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MINIREVIEW

The molecular mechanism of azole resistance in *Aspergillus fumigatus*: from bedside to bench and back

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The growing use of immunosuppressive therapies has resulted in a dramatic increased incidence of invasive fungal infections (IFIs) caused by *Aspergillus fumigatus*, a common pathogen, and is also associated with a high mortality rate. Azoles are the primary guideline-recommended therapy agents for first-line treatment and prevention of IFIs. However, increased azole usage in medicinal and agricultural settings has caused azole-resistant isolates to repeatedly emerge in the environment, resulting in a significant threat to human health. In this review, we present and summarize current research on the resistance mechanisms of azoles in *A. fumigatus* as well as efficient susceptibility testing methods. Moreover, we analyze and discuss the putative clinical (bedside) indication of these findings from bench work.

Keywords: azole resistance, pathogenic fungi, *Aspergillus fumigatus*, antifungal susceptibility testing

Introduction

In recent years, the prevalence of invasive fungal infections (IFIs) that are caused by *Aspergillus fumigatus* and associated with a high mortality rate has increased dramatically, due primarily to the increased use of immunosuppressive therapies (Richardson, 2005; Shao *et al.*, 2007; Mayr and Lass-Florl, 2011). There are currently three classes of antifungal agents, azoles, polyenes and echinocandins, each with a different target, that are used in the clinical treatment of IFIs (Walsh *et al.*, 2008; Galimberti *et al.*, 2012). To date, azoles are the primary guideline-recommended therapy agents for first-line treatment and prevention of IFIs because azoles have fewer side effects for the host than the polyene amphotericin and a broader antifungal spectrum than echinocandins (Traunmuller *et al.*, 2011; Becher and Wirsel, 2012). Imidazole-based chlormidazole became widely available in the clinic in 1958. Since then, a variety of imidazoles (e.g., clotrimazole, miconazole, and ketoconazole) and triazoles (e.g., fluconazole, itraconazole, voriconazole, and posaconazole) have been successively developed for antifungal therapy (Shao *et al.*, 2007; Parker *et al.*, 2014). In addition to their use in medicine, azoles are increasingly used in agricultural settings, resulting in the emergence of azole-resistant isolates. Since 1997, when the first azole-resistant isolate of *A. fumigatus* was found, an increasing number of resistant isolates have appeared in the Netherlands, the UK, France, Spain, and China, and other countries (Denning *et al.*, 1997; Mayr and Lass-Florl, 2011; Chowdhary *et al.*, 2012; Kikuchi *et al.*, 2014; Wang *et al.*, 2014). Moreover, the azole-resistant isolates are likely leading to therapy failure, with the mortality rate for immunosuppressed patients infected by azole-resistant isolates reaching 88% in some studies (Howard *et al.*, 2009; Denning and Perlin, 2011; van der Linden *et al.*, 2011). Thus, this phenomenon poses a serious threat to human health.

A number of studies have provided convincing evidence that azoles work as antifungals by inhibiting ergosterol biosynthesis through interference with the activity of 14-α-lanosterol demethylase, which is encoded by *cyp51A* and *cyp51B* (Ferreira *et al.*, 2005; Warrilow *et al.*, 2010; Snelders *et al.*, 2011). To date, most clinical *A. fumigatus* azole-resistant isolates are associated with *cyp51A* mutations. To explain why multiple missense mutations in *cyp51A* are linked to azole resistance, it is postulated that *cyp51A* contributes the major sterol 14-alpha-demethylase activity required for growth. However, multiple mechanisms of resistance with differing degrees of azole cross-resistance have been identified, and clinicians and microbiologists have devoted themselves to elucidating the molecular mechanisms underlying azole resistance in *A. fumigatus* (Chowdhary *et al.*, 2014; Cowen *et al.*, 2014). In this review, we present a progress report on the development of efficient susceptibility testing methods and summarize identified azole resistance mechanisms in *A. fumigatus*. Moreover, we discuss the significant clinical (bedside) indications of these findings from bench work.

Antifungal susceptibility testing of azole agents to *A. fumigatus*

To identify both intrinsic and emergent antifungal drug resistance encountered in invasive fungal infections and to improve clinical efficacy of treatment, it is necessary to establish accurate and predictive susceptibility testing of fungal

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pathogens *in vitro*. Various testing methods have been proposed, including broth-based assays, disk-based assays, commercial kits utilizing macrodilution and microdilution, agar diffusion, disk diffusion and Etest (Espinel-Ingroff *et al.*, 2007; Lass-Florl *et al.*, 2010). Two standard methods, M38-A2 and E.DEF 9.1, have been proposed by the Clinical Laboratory Standards Institute (CLSI) and the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (EUCAST-AFST), respectively, specifically for susceptibility testing of *Aspergillus* spp.. These two broth-based microdilution methods are highly reproducible; even though they differ in inoculum amount, inoculum method and tested media (Table 1), the susceptibility results obtained by these methods are consistent (Chryssanthou and Cuenca-Estrella, 2006; Lass-Flörl *et al.*, 2006, 2010; Fothergill, 2012). To establish a gold standard for detecting resistant clinical isolates, EUCAST and CLSI have noted clinical breakpoints and epidemiological cut-off values (ECVs), which determine whether detected strains are azole resistant or susceptible (Table 2) (Pfaller *et al.*, 2011; Arendrup *et al.*, 2012). Clinical breakpoints are established based on the standardized approach, pharmacokinetic and pharmacodynamics (PK/PD) characteristics and animal and clinic trials (Espinel-Ingroff *et al.*, 2010). In addition, ECVs, which indicate the drug concentration at which 95% of the detected fungal cells are killed, are used to distinguish wild-type strains from strains with mutational or acquired resistance (Posteraro and Sanguinetti, 2014; Seyedmousavi *et al.*, 2014). However, the proposed standard methods are complex, which makes the detection procedure time-consuming and difficult to use routinely (Posteraro *et al.*, 2014).

Compared with the above standard methods, some of the commercially available protocols are more convenient and timesaving. Among them, the agar-based Etest and the broth-based Sensititre YeastOne (SYO) are currently the two most popular commercial methods for antifungal susceptibility testing of the *Aspergilli* (Posteraro *et al.*, 2014). The advantages of Etest are its speed and simplicity; the Minimal Inhibitory Concentration (MIC) can be directly determined from the position where the elliptical inhibition zone intersects the drug strip. In addition, the Etest is easily performed, requiring only the placement of gradient strips infused with different drugs in RPMI agar plates followed by incubation at 35°C for 48 h (Pfaller *et al.*, 2000; Espinel-Ingroff and Rezusta, 2002; Messer *et al.*, 2007). In comparison, the SYO method uses a commercially prepared panel comprising serial two-fold dilutions of drugs and the Alamar Blue indicator for endpoints (Castro *et al.*, 2004). The susceptibility of *A. fumigatus* to itraconazole and voriconazole as determined by SYO is in close agreement with the results derived from CLSI methods (Guinea *et al.*, 2006). Another novel approach for the *Aspergilli* susceptibility testing is isothermal microcalorimetry (IMC). Unlike conventional methods, IMC is performed in real time by measuring the heat production from growth and metabolism. In this method, minimal heat inhibitory concentration (MHIC), defined as the lowest drug concentration that results in a 50% reduction of the total heat produced by the growth control at 48 h (except for echinocandins), is the susceptibility endpoint of IMC (Furustrand Tafin *et al.*, 2012). However, this method for real-time antifungal susceptibility testing is still in preliminary form and requires further experimentation to improve and standardize the protocol.

Table 1. Cultural conditions for antifungal susceptibility testing in *A. fumigatus*

Characteristic	CLSI M38-A2	EUCAST-E. DEF 9.1
Test medium	RPMI 1640	RPMI 2% G
96 well microdilution plates	U-shaped wells	flat-bottom wells
inoculum	$0.4–5 \times 10^4$ CFU/ml	$2–5 \times 10^5$ CFU/ml
Inoculum standardisation	spectrophotometrically	haemocytometer
Temperature	35°C	35 ± 2°C
Incubation time	48 h	48 h
MIC endpoint	No growth	No growth

Detection of molecular resistance to azole agents

Compared to our understanding of resistance in the yeast-type pathogen *Candida albicans*, our knowledge of the molecular mechanisms underlying azole resistance in *A. fumigatus* is relatively limited and superficial. Based on published and clinical reports, the molecular mechanisms causing azole resistance can be divided into the following three general categories: (i) alteration or overexpression of the drug target, which decreases the affinity of the drug for its target or leads to an increase in the quantity of drug necessary to inhibit fungal growth, (ii) activation of the drug efflux pump, resulting in reduced accumulation of the drug within the fungal cell, and (iii) modifications of the cellular stress-response pathway to counterbalance the drug effect (Shapiro *et al.*, 2011; Liu *et al.*, 2014). We will summarize the resistance mechanisms from the three different classes that have been identified in *A. fumigatus* separately.

Azole targeted Cyp51-mediated azole resistance

Over the last few decades, the most common resistance mechanism observed in *A. fumigatus* has been mutation of the azole target Cyp51, which encodes lanosterol demethylase (Chowdhary *et al.*, 2014). Cyp51 consists of two paralogous proteins, named Cyp51A and Cyp51B. Either *cyp51A* or *cyp51B* can be knocked out individually, but a double deletion is lethal. Clinical findings suggest that *cyp51A* plays the dominant role in the regulation of the 14α-demethylase activity, while *cyp51B* either is a redundant gene that only functions in the absence of *cyp51A* or has other unknown functions under particular conditions (Mellado *et al.*, 2005; Hu *et al.*, 2007).

Most azole-resistant isolates of *A. fumigatus* identified in previous studies harbor mutations only in *cyp51A* (Lelievre *et al.*, 2013). Point mutations in *cyp51A* that alter drug-enzyme interaction or cause overexpression of *cyp51A* have

Table 2. EUCAST breakpoints and CLSI ECVs

Antifungal agent	MIC breakpoint		ECVs	
	Susceptible ≤	Resistant >	Wild-type ≤	Non-wild-type ≥
Itraconazole	1	2	1	2
Voriconazole	1	2	1	2
Posaconazole	0.12	0.25	0.25	0.5

Table 3. Mutations related to azole resistance in the *cyp51A* gene of *A. fumigatus*

Genotype	Amino acid substitution	MIC (μg/ml)		
		Itraconazole	Voriconazole	Posaconazole
Hotspot				
G54	E	>16.0	0.25	1
	R	>16.0	0.12	0.5
	W	>16.0	0.25	4
G138	C	>16.0	8	>16.0
	R	2	16	0.25
M220	I	>16.0	1	0.5
	K	>16.0	2	>16.0
	R	>16.0	2	2
	V	>16.0	1	0.5
G448	S	0.5	8	0.25
Other mutations				
N22D, S52T, Q88H, V101F, N125I, Q141H, H147Y, P216L, F219C, M236K/T/V, A284T, S297T, P394L, Y431C, G434C, T440A, Y491H, F495I, TR34/L98H, TR46/Y121F/T289A				

been associated with azole drug resistance in clinical isolates. Lanosterol demethylase is one of a large class of monooxygenases called P450 proteins because of the spectral absorbance of a cysteine-linked heme molecule found in their active site (Chen *et al.*, 2014; Parker *et al.*, 2014). Cyp51A has two ligand channels through which azoles and the lanosterol substrate enter the active site of the protein. Mutations in Cyp51A may directly restrict the docking of the drug, reduce affinity for the target by altering the structure of the opening in each channel or change the position of the heme molecule. The most commonly reported sites of mutation in *cyp51A*, called hot spots, are G54, G138, M220, and G448, as shown in Table 3 (Diaz-Guerra *et al.*, 2003; Verweij *et al.*, 2009; Snelders *et al.*, 2010). Based on information from models of *Mycobacterium tuberculosis* and human Cyp51A orthologs, the *A. fumigatus* Cyp51A protein structure has been deduced. In models of the *A. fumigatus* Cyp51A protein, the hot spots are positioned near the opening of the ligand access channels or close to the active site (Xiao *et al.*, 2004; Fraczek *et al.*, 2011). To a certain extent, the locations of the hot spots explain the drug resistance conferred to *A. fumigatus* by mutations occurring at these sites. The identity of the substituted amino acid also plays an important role in azole resistance. For instance, valine, lysine, isoleucine, and arginine substitutions at M220 cause azole resistances of varying strengths (Mellado *et al.*, 2004; Becher and Wirsel, 2012). To date, more than 30 different Cyp51A point mutations conferring azole resistance in *A. fumigatus* have been identified (Table 3) (Bueid *et al.*, 2010; reviewed in Becher and Wirsel, 2012; Bader *et al.*, 2013).

Another major mechanism of azole resistance is the combination of a mutation in the *cyp51A* gene with a tandem repeat in the *cyp51A* promoter, which acts as a transcriptional enhancer. Although TR34/L98H was first reported in the Netherlands in 1998, the mutant is now frequently identified in many countries (Chowdhary *et al.*, 2012; Rath *et al.*, 2012; Prigitano *et al.*, 2014). These mutations cause high-level resistance to itraconazole and intermediate-level susceptibility or resistance to voriconazole and/or posaconazole (Chowdhary *et al.*, 2014; Prigitano *et al.*, 2014). The appearance of this combined mutation resistance mechanism may be due to the widespread usage of demethylase inhibitors (DMIs) in agriculture (Snelders *et al.*, 2012; Cuenca-Estrella, 2014). A similar resistant isolate, the TR46/Y121F/T289A mutation in *cyp51A*, has been detected in recent years (Vermeulen *et al.*, 2013). The TR46/Y121F/T289A mutation contributes to high-level resistance to voriconazole (van der Linden *et al.*, 2013; van Ingen *et al.*, 2014). The increase in *cyp51A* transcript levels observed in these two mutants may underlie their azole resistance phenotypes (Mellado *et al.*, 2007; Spiess *et al.*, 2014).

Transcriptional regulation of *cyp51A* may be mediated by transcription factors in addition to tandem repeats in the promoter. SrbA, a transcriptional regulator in the sterol regulator element binding protein (SREBP) family, is crucial for growth during hypoxia, sterol biosynthesis, cell polarity, hyphal morphogenesis and virulence in *A. fumigatus* (Blatzer *et al.*, 2011). A SrbA null mutation was reported to increase susceptibility to fluconazole and voriconazole. A plausible explanation for this phenotype is that SrbA binds Cyp51A and possibly regulates its activity (Willger *et al.*, 2008; Blosser and Cramer, 2011). Camps *et al.* (2012) showed that a mutation in the CCAAT-binding transcription factor complex subunit HapE is also capable of inducing an azole-resistance phenotype. The single amino acid-P88, which located at the fourth exon of HapE, belongs to a conserved core domain, and is very important for binding the regulatory CCAAT element. Coincidentally, a CCAAT-box is present in the promoter of *cyp51A* whilst the *hapE* mutant indeed results in over-expression of *cyp51A* mRNA, indicating transcription factor may regulate *cyp51A* expression in bench studies (Camps *et al.*, 2012). However, clinical identifications of similar mutations in SrbA and HapE, have not been reported. Thus, the relationship between transcription factors and clinical azole resistance phenotypes requires further study.

As mentioned above, Cyp51B can replace Cyp51A to some extent. Sequence analysis indicates that *cyp51B* has 63% identity to *cyp51A*, and azoles bind more tightly to Cyp51B than Cyp51A. Bueid *et al.* (2013) found that, out of 12 clinical azole-resistant isolates without mutations in Cyp51A, one isolate has 18.7-fold higher expression of *cyp51B* than is observed in wild-type strain AF293 after exposure to 1 mg/L itraconazole, and another has higher basal expression of *cyp51B*. However, data on the function of *cyp51B* in azole resistance are limited.

Efflux pump-mediated azole resistance

Many azole-resistant isolates of *A. fumigatus* have wild-type Cyp51A and Cyp51B sequences, indicating there are mechanisms of azole resistance other than those described above (Bueid *et al.*, 2010; Vermeulen *et al.*, 2013). Another well-known azole resistance mechanism is overexpression of efflux pumps, which can reduce the accumulation of drugs in a cell by pumping the drugs out of the cell (Lamping *et al.*, 2010; Morschhauser, 2010).

ATP-binding cassette (ABC) transporters and members of the major facilitator superfamily (MFS) constitute the two main types of efflux pumps. ABC transporters hydrolyze ATP for energy, and the core structure of ABC transporters

is composed of two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs). MFS pumps use proton gradients across the cell membrane to drive the pump and have multiple TMDs (Coleman and Mylonakis, 2009). Compared with MFS pumps, ABC transporters have broader substrate specificity and play more significant roles in clinical studies (Cannon *et al.*, 2009). The most extensive research on ABC transporters focuses on the yeast *Saccharomyces cerevisiae* model (Morschhauser, 2010). Based on bioinformatic conserved domains analysis, there are 278 different MFS pumps and 49 ABC transporters in *A. fumigatus* (Rajendran *et al.*, 2011). The first ABC transporter to be associated with drug resistance in *A. fumigatus* was AtrF, which is significantly up-regulated when *A. fumigatus* is grown in the presence of itraconazole in the clinical resistant isolate AF72 (Slaven *et al.*, 2002). To search for homologs of AtrF in *A. fumigatus*, we used the AtrF protein from AF293 as the query in a NCBI BLASTp search (http://www.ncbi.nlm.nih.gov/BLAST/) against the AF293 genome. We obtained 28 protein homolog candidates with an e-value less than 10^{-5} (Table 4). In addition, mutants with up-regulation of the ABC transporter gene *mdr4* show hyper-resistance to itraconazole (Nascimento *et al.*, 2003). Several studies indicate that isolates having high basal expression of the ABC transporter *abcB* (renamed *cdr1B*) display stronger azole resistance than do wild-type controls. Moreover, the *abcB* knockout strain consistently displays low azole tolerance (Fraczek *et al.*, 2013; Paul *et al.*, 2013).

The first efflux pump to be identified as a multidrug MFS transporter in pathogenic fungus was CaMdr1 (Sun *et al.*, 2013; Costa *et al.*, 2014). Studies have shown that over-expression of *mdr1* is tightly correlated with clinical azole resistance in *C. albicans* (Prasad and Rawal, 2014). *Mdr3*, a MFS transporter gene, is constitutively overexpressed in itraconazole-resistant *A. fumigatus* isolates even in the absence of stimulation by drugs (Nascimento *et al.*, 2003). In conclusion, a number of *in vitro* studies have shown that over-expression of efflux pumps is tightly linked to drug resistance. However, our understanding of efflux pump-mediated azole resistance comes solely from bench work; the role of efflux pumps in clinical cases of *A. fumigatus* azole resistance is not yet clear.

Stress adaptation-mediated azole resistance

Many lines of evidence have shown that fungi are able to develop drug resistance by modifying intracellular signaling pathways involved in stress response (Cowen, 2008, 2009; Epp *et al.*, 2010). Several stress responses capable of contributing to elevated azole resistance have been reported, including unfolded protein response (UPR), oxidative stress response, and membrane and cell wall stress responses in *A. fumigatus*. UPR is one of the cellular response pathways that protect cells from the burden of unfolded proteins on the endoplasmic reticulum (ER) (Krishnan and Askew, 2014).

Table 4. The predicted AtrF analogs in *A. fumigatus* AF293

Number	Description	Length (aa)	E value	Identity	Accession
1	ABC drug exporter AtrF	1547	0.0	100%	XP_747642.1
2	ABC multidrug transporter	1453	0.0	44%	XP_746942.1
3	ABC transporter	1526	0.0	41%	XP_750693.1
4	ABC transporter	1485	0.0	40%	XP_753693.1
5	ABC multidrug transporter	1424	0.0	40%	XP_748461.2
6	ABC multidrug transporter	1472	0.0	38%	XP_754878.1
7	ABC multidrug transporter	1349	0.0	41%	XP_746352.2
8	ABC multidrug transporter	1499	0.0	39%	XP_755847.1
9	ABC transporter	1497	0.0	38%	XP_752803.1
10	ABC multidrug transporter	1469	0.0	37%	XP_748116.1
11	ABC drug exporter AbcA	1452	0.0	37%	XP_753111.1
12	ABC multidrug transporter	1471	0.0	38%	XP_748261.1
13	ABC efflux transporter	1299	6e-72	24%	XP_754651.1
14	ABC transporter	628	9e-48	29%	XP_755735.2
15	ABC transporter (Adp1)	1088	2e-44	40%	XP_750621.1
16	ABC multidrug transporter	1297	8e-09	29%	XP_751419.1
17	ABC transporter	1604	2e-08	28%	XP_753691.1
18	ABC multidrug transporter	1285	7e-08	27%	XP_747730.1
19	ABC multidrug transporter Mdr1	1349	7e-08	31%	XP_754025.1
20	ABC transporter	1493	2e-07	23%	XP_753593.1
21	ABC multidrug transporter SitT	1315	2e-07	29%	XP_748663.1
22	ABC multidrug transporter	1314	7e-07	24%	XP_748687.1
23	ABC metal ion transporter	1540	1e-06	26%	XP_753839.1
24	ABC multidrug transporter Mdr4	1330	1e-06	28%	XP_752639.1
25	RNase L inhibitor of the ABC superfamily	703	1e-06	24%	XP_752397.1
26	ABC multidrug transporter	1287	2e-06	26%	XP_747768.1
27	ABC iron exporter Atm1	727	3e-06	25%	XP_751165.1
28	Vacuolar ABC heavy metal transporter (Hmt1)	873	4e-06	25%	XP_749444.1

Studies have shown that the E3-ubiquitin ligase Hrd1 contributes to ER-associated degradation (ERAD) in yeast (Carvalho *et al.*, 2006; Horn *et al.*, 2009). Deletion of *hrdA*, the gene encoding the *A. fumigatus* ortholog of Hrd1, stimulates UPR and results in slightly higher resistance to the azole antifungal voriconazole (Krishnan *et al.*, 2013). Similarly, Yap1 is involved in oxidative stress response in *A. fumigatus,* and mutants that harbor multiple-copy truncated Yap1 protein lacking the c-CRD domain show higher resistance to voriconazole than controls (Qiao *et al.*, 2010). The heat shock protein 90 (Hsp90) is a highly conserved molecular chaperone protein in eukaryotes that orchestrates stress response *via* interacting with numerous client proteins (Lamoth *et al.*, 2012; Cowen, 2013; Lamoth *et al.*, 2014b). As a client protein of Hsp90, calcineurin, a central component of the cellular calcium-signaling pathway, is essential for surviving the membrane stress induced by azole antifungals (Steinbach *et al.*, 2007). Calcineurin inhibitors reduce azole resistance, and the combination of the Hsp90 inhibitor geldanamycin and the calcineurin inhibitor FK506 has higher antifungal efficacy against azole-resistant isolates than geldanamycin alone (Lamoth *et al.*, 2013). Moreover, another study indicated that the Hsp90 K27 residue plays an important role in voriconazole resistance, such that either deletion of K27 or acetylationmimetic mutation (K27A) can confer resistance to voriconazole in *A. fumigatus* (Lamoth *et al.*, 2014a). Likewise, mutants lacking Mkk2, a cell wall integrity (CWI) signaling kinase involved in the cell wall stress response, show increased susceptibility to posaconazole and voriconazole (Dirr *et al.*, 2010). Altogether, these findings support the existence of a less common azole resistance mechanism mediated by stress adaptation, and suggest that intracellular signaling pathways and their regulators may be used as anti-fungal target candidates in the near future.

Although all of the drug resistances discussed above are stable, transient resistance in fungi is also observed, and it has been gradually garnering attention, especially in recent years. Unstable drug resistance exists in some isolates, in which drug resistance reverses when the isolates are grown on drug-free media. Reversible changes in phenotype result from epigenetic modification, which can regulate gene expression but not directly change DNA sequences. Epigenetic modification, including DNA methylation and RNA interference (RNAi), has been studied in fungi, plants, and animals, particularly with respect to its role in human diseases (Chung and Sidhu, 2008; Gu *et al.*, 2014; Smialowska *et al.*, 2014). The rearrangement induced premeiotically (RIP) epigenetic mutation was first found in *Neurospora crassa* in 1986; subsequently, scientists launched extensive studies on epigenetics in fungi (Montiel *et al.*, 2006). Recently, Calo *et al.* (2014) found that FK506-resistant isolates could be transformed to FK506-sensitivity when growth and reproduction occurred without drug-selective pressure. With genetic and biochemical techniques and drug susceptibility analysis, they demonstrated that these mutants conferred drug resistance *via* an epigenetic RNA interference (RNAi)-mediated mechanism, referred to as epimutation or epigenetic resistance. The existence of epimutants may answer long-standing questions about why drug resistance is not stable and changes under different environmental conditions. Moreover, gaining insight into RNAi-mediated transient resistance mechanisms may suggest new ways to fight clinical drug resistance in fungi.

Conclusion

Various antifungal susceptibility-testing methods have been promoted by a number of governments and commercial companies. Every method has advantages and limitations,

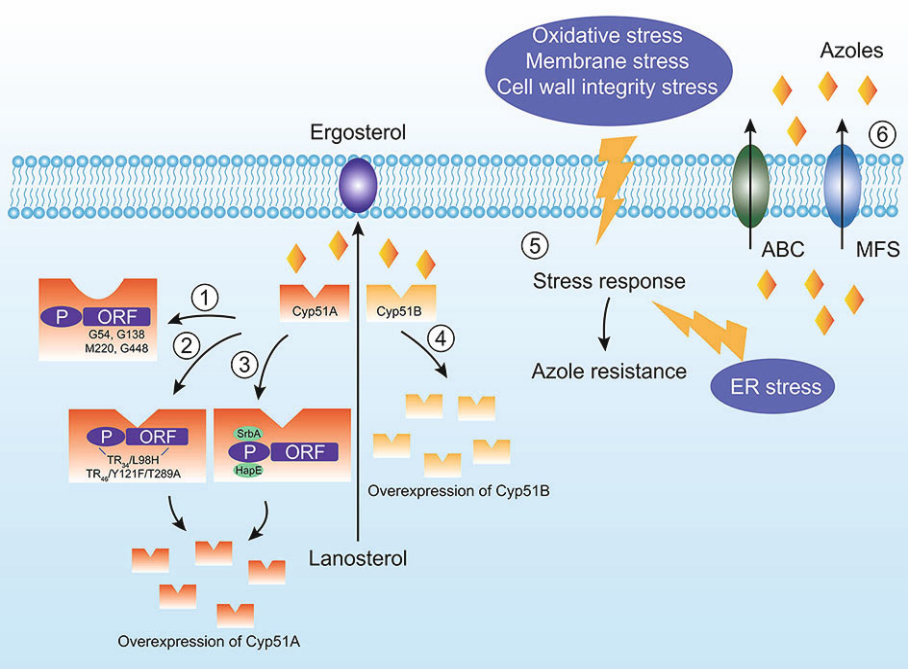

Fig. 1. Azole resistance pathways currently identified in *A. fumigatus*. (1) point-mutations in *cyp51A*, (2) site-mutated *cyp51A* gene plus a tandem repeat in *cyp51A* promoter, (3) overexpression of Cyp51A through transcription factors, (4) overexpression of Cyp51B, (5) stress adaptation, (6) up-regulation of efflux transporters.

suggesting microbiologists and clinicians need to select an appropriate method based on individual requirements and conditions.

Molecular mechanisms of resistance to azole agents in *A. fumigatus* have been summarized and divided into three categories: azole-targeted Cyp51-based mutants, efflux pump-based resistance and stress adaptation-based mechanisms (Fig. 1). Most recent findings have focused on the transient resistance caused by epigenetic modification-referred to as epimutation or epigenetic resistance-such as DNA methylation and RNAi. Gaining insight into the molecular mechanisms underlying azole resistance at the bench could lead to efficient indications for addressing fungal drug resistances at the bedside.

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References

Arendrup, M.C., Cuenca-Estrella, M., Lass-Florl, C., Hope, W.W., and European Committee on Antimicrobial Susceptibility Testing Subcommittee on Antifungal Susceptibility, T. 2012. EUCAST technical note on Aspergillus and amphotericin B, itraconazole, and posaconazole. *Clin. Microbiol. Infect.* **18**, E248–250.

Bader, O., Weig, M., Reichard, U., Lugert, R., Kuhns, M., Christner, M., Held, J., Peter, S., Schumacher, U., Buchheidt, D., *et al.* 2013. cyp51A-Based mechanisms of *Aspergillus fumigatus* azole drug resistance present in clinical samples from Germany. *Antimicrob. Agents Chemother.* **57**, 3513–3517.

Becher, R. and Wirsel, S.G. 2012. Fungal cytochrome P450 sterol 14alpha-demethylase (Cyp51) and azole resistance in plant and human pathogens. *Appl. Microbiol. Biotechnol.* **95**, 825–840.

Blatzer, M., Barker, B.M., Willger, S.D., Beckmann, N., Blosser, S.J., Cornish, E.J., Mazurie, A., Grahl, N., Haas, H., and Cramer, R.A. 2011. SREBP coordinates iron and ergosterol homeostasis to mediate triazole drug and hypoxia responses in the human fungal pathogen *Aspergillus fumigatus*. *PLoS Genet.* **7**, e1002374.

Blosser, S.J. and Cramer, R.A. 2011. SREBP-dependent triazole susceptibility in *Aspergillus fumigatus* is mediated through direct transcriptional regulation of *erg11A* (*cyp51A*). *Antimicrob. Agents Chemother.* **56**, 248–257.

Bueid, A., Howard, S.J., Moore, C.B., Richardson, M.D., Harrison, E., Bowyer, P., and Denning, D.W. 2010. Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. *J. Antimicrob. Chemother.* **65**, 2116–2118.

Buied, A., Moore, C.B., Denning, D.W., and Bowyer, P. 2013. High-level expression of cyp51B in azole-resistant clinical *Aspergillus fumigatus* isolates. *J. Antimicrob. Chemother.* **68**, 512–514.

Calo, S., Shertz-Wall, C., Lee, S.C., Bastidas, R.J., Nicolas, F.E., Granek, J.A., Mieczkowski, P., Torres-Martinez, S., Ruiz-Vazquez, R.M., Cardenas, M.E., *et al.* 2014. Antifungal drug resistance evoked via RNAi-dependent epimutations. *Nature* **513**, 555–558.

Camps, S.M., Dutilh, B.E., Arendrup, M.C., Rijs, A.J., Snelders, E., Huynen, M.A., Verweij, P.E., and Melchers, W.J. 2012. Discovery of a HapE mutation that causes azole resistance in *Aspergillus fumigatus* through whole genome sequencing and sexual crossing. *PLoS One* **7**, e50034.

Cannon, R.D., Lamping, E., Holmes, A.R., Niimi, K., Baret, P.V., Keniya, M.V., Tanabe, K., Niimi, M., Goffeau, A., and Monk, B.C. 2009. Efflux-mediated antifungal drug resistance. *Clin. Microbiol. Rev.* **22**, 291–321, Table of Contents.

Carvalho, P., Goder, V., and Rapoport, T.A. 2006. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* **126**, 361–373.

Castro, C., Serrano, M.C., Flores, B., Espinel-Ingroff, A., and Martin-Mazuelos, E. 2004. Comparison of the sensititre yeastone colorimetric antifungal panel with a modified NCCLS M38-A method to determine the activity of voriconazole against clinical isolates of *Aspergillus* spp. *J. Clin. Microbiol.* **42**, 4358–4360.

Chen, W., Lee, M.K., Jefcoate, C., Kim, S.C., Chen, F., and Yu, J.H. 2014. Fungal cytochrome p450 monooxygenases: their distribution, structure, functions, family expansion, and evolutionary origin. *Genome Biol. Evol.* **6**, 1620–1634.

Chowdhary, A., Kathuria, S., Xu, J., Sharma, C., Sundar, G., Singh, P.K., Gaur, S.N., Hagen, F., Klaassen, C.H., and Meis, J.F. 2012. Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR(3)(4)/L98H mutations in the cyp51A gene in India. *PLoS One* **7**, e52871.

Chowdhary, A., Sharma, C., Hagen, F., and Meis, J.F. 2014. Exploring azole antifungal drug resistance in *Aspergillus fumigatus* with special reference to resistance mechanisms. *Future Microbiol.* **9**, 697–711.

Chryssanthou, E. and Cuenca-Estrella, M. 2006. Comparison of the EUCAST-AFST broth dilution method with the CLSI reference broth dilution method (M38-A) for susceptibility testing of posaconazole and voriconazole against *Aspergillus* spp. *Clin. Microbiol. Infect.* **12**, 901–904.

Chung, H. and Sidhu, K.S. 2008. Epigenetic modifications of embryonic stem cells: current trends and relevance in developing regenerative medicine. *Stem Cells Cloning* **1**, 11–21.

Coleman, J.J. and Mylonakis, E. 2009. Efflux in fungi: la piece de resistance. *PLoS Pathog.* **5**, e1000486.

Costa, C., Dias, P.J., Sa-Correia, I., and Teixeira, M.C. 2014. MFS multidrug transporters in pathogenic fungi: do they have real clinical impact? *Front. Physiol.* **5**, 197.

Cowen, L.E. 2008. The evolution of fungal drug resistance: modulating the trajectory from genotype to phenotype. *Nature Rev. Microbiol.* **6**, 187–198.

Cowen, L.E. 2009. Hsp90 orchestrates stress response signaling governing fungal drug resistance. *PLoS Pathog.* **5**, e1000471.

Cowen, L.E. 2013. The fungal Achilles' heel: targeting Hsp90 to cripple fungal pathogens. *Curr. Opin. Microbiol.* **16**, 377–384.

Cowen, L.E., Sanglard, D., Howard, S.J., Rogers, P.D., and Perlin, D.S. 2014. Mechanisms of antifungal drug resistance. *Cold Spring Harb. Perspect. Med.* Doi: 10.1101/cshperspect.a019752.

Cuenca-Estrella, M. 2014. Antifungal drug resistance mechanisms in pathogenic fungi: from bench to bedside. *Clin. Microbiol. Infect.* **20 Suppl 6**, 54–59.

Denning, D.W. and Perlin, D.S. 2011. Azole resistance in Aspergillus: a growing public health menace. *Future Microbiol.* **6**, 1229–1232.

Denning, D.W., Venkateswarlu, K., Oakley, K.L., Anderson, M.J., Manning, N.J., Stevens, D.A., Warnock, D.W., and Kelly, S.L. 1997. Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **41**, 1364–1368.

Diaz-Guerra, T.M., Mellado, E., Cuenca-Estrella, M., and Rodriguez-Tudela, J.L. 2003. A point mutation in the 14α-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **47**, 1120–1124.

Dirr, F., Echtenacher, B., Heesemann, J., Hoffmann, P., Ebel, F.,

and Wagener, J. 2010. AfMkk2 is required for cell wall integrity signaling, adhesion, and full virulence of the human pathogen *Aspergillus fumigatus*. *Int. J. Med. Microbiol.* **300**, 496–502.

Epp, E., Vanier, G., Harcus, D., Lee, A.Y., Jansen, G., Hallett, M., Sheppard, D.C., Thomas, D.Y., Munro, C.A., Mullick, A., and Whiteway, M. 2010. Reverse genetics in *Candida albicans* predicts ARF cycling is essential for drug resistance and virulence. *PLoS Pathog.* **6**, e1000753.

Espinel-Ingroff, A., Arthington-Skaggs, B., Iqbal, N., Ellis, D., Pfaller, M.A., Messer, S., Rinaldi, M., Fothergill, A., Gibbs, D.L., and Wang, A. 2007. Multicenter evaluation of a new disk agar diffusion method for susceptibility testing of filamentous fungi with voriconazole, posaconazole, itraconazole, amphotericin B, and caspofungin. *J. Clin. Microbiol.* **45**, 1811–1820.

Espinel-Ingroff, A., Diekema, D.J., Fothergill, A., Johnson, E., Pelaez, T., Pfaller, M.A., Rinaldi, M.G., Canton, E., and Turnidge, J. 2010. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *J. Clin. Microbiol.* **48**, 3251–3257.

Espinel-Ingroff, A. and Rezusta, A. 2002. E-test method for testing susceptibilities of *Aspergillus* spp. to the new triazoles voriconazole and posaconazole and to established antifungal agents: comparison with NCCLS broth microdilution method. *J. Clin. Microbiol.* **40**, 2101–2107.

Ferreira, M.E., Colombo, A.L., Paulsen, I., Ren, Q., Wortman, J., Huang, J., Goldman, M.H., and Goldman, G.H. 2005. The ergosterol biosynthesis pathway, transporter genes, and azole resistance in *Aspergillus fumigatus*. *Med. Mycol.* **43 Suppl 1**, S313–319.

Fothergill, A.W. 2012. Antifungal susceptibility testing: Clinical Laboratory and Standards Institute (CLSI) Methods. pp. 65–74.

Fraczek, M.G., Bromley, M., and Bowyer, P. 2011. An improved model of the *Aspergillus fumigatus* Cyp51A protein. *Antimicrob. Agents Chemother.* **55**, 2483–2486.

Fraczek, M.G., Bromley, M., Buied, A., Moore, C.B., Rajendran, R., Rautemaa, R., Ramage, G., Denning, D.W., and Bowyer, P. 2013. The cdr1B efflux transporter is associated with non-cyp51a-mediated itraconazole resistance in *Aspergillus fumigatus*. *J. Antimicrob. Chemother.* **68**, 1486–1496.

Furustrand Tafin, U., Clauss, M., Hauser, P.M., Bille, J., Meis, J.F., and Trampuz, A. 2012. Isothermal microcalorimetry: a novel method for real-time determination of antifungal susceptibility of Aspergillus species. *Clin. Microbiol. Infect.* **18**, E241–245.

Galimberti, R., Torre, A.C., Baztan, M.C., and Rodriguez-Chiappetta, F. 2012. Emerging systemic fungal infections. *Clin. Dermatol.* **30**, 633–650.

Gu, L., Frommel, S.C., Oakes, C.C., Simon, R., Grupp, K., Gerig, C.Y., Bar, D., Robinson, M.D., Baer, C., Weiss, M., *et al.* 2014. BAZ2A (TIP5) is involved in epigenetic alterations in prostate cancer and its overexpression predicts disease recurrence. *Nat. Genet.* **47**, 22–30.

Guinea, J., Pelaez, T., Alcala, L., and Bouza, E. 2006. Comparison of Sensititre YeastOne with the NCCLS M38-A microdilution method to determine the activity of amphotericin B, voriconazole, and itraconazole against clinical isolates of *Aspergillus fumigatus*. *Diagn. Microbiol. Infect. Dis.* **56**, 53–55.

Horn, S.C., Hanna, J., Hirsch, C., Volkwein, C., Schutz, A., Heinemann, U., Sommer, T., and Jarosch, E. 2009. Usa1 functions as a scaffold of the HRD-ubiquitin ligase. *Mol. Cell* **36**, 782–793.

Howard, S.J., Cerar, D., Anderson, M.J., Albarrag, A., Fisher, M.C., Pasqualotto, A.C., Laverdiere, M., Arendrup, M.C., Perlin, D.S., and Denning, D.W. 2009. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerging Infect. Dis.* **15**, 1068–1076.

Hu, W., Sillaots, S., Lemieux, S., Davison, J., Kauffman, S., Breton, A., Linteau, A., Xin, C., Bowman, J., Becker, J., *et al.* 2007. Essential gene identification and drug target prioritization in *Aspergillus fumigatus*. *PLoS Pathog.* **3**, e24.

Kikuchi, K., Watanabe, A., Ito, J., Oku, Y., Wuren, T., Taguchi, H., Yarita, K., Muraosa, Y., Yahiro, M., Yaguchi, T., *et al.* 2014. Antifungal susceptibility of *Aspergillus fumigatus* clinical isolates collected from various areas in Japan. *J. Infect. Chemother.* **20**, 336–338.

Krishnan, K. and Askew, D.S. 2014. The fungal UPR: a regulatory hub for virulence traits in the mold pathogen *Aspergillus fumigatus*. *Virulence* **5**, 334–340.

Krishnan, K., Feng, X., Powers-Fletcher, M.V., Bick, G., Richie, D.L., Woollett, L.A., and Askew, D.S. 2013. Effects of a defective endoplasmic reticulum-associated degradation pathway on the stress response, virulence, and antifungal drug susceptibility of the mold pathogen *Aspergillus fumigatus*. *Eukaryot. Cell* **12**, 512–519.

Lamoth, F., Juvvadi, P.R., Fortwendel, J.R., and Steinbach, W.J. 2012. Heat shock protein 90 is required for conidiation and cell wall integrity in *Aspergillus fumigatus*. *Eukaryotic Cell* **11**, 1324–1332.

Lamoth, F., Juvvadi, P.R., Gehrke, C., and Steinbach, W.J. 2013. *In vitro* activity of calcineurin and heat shock protein 90 inhibitors against *Aspergillus fumigatus* azole- and echinocandin-resistant strains. *Antimicrob. Agents Chemother.* **57**, 1035–1039.

Lamoth, F., Juvvadi, P.R., Soderblom, E.J., Moseley, M.A., Asfaw, Y.G., and Steinbach, W.J. 2014a. Identification of a key lysine residue in heat shock protein 90 required for azole and echinocandin resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **58**, 1889–1896.

Lamoth, F., Juvvadi, P.R., and Steinbach, W.J. 2014b. Heat shock protein 90 (Hsp90): A novel antifungal target against *Aspergillus fumigatus*. *Crit. Rev. Microbiol.* **22**, 1–12.

Lamping, E., Baret, P.V., Holmes, A.R., Monk, B.C., Goffeau, A., and Cannon, R.D. 2010. Fungal PDR transporters: Phylogeny, topology, motifs and function. *Fungal Genet. Biol.* **47**, 127–142.

Lass-Flörl, C., Cuenca-Estrella, M., Denning, D.W., and Rodriguez-Tudela, J.L. 2006. Antifungal susceptibility testing in *Aspergillus* spp. according to EUCAST methodology. *Med. Mycol.* **44**, 319–325.

Lass-Florl, C., Perkhofer, S., and Mayr, A. 2010. *In vitro* susceptibility testing in fungi: a global perspective on a variety of methods. *Mycoses* **53**, 1–11.

Lelievre, L., Groh, M., Angebault, C., Maherault, A.C., Didier, E., and Bougnoux, M.E. 2013. Azole resistant *Aspergillus fumigatus*: an emerging problem. *Med. Mal. Infect.* **43**, 139–145.

Liu, F., Pu, L., Zheng, Q., Zhang, Y., Gao, R., Xu, X., Zhang, S., and Lu, L. 2014. Calcium signaling mediates antifungal activity of triazole drugs in the Aspergilli. *Fungal Genet. Biol.* Doi: 10.1016/j.fgb.2014.12.005.

Mayr, A. and Lass-Florl, C. 2011. Epidemiology and antifungal resistance in invasive Aspergillosis according to primary disease: review of the literature. *Eur. J. Med. Res.* **16**, 153–157.

Mellado, E., Garcia-Effron, G., Alcazar-Fuoli, L., Cuenca-Estrella, M., and Rodriguez-Tudela, J.L. 2004. Substitutions at methionine 220 in the 14alpha-sterol demethylase (Cyp51A) of *Aspergillus fumigatus* are responsible for resistance *in vitro* to azole antifungal drugs. *Antimicrob. Agents Chemother.* **48**, 2747–2750.

Mellado, E., Garcia-Effron, G., Alcazar-Fuoli, L., Melchers, W.J.G., Verweij, P.E., Cuenca-Estrella, M., and Rodriguez-Tudela, J.L. 2007. A new *Aspergillus fumigatus* resistance mechanism conferring *in vitro* cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. *Antimicrob. Agents Chemother.* **51**, 1897–1904.

Mellado, E., Garcia-Effron, G., Buitrago, M.J., Alcazar-Fuoli, L., Cuenca-Estrella, M., and Rodriguez-Tudela, J.L. 2005. Targeted gene disruption of the 14-alpha sterol demethylase (*cyp51A*) in *Aspergillus fumigatus* and its role in azole drug susceptibility. *Antimicrob. Agents Chemother.* **49**, 2536–2538.

Messer, S.A., Diekema, D.J., Hollis, R.J., Boyken, L.B., Tendolkar, S., Kroeger, J., and Pfaller, M.A. 2007. Evaluation of disk diffusion

and Etest compared to broth microdilution for antifungal susceptibility testing of posaconazole against clinical isolates of filamentous fungi. *J. Clin. Microbiol.* **45**, 1322–1324.

Montiel, M.D., Lee, H.A., and Archer, D.B. 2006. Evidence of RIP (repeat-induced point mutation) in transposase sequences of *Aspergillus oryzae*. *Fungal Genet. Biol.* **43**, 439–445.

Morschhauser, J. 2010. Regulation of multidrug resistance in pathogenic fungi. *Fungal Genet. Biol.* **47**, 94–106.

Nascimento, A.M., Goldman, G.H., Park, S., Marras, S.A.E., Delmas, G., Oza, U., Lolans, K., Dudley, M.N., Mann, P.A., and Perlin, D.S. 2003. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrob. Agents Chemother.* **47**, 1719–1726.

Parker, J.E., Warrilow, A.G., Price, C.L., Mullins, J.G., Kelly, D.E., and Kelly, S.L. 2014. Resistance to antifungals that target Cyp51. *J. Chem. Biol.* **7**, 143–161.

Paul, S., Diekema, D., and Moye-Rowley, W.S. 2013. Contributions of *Aspergillus fumigatus* ATP-binding cassette transporter proteins to drug resistance and virulence. *Eukaryotic Cell* **12**, 1619–1628.

Pfaller, M., Boyken, L., Hollis, R., Kroeger, J., Messer, S., Tendolkar, S., and Diekema, D. 2011. Use of epidemiological cutoff values to examine 9-year trends in susceptibility of *Aspergillus* species to the triazoles. *J. Clin. Microbiol.* **49**, 586–590.

Pfaller, M.A., Messer, S.A., Mills, K., and Bolmstrom, A. 2000. *In vitro* susceptibility testing of filamentous fungi: comparison of Etest and reference microdilution methods for determining itraconazole MICs. *J. Clin. Microbiol.* **38**, 3359–3361.

Posteraro, B. and Sanguinetti, M. 2014. The future of fungal susceptibility testing. *Future Microbiol.* **9**, 947–967.

Posteraro, B., Torelli, R., De Carolis, E., Posteraro, P., and Sanguinetti, M. 2014. Antifungal susceptibility testing: current role from the clinical laboratory perspective. *Mediterr. J. Hematol. Infect. Dis.* **6**, e2014030.

Prasad, R. and Rawal, M.K. 2014. Efflux pump proteins in antifungal resistance. *Front. Pharmacol.* **5**, 202.

Prigitano, A., Venier, V., Cogliati, M., De Lorenzis, G., Esposto, M.C., and Tortorano, A.M. 2014. Azole-resistant *Aspergillus fumigatus* in the environment of northern Italy, May 2011 to June 2012. *Euro Surveill.* **19**, 20747.

Qiao, J., Liu, W., and Li, R. 2010. Truncated Afyap1 attenuates antifungal susceptibility of *Aspergillus fumigatus* to voriconazole and confers adaptation of the fungus to oxidative stress. *Mycopathologia* **170**, 155–160.

Rajendran, R., Mowat, E., McCulloch, E., Lappin, D.F., Jones, B., Lang, S., Majithiya, J.B., Warn, P., Williams, C., and Ramage, G. 2011. Azole resistance of *Aspergillus fumigatus* biofilms is partly associated with efflux pump activity. *Antimicrob. Agents Chemother.* **55**, 2092–2097.

Rath, P.M., Buchheidt, D., Spiess, B., Arfanis, E., Buer, J., and Steinmann, J. 2012. First reported case of azole-resistant *Aspergillus fumigatus* due to the TR/L98H mutation in Germany. *Antimicrob. Agents Chemother.* **56**, 6060–6061.

Richardson, M.D. 2005. Changing patterns and trends in systemic fungal infections. *J. Antimicrob. Chemother.* **56 Suppl 1**, i5–i11.

Seyedmousavi, S., Mouton, J.W., Melchers, W.J., Bruggemann, R.J., and Verweij, P.E. 2014. The role of azoles in the management of azole-resistant aspergillosis: from the bench to the bedside. *Drug Resist. Updat.* **17**, 37–50.

Shao, P.L., Huang, L.M., and Hsueh, P.R. 2007. Recent advances and challenges in the treatment of invasive fungal infections. *Int. J. Antimicrob. Agents* **30**, 487–495.

Shapiro, R.S., Robbins, N., and Cowen, L.E. 2011. Regulatory circuitry governing fungal development, drug resistance, and disease. *Microbiol. Mol. Biol. Rev.* **75**, 213–267.

Slaven, J.W., Anderson, M.J., Sanglard, D., Dixon, G.K., Bille, J., Roberts, I.S., and Denning, D.W. 2002. Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, *atrF*, in the presence of itraconazole in an itraconazole resistant clinical isolate. *Fungal Genet. Biol.* **36**, 199–206.

Smialowska, A., Djupedal, I., Wang, J., Kylsten, P., Swoboda, P., and Ekwall, K. 2014. RNAi mediates post-transcriptional repression of gene expression in fission yeast *Schizosaccharomyces pombe*. *Biochem. Biophys. Res. Commun.* **444**, 254–259.

Snelders, E., Camps, S.M., Karawajczyk, A., Schaftenaar, G., Kema, G.H., van der Lee, H.A., Klaassen, C.H., Melchers, W.J., and Verweij, P.E. 2012. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. *PLoS One* **7**, e31801.

Snelders, E., Karawajczyk, A., Schaftenaar, G., Verweij, P.E., and Melchers, W.J. 2010. Azole resistance profile of amino acid changes in *Aspergillus fumigatus* Cyp51A based on protein homology modeling. *Antimicrob. Agents Chemother.* **54**, 2425–2430.

Snelders, E., Karawajczyk, A., Verhoeven, R.J., Venselaar, H., Schaftenaar, G., Verweij, P.E., and Melchers, W.J. 2011. The structure-function relationship of the *Aspergillus fumigatuscyp*51A L98H conversion by site-directed mutagenesis: the mechanism of L98H azole resistance. *Fungal Genet. Biol.* **48**, 1062–1070.

Spiess, B., Postina, P., Reinwald, M., Cornely, O.A., Hamprecht, A., Hoenigl, M., Lass-Florl, C., Rath, P.M., Steinmann, J., Miethke, T., *et al.* 2014. Incidence of Cyp51 A key mutations in *Aspergillus fumigatus*-a study on primary clinical samples of immunocompromised patients in the period of 1995-2013. *PLoS One* **9**, e103113.

Steinbach, W.J., Reedy, J.L., Cramer, R.A. Jr., Perfect, J.R., and Heitman, J. 2007. Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. *Nat. Rev. Microbiol.* **5**, 418–430.

Sun, N., Li, D., Fonzi, W., Li, X., Zhang, L., and Calderone, R. 2013. Multidrug-resistant transporter mdr1p-mediated uptake of a novel antifungal compound. *Antimicrob. Agents Chemother.* **57**, 5931–5939.

Traunmuller, F., Popovic, M., Konz, K.H., Smolle-Juttner, F.M., and Joukhadar, C. 2011. Efficacy and safety of current drug therapies for invasive aspergillosis. *Pharmacology* **88**, 213–224.

van der Linden, J.W., Camps, S.M., Kampinga, G.A., Arends, J.P., Debets-Ossenkopp, Y.J., Haas, P.J., Rijnders, B.J., Kuijper, E.J., van Tiel, F.H., Varga, J., *et al.* 2013. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clin. Infect. Dis.* **57**, 513–520.

van der Linden, J.W., Snelders, E., Kampinga, G.A., Rijnders, B.J., Mattsson, E., Debets-Ossenkopp, Y.J., Kuijper, E.J., Van Tiel, F.H., Melchers, W.J., and Verweij, P.E. 2011. Clinical implications of azole resistance in *Aspergillus fumigatus,* The Netherlands, 2007–2009. *Emerg. Infect. Dis.* **17**, 1846–1854.

van Ingen, J., van der Lee, H.A., Rijs, T.A., Zoll, J., Leenstra, T., Melchers, W.J., and Verweij, P.E. 2014. Azole, polyene and echinocandin MIC distributions for wild-type, TR34/L98H and TR46/Y121F/T289A *Aspergillus fumigatus* isolates in the Netherlands. *J. Antimicrob. Chemother.* **70**, 178–181.

Vermeulen, E., Lagrou, K., and Verweij, P.E. 2013. Azole resistance in *Aspergillus fumigatus*: a growing public health concern. *Curr. Opin. Infect. Dis.* **26**, 493–500.

Verweij, P.E., Howard, S.J., Melchers, W.J., and Denning, D.W. 2009. Azole-resistance in Aspergillus: proposed nomenclature and breakpoints. *Drug Resist. Updat.* **12**, 141–147.

Walsh, T.J., Anaissie, E.J., Denning, D.W., Herbrecht, R., Kontoyiannis, D.P., Marr, K.A., Morrison, V.A., Segal, B.H., Steinbach, W.J., Stevens, D.A., *et al.* 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. *Clin. Infect. Dis.* **46**, 327–360.

Wang, D.Y., Gricourt, M., Arne, P., Thierry, S., Seguin, D., Chermette, R., Huang, W.Y., Dannaoui, E., Botterel, F., and Guillot, J. 2014. Mutations in the *Cyp51A* gene and susceptibility to itraconazole

in *Aspergillus fumigatus* isolated from avian farms in France and China. *Poult. Sci.* **93**, 12–15.

Warrilow, A.G., Melo, N., Martel, C.M., Parker, J.E., Nes, W.D., Kelly, S.L., and Kelly, D.E. 2010. Expression, purification, and characterization of *Aspergillus fumigatus* sterol 14-alpha demethylase (Cyp51) isoenzymes A and B. *Antimicrob. Agents Chemother.* **54**, 4225–4234.

Willger, S.D., Puttikamonkul, S., Kim, K.H., Burritt, J.B., Grahl, N., Metzler, L.J., Barbuch, R., Bard, M., Lawrence, C.B., and Cramer, R.A. Jr. 2008. A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. *PLoS Pathog.* **4**, e1000200.

Xiao, L., Madison, V., Chau, A.S., Loebenberg, D., Palermo, R.E., and McNicholas, P.M. 2004. Three-dimensional models of wild-type and mutated forms of cytochrome P450 14α-sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into posaconazole binding. *Antimicrob. Agents Chemother.* **48**, 568–574.