and *ERG6* might not be involved in polyene resistance in *A. fumigatus*.

Most *A. terreus* isolates are resistant to amphotericin B *in vitro*, consistent with the poor outcome of this drug (72% mortality) *in vivo*⁵⁸. The mechanisms of the intrinsic resistance of *A. terreus* to amphotericin B are not clear, and it has been postulated that much less ergosterol content in the cell membrane of *A. terreus* partially accounts for the poor activity of amphotericin B against this fungus^{6, 53}.

Because the antifungal activity of amphotericin B is partially attributable to its action as an oxidizing agent, resistance of *A. fumigatus* to this drug might be induced through increased production of neutralizing enzymes that confer resistance to oxidative stress⁶. It has been demonstrated that amphotericin B-resistant *C. albicans* strains that overproduce catalase and superoxide dismutase were less sensitive to oxidative agents than amphotericin B-sensitive *C. albicans*⁶. So the relation between overexpression of the neutralizing enzymes and polyene resistance in *Aspergillus* should be established.

Resistance to terbinafine

Extra copies of *A. fumigatus ERG1* gene, the target gene of terbinafine, could result in resistance to terbinafine in *A. fumigatus* transformants⁵⁹. Terbinafine is not approved for the treatment of invasive aspergillosis, so this phenomenon is of little clinical importance.

Resistance to echinocandins

Until last year there were no reports of clinical failure of IA due to echinocandin resistance to *Aspergillus*. A caspofungin-resistant *A. flavus* from a patient after heart transplantation and mechanical circulatory support was reported recently⁶⁰. The MIC of the isolate to caspofungin was determined with Etest; however, cross resistance among echinocandins as well as the sequence of the *FKS1* gene encoding the target for echinocandins were not reported in this paper⁶⁰. Perlin's group found that modification of Fks1p is sufficient to lead to echinocandin resistance in a laboratory strain of *A. fumigatus*⁶¹.

Conclusions and future perspectives

Compared to *C. albicans*, available data for the antifungal resistance in *Aspergillus* is limited. Clinicians' concern is the definition of the category (susceptible, dose-dependent susceptible or resistant) for an isolate. Although the CLSI has published a standardized method for *in vitro* antifungal susceptibility testing of filamentous fungi, it is necessary to establish the breakpoint for the antifungals in the future. Certain resistant mechanisms of azoles and polyenes have been defined for *A. fumigatus*, however, further understanding is strongly needed in *A. fumigatus* and non-*fumigatus Aspergillus*. There is some evidence regarding the correlation between overexpression of the transporter genes and triazole resistance in *A. fumigatus*, but the exact role of these genes in antifungal resistance needs further investigation. Knowledge of

drug-resistance mechanisms should maximize the utility of current drugs, and assist in the development of new antifungal agents and new treatment strategies. The *Aspergillus* genome, both the finished and that in process, may facilitate understanding of the resistance mechanisms.

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