Resistance of human fungal pathogens to antifungal drugs

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Resistance mechanisms can be engaged in clinically relevant fungal pathogens under different conditions when exposed to antifungal drugs. Over past years, active research was undertaken in the understanding of the molecular basis of antifungal drug resistance in these pathogens, and especially against the class of azole antifungals. The isolation of various alleles of the gene encoding the target of azoles has enabled correlation of the appearance of resistance with distinct mutations. Resistance mechanisms to azoles also converge to the upregulation of multidrug transporter genes, whose products have the capacity to extrude from cells several chemically unrelated antifungal agents and toxic compounds. Genome-wide studies of azole-resistant isolates are now permitting a more comprehensive analysis of the impact of resistance on gene expression, and may deliver new clues to their mechanisms. Several laboratories are also exploring, as well as possible alternative resistance pathways, the role of biofilm formation by several fungal species in the development of resistance to various antifungals, including azoles.

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Abbreviations

ABC	ATP-binding cassette
CaMDR1	*Candida albicans* multidrug resistance 1
CDR	*Candida* drug resistance
DRE	drug-responsive element
HFAR	high-frequency azole resistance
MIC	minimum inhibitory concentration

Introduction

When faced with antifungal drugs, fungal pathogens have, in principle, the capacity to overcome their inhibitory action through specific resistance mechanisms. This biological response, reflected *in vitro* by the ability to select mutants resistant to antifungal drugs, has even been used on several occasions to identify the cellular targets of antifungal drugs [1,2]. In a clinical context, whenever antifungal agents are used to combat fungal infections, the exposure of fungal pathogens to these agents is therefore expected to give rise to resistant isolates. The increasing number of fungal infections documented in several hospital sites around the world could potentially favor the occurrence of this phenomenon, as the number of antifungal treatments will be higher [3]. The occurrence of resistance will be, of course, dependent on the type of fungal pathogen to be treated and the type of antifungal agents applied. An outlook on the different classes of antifungals is given in Table 1, which summarizes their use against major fungal pathogens and the occurrence of resistance among clinical isolates. The facts show that resistance to antifungal drugs, which is measured as an increase in minimum inhibitory concentration (MIC) as compared to values measured in susceptible reference organisms, has been reported in clinical use for three classes of antifungal drugs up to now: the polyenes, pyrimidine analogues (5-fluorocytosine) and the azoles (Table 1) [4•].

Resistance to azoles has a leading position in published reports. The repeated use of azoles (especially fluconazole) in treatments of HIV-positive patients with mucosal fungal infections in the period preceding the introduction of highly active antiretroviral therapy has favored the acquisition of azole resistance in several fungal pathogens. These were mostly *Candida* species, including (with decreasing importance) *C. albicans*, *C. glabrata*, *C. dubliniensis* and *C. tropicalis*, and (less frequently) *Cryptococcus* species [5]. Azole resistance in systemic fungal infections of severely immunocompromised patients is much less frequent and has been described mainly for *C. albicans* [6] and *A. fumigatus* species [7]. Mechanisms of azole resistance have been most extensively investigated in recent years, as a large number of yeast isolates were available to research laboratories. Several reviews are available that describe in detail the different mechanisms resulting in resistance to the azoles [8–10]. In this review, I summarize the most recent findings that deal with the molecular basis of the mechanisms of azole resistance in yeast pathogens, including *Candida*, *Cryptococcus* and *Aspergillus* species.

Resistance mechanisms involving target alterations

The major cellular target of azole compounds in yeast and fungi is a cytochrome P450 (Erg11p) involved in the demethylation of the lanosterol molecule in position 14α. This step is necessary for the biosynthesis of ergosterol, a fungal-specific sterol that maintains membrane functions. Inhibiting the activity of Erg11p by azoles leads to ergosterol depletion and accumulation of 14α-methylated sterols (lanosterol and 14α-methyl-3-6-diol) [11]. Both effects result in growth arrest and not in cell death in most yeast and fungal species, although, in some other species (*C. neoformans* and *A. fumigatus*), specific azoles such as itraconazole have a fungicidal effect [12–14]. A first method for fungal pathogens to overcome the inhibition of azoles is to increase the content of the target enzyme molecules either by gene amplification or upregulation of the corresponding gene. Besides a single example of gene amplification of the *ERG11* gene in *C. glabrata* [15], upregulation of *ERG11* genes in fungal pathogens is thought to have a limited impact in azole resistance, particularly when resistance must reach high levels [16]. Some fungal

Table 1

Antifungal agents: activities against principal modes of action and resistance mechanisms of fungal pathogens.

Antifungal	Spectrum/comments	Mode of action	Mechanism of resistance observed in clinical isolates
Polyenes			
Amphotericin B	Broad activity against *Candida* spp (except *C. lusitaniae*), *Cryptococcus neoformans* and filamentous fungi (except, of the *Aspergillus* spp, *A. terreus* and *A. flavus*).	Binding to ergosterol and destabilization of cell membrane functions	Alteration in specific steps of ergosterol biosynthesis
Pyrimidines analogues			
5-fluorocytosine (5-FC)	Active against *Candida* spp and *Cryptococcus* spp; however, rapid emergence of resistance can appear when 5-FC is used as monotherapy	Impairment of nucleic acid biosynthesis by formation of toxic fluorinated pyrimidine antimetabolites	Decreased uptake of 5-FC; decreased formation of toxic antimetabolites.
Azoles			
Fluconazole	Active against *Candida* spp and *Cryptococcus* spp, less active against *C. glabrata* and no activity against *C. krusei*; no activity against filamentous fungi	Inhibition of cytochrome P450 14α-lanosterol demethylase	Enhanced efflux by upregulation of multidrug transporter genes.
Itraconazole	Like fluconazole, but enhanced activity against filamentous fungi		Target alterations by occurrence of mutations
Voriconazole	Like fluconazole, but enhanced activity against filamentous fungi, including *Aspergillus* and *Fusarium* spp		Alteration of specific steps in the ergosterol biosynthetic pathway
Posaconazole	Closely related to itraconazole, but more active		
Allylamines			
Terbinafine	Active against most dermatophytes, poor activity against *Candida* spp	Inhibition of squalene epoxidase	Unknown
Morpholines			
Amorolfine	Active against most dermatophytes, poor activity against *Candida* spp	Inhibition of sterol Δ^{14} reductase and $\Delta^{7,8}$ isomerase	Unknown
Echinocandins			
Caspofungin	Active against *Candida* spp with fungicidal activity, moderately active against *Aspergillus* spp, poor activity against *C. neoformans*	Inhibition of the cell wall synthesis enzyme β-1,3 glucan synthase	Unknown

species, particularly *Candida krusei* and *A. fumigatus*, show low susceptibility to fluconazole, but lower susceptibility to other azoles such as itraconazole and voriconazole. The low specific susceptibility to fluconazole is believed to be caused by weak inhibition of the 14α lanosterol demethlylase enzyme from both organisms [17–19]. Following early reports that azole resistance could be caused in *C. albicans* by a reduction of affinity to the cytochrome P450 protein, *ERG11* alleles from azole-resistant isolates have been sequenced and compared to alleles of matched azole-susceptible isolates. Because sequence alterations may simply be due to allelic variations — well known in yeast species such as *C. albicans* — the involvement of such sequence alterations in azole resistance is difficult to determine. To circumvent this problem, the functional expression of *ERG11* alleles in *Saccharomyces cerevisiae* was attempted and probably represents a better predictor for the involvement of specific mutations in azole resistance. Several *ERG11* mutations have been reported using this method [20]. Nevertheless, *in vitro* assays with the mutated proteins in reconstituted systems will still be needed to confirm the results of phenotypic assays performed with yeasts. A structure model for the *C. albicans* Erg11 protein has been recently proposed, and the effect of some described mutations on binding interference with fluconazole has also been investigated [21••]. The main conclusion of the authors was that the amino acid positions of some mutations, rather than directly affecting azole binding, were most probably altering the different conformational stages along the catalytic cycle of the protein. The availability of a crystal structure of a eukaryotic Erg11p, which still represents a very difficult challenge, would unequivocally help to refine our current understanding of the effects of the mutations so far described.

Resistance mechanisms affecting antifungal transport

A prerequisite for the inhibitory action of azoles is to reach intracellular concentration levels that are able to block the function of every Erg11p molecule present in the membrane of the endoplasmic reticulum. The transport of azoles into the fungal cells is still not totally understood. Current models support the idea of passive transport through cell wall and cell membrane barriers, given that: first, modification of cell wall structures by altering the glycosylation of surface proteins leads to modification of azole susceptibility [22]; and second, a mutant in a specific step of ergosterol biosynthetic pathway (*ERG6*, sterol

methyltransferase) shows enhanced permeability to different growth inhibitors, including azoles [23]. However, fungal cells possess in their plasma membranes efflux systems that have the capacity to extrude from cells a large variety of compounds. It became clear from the studies of several laboratories that these efflux systems could operate in azole resistance. In azole-resistant yeasts, genes encoding ATP-binding cassette (ABC) transporters were upregulated, as compared to the corresponding azole-susceptible species. So far, only *CDR1* (*Candida d*rug *r*esistance 1) and *CDR2* in *C. albicans* [24,25], *CdCDR1* in *C. dubliniensis* [26], a *CDR1* homologue in *C. tropicalis* [27], *CgCDR1* and *CgCDR2* in *C. glabrata* [28,29•] and *CnDR* in *C. neoformans* (B Posteraro, personal communication) have been identified as ABC-transporter genes upregulated in azole-resistant isolates. In *A. fumigatus*, itraconazole is able to induce an ABC-transporter gene, *atrF*, though the role of this gene in the resistance of clinical isolates to itraconazole is still not known in detail [30]. Heterologous expression of ABC-transporter genes, such as *CDR1*, *CDR2*, *CdCDR1*, *CgCDR1* and *CgCDR2*, in *S. cerevisiae* conferred not only resistance to several azole derivatives (fluconazole, itraconazole and ketoconazole) but also to a wide range of compounds, including antifungals and metabolic inhibitors [25,28,31•]. With the sequence data available from the genomes of yeast pathogens (*C. albicans*, *C. glabrata* and *C. tropicalis*), it is possible that other multidrug transporter genes involved in antifungal resistance will still be characterized in the future.

Several laboratories have also observed that, besides upregulation of ABC-transporter genes, a multidrug transporter gene named *CaMDR1* (for *C. albicans m*ulti*d*rug *r*esistance 1, previously known as *BEN*^{r} for *ben*omyl *r*esistance) and belonging to the family of major facilitators (proteins with 10–14 transmembrane domains that use proton motive force for compound extrusion) was upregulated in some *C. albicans* azole-resistant yeast clinical isolates. Deletion of *CaMDR1* in *C. albicans* but also in *C. dubliniensis* isolates with acquired azole resistance by *CaMDR1* upregulation resulted in a sharp increase of azole susceptibility, thus supporting by a genetical approach the involvement of this specific gene in azole resistance [32•,33]. Deletion of *CaMDR1* in an azole-susceptible laboratory strain did not result in a significant increase of azole susceptibility, thus agreeing with the fact that *CaMDR1* is almost not expressed in this type of strain and, more generally, in azole-susceptible clinical isolates [34]. Upregulation of a *CaMDR1*-like gene has also been observed in a fluconazole-exposed *C. tropicalis* isolate, which acquired cross-resistance to fluconazole and itraconazole [27]. This type of transient upregulation has been observed in clinical situations with *C. albicans* isolates exposed to fluconazole. In this case, only the expression of *CDR* genes was enhanced when the drug was given *in vitro* or *in vivo* but was decreased by drug removal [35]. In a recent report, this particular phenomenon of reversible expression was linked to homozygocity at the mating type locus (MTL) of *C. albicans* [36]. Transient upregulation of multidrug transporter genes, beside constitutive upregulation in azole-resistant clinical isolates, is also known when yeast or other fungal pathogens are exposed to different drugs. In *C. albicans*, the ABC-transporter genes *CDR1* and *CDR2* can be upregulated by short-term exposure to steroids, alternative antifungals (terbinafine and amorolfine) or other metabolic inhibitors [37•]. In the same species, expression of the major facilitator gene *CaMDR1* can be stimulated by the addition of agents like benomyl or compounds exerting oxidative stress (H_2O_2), which themselves cannot stimulate *CDR* gene expression [38].

Molecular basis of multidrug transporter upregulation

The molecular basis of the upregulation of multidrug transporters belonging to the ABC and major facilitator families is being actively investigated in yeast pathogens. Several questions must be addressed. Are mutations responsible for upregulation *in cis* or *in trans*? Which are the regulatory elements present in the promoters of the genes? Which are the transcription factors responsible for gene upregulation? What are the pathways (*CDR*- and *CaMDR1*-specific) that start from drug exposure to multidrug transporter upregulation? Are there basic differences with known regulatory circuits of multidrug transporter genes described in *S. cerevisiae*?

Recently published work answers part of these questions. It is believed that the mutation(s) leading to gene upregulation might be caused by alterations *in trans* (those involving transcription factors). Using the *Renilla* luciferase reporter system fused to *CDR1* and *CDR2* promoters cloned from azole-susceptible isolates, de Micheli *et al.* [37•] showed that their expression was enhanced in an azole-resistant strain, in which these genes are constitutively upregulated. With another reporter system (green fluorescent protein [GFP] fused to the *CaMDR1* promoter from an azole-susceptible strain), Wirsching *et al.* [39] showed that high fluorescence could be obtained when the chimeric construct was introduced in an azole-resistant strain upregulating *CaMDR1*. Until now, only the *CDR1* and *CDR2* promoters have been dissected systematically for the presence of regulatory elements. A common drug-responsive element (DRE) in both promoters could be experimentally delimited with the consensus 5′-CGGA(A/T)ATCGGATATTTTTTTT-3′, which has no equivalent in eukaryotic promoter databases [37•]. This DRE is necessary for *CDR1* and *CDR2* transient upregulation by drugs and for constitutive upregulation in an azole-resistant isolate. However, the detailed pathway resulting in *CDR* gene upregulation and the identity of proteins binding to the DRE have still to be determined.

No published data are yet available on the dissection of the *CaMDR1* promoter. In this promoter, however, an AP1-like binding site (TTAGTAA) is present at –470 from the ATG start codon, suggesting that an AP1-like transcription factor interacts with the regulation of *CaMDR1*. *S. cerevisiae*

possesses an AP1-like transcription factor, *YAP1*, which mediates the expression of *FLR1* (*fl*uconazole *r*esistance 1), a gene similar to *CaMDR1* in *S. cerevisiae* [40]. This transcription factor shuffles from the cytoplasm to the nucleus under oxidative stress conditions and, thus, activates the transcription of target genes [41]. A *C. albicans* homologue of *YAP1*, *CAP1*, has been isolated and its disruption affects survival under oxidative stress stimulated by exposure to H_2O_2 [42]. Deletion of *CAP1* also affects, but not abolishes, responsiveness of *CaMDR1* in the presence of benomyl, suggesting that *CAP1* and yet unknown factors have the ability to interact with *CaMDR1*. *CAP1* deletion has been performed in an azole-resistant isolate in which *CaMDR1* upregulation was detected. In this case, however, no decrease of *CaMDR1* expression could be measured, suggesting that *CaMDR1* upregulation can be caused by *CAP1*-independent upregulation pathway(s) (D Sanglard, unpublished data).

Multidrug transporter genes can be upregulated by another phenomenon, described as 'high-frequency azole resistance' (HFAR), in *C. glabrata*. HFAR strains appear at frequencies of 10^{-3} to 10^{-4} on plates containing fluconazole [29•]. Phenotypic analysis demonstrated that HFAR strains were devoid of mitochondrial DNA and resembled the 'petite' phenotype in *S. cerevisiae*, a name originating from the smaller size of such colonies on growth agar that occurs because of their inability to completely metabolize carbon sources. HFAR strains show high constitutive expression of the ABC-transporters *CgCDR1* and *CgCDR2*, attributing to the high level of fluconazole resistance measured in these strains. *CgCDR1* and *CgCDR2* promoters contain DNA regulatory elements, so called PDRE (pleotropic drug-responsive elements) that were first described in the promoter of the *S. cerevisiae PDR5* gene, the product of which is an important ABC transporter with similar functions to the *C. albicans CDR1* or the *C. glabrata CgCDR1* [43]. The regulatory circuit of *PDR5* involves two transcription factors (*PDR1* and *PDR3*) and it is therefore likely that similar factors function in the upregulation of *CgCDR1* and *CgCDR2* in *C. glabrata*. Taken together, these data show that multidrug transporter upregulation in *C. glabrata* can be considered to be basically different from our current knowledge of that in *C. albicans*, given that *CDR* genes do not possess PDRE-like sequences but other unrelated DRE sequences in their promoters. It is therefore likely that different transcription factors will bind to these sequences. Surprisingly enough, *C. albicans* genes related to *PDR1* and *PDR3* have been isolated by functional complementation in *S. cerevisiae* and can activate the transcription of *PDR5* via the PDRE sequences [42,44]. Currently available data support the fact that the transcription factors encoded by these genes are not involved in *CDR1* and *CDR2* upregulation (D Sanglard, unpublished data).

Resistance mechanisms and their combinations in clinical isolates

In some studies investigating resistance mechanisms to azoles in clinical isolates, it was possible to recover from patients treated with these compounds sequential isolates showing stepwise increase in azole resistance, as measured by susceptibility testing. The stepwise increase in azole resistance indicated that different resistance mechanisms probably operate and, through their sequential addition, explains the increase in azole MIC values. Several examples have been reported that document the multifactorial basis of azole resistance in clinical isolates. The combination of resistance mechanisms seems to be associated with a high level of azole resistance, for example, MIC values for fluconazole exceeding 64 μg/ml [45]. Alterations of the target enzymes by several distinct single or multiple mutations and upregulation of multidrug transporters from two different families provide much flexibility in the combination of resistance mechanisms. Molecular epidemiology of azole resistance performed mainly with *C. albicans* isolates demonstrated that the diversity of resistance mechanism combinations was high enough for there to be only very few azole-resistant isolates with identical patterns of *ERG11* mutations and profiles of multidrug transporter genes expression. The relative frequency of resistance mechanisms in a large population of azole-resistant isolates has been investigated in only a few studies. Perea *et al.* [45] showed that 85% of azole-resistant isolates upregulated multidrug transporter genes and that 65% contained *ERG11* mutations linked to azole resistance. Overall, 75% of the azole-resistant isolates showed combined resistance mechanisms. These numbers matched our own data, including the isolates of 18 HIV-positive patients from whom azole-resistant isolates could be recovered (D Sanglard, unpublished data). Of these isolates, 82% showed upregulation of multidrug transporter genes, 63% contained *ERG11* mutations linked to azole resistance, and 50% showed combinations of resistance mechanisms. The relative distribution of the type of multidrug transporter genes upregulated in these populations is in favor of the ABC transporters *CDR1* and *CDR2*; these transporters are upregulated in approximately twice as many azole-resistant isolates than is observed for isolates with *CaMDR1* upregulation.

Combinations of resistance mechanisms are not always linked with high levels of resistance. In *C. glabrata* azole-resistant isolates, a single resistance mechanism (upregulation of the *CgCDR1* ABC-transporter gene) is responsible for high levels of azole resistance. Genetic evidence has also been provided for the occurrence of this single resistance mechanism by deletion of *CgCDR1* in an azole-resistant strain, which results in a decrease in fluconazole MIC values near to those obtained in the parental azole-susceptible isolate [28].

Alternative mechanisms of azole resistance

Besides the two main resistance mechanisms described above, alternative pathways for azole resistance can be used by fungi. One of these alternative pathways targets specific steps in ergosterol biosynthesis. For example, mutation in the gene *ERG3*, which encodes the enzyme $\Delta^{5,6}$ desaturase (Erg3p) is linked to azole resistance in

C. albicans clinical isolates. Azole resistance is thought to be caused by the inability of cells to produce a sterol metabolite (3,6-diol) from 14α-methylfecosterol, a conversion normally catalyzed by Erg3p. The ergosterol content of normal cells exposed to azoles is depleted and, together with the production of the toxic metabolite 3,6-diol, growth is arrested. Interestingly, this type of resistance is paralleled with resistance to amphotericin B, as ergosterol is absent from cell membranes in these mutants. This specific resistance mechanism is, however, poorly exploited by fungal pathogens, suggesting that it probably has a selective disadvantage, compared to others, when engaged in host conditions [46].

Another interesting alternative for developing azole resistance has recently been described. It uses the ability of fungal pathogens to build biofilms on synthetic or natural surfaces. Biofilms are organized as a dense network of differentiated cells onto which a layer of extracellular matrix can form. Biofilms can constitute a physical barrier for the efficient penetration of antifungals, which could explain that cells embedded in these structures can become recalcitrant to their action. Measurement of drug susceptibilities in biofilms of *C. albicans* or *C. dubliniensis* yielded high MIC values for azoles and amphotericin B as compared to planktonic cells [47,48•]. As reported in *C. albicans*, the expression of genes involved in azole resistance (multidrug transporter genes) can also be altered in biofilms and may contribute to the relatively high azole resistance measured in the cell population of these dense structures [49].

Genome-wide studies with azole-resistant isolates

As mentioned above, the ABC-transporter genes *CDR1* and *CDR2* and the major facilitator *CaMDR1* respond to the presence of distinct drugs. Azole resistance is also mostly correlated with the upregulation of genes of a single family in individual isolates, meaning that it is only on rare occasions that genes of both families are co-regulated in the same azole-resistant isolate. Microarray experiments, with their ability to deliver collections of genes differentially expressed in a genome, represent an attractive tool to identify clusters of genes co-regulated between azole-susceptible and azole-resistant isolates. The expression of co-regulated genes might be controlled by common regulatory circuits converging to similar regulatory sequences in the promoters of these genes. In a study by Cowen *et al.* [50••], the expression profiles of individual *C. albicans* isolates with reduced azole susceptibility were investigated, each isolate upregulating either *CDR1/CDR2* or *CaMDR1*. Interestingly, in an isolate upregulating *CDR2*, other upregulated genes were found and, of them, three (*YPL88 (LPG20), YOR49* and *YLR63*) contained in their promoter a consensus for a DRE, the presence of which is necessary for *CDR1* and *CDR2* upregulation (see above). In the other isolates upregulating *CaMDR1*, several genes involved in oxidative stress response were also upregulated, suggesting that the oxidative stress pathway, to which *CAP1* also belongs, contributes to *CaMDR1* upregulation. It is likely that, in the future, additional microarray experiments will be reported that use other azole-resistant isolates as tester strains for expression profile analysis. These analyses will be helpful not only because they may cluster genes under the control of specific regulatory pathways, but also because they might reveal still unmasked azole resistance mechanisms.

Conclusions

Studies on resistance mechanisms to the azoles have delivered the many different resources utilized by simple microorganisms to circumvent the effect of growth inhibitory substances. Although antifungal resistance is now less of a problem than it was several years ago, before the introduction of highly active antiretroviral therapy to combat HIV, several basic biological processes that emerged from these studies will continue to be investigated and can be used when screening new antifungal drugs. One of the promising fields of investigation is dissection of the pathways that control the regulation of multidrug transporter genes in yeast pathogens. More practically, screening for novel antifungal substances can integrate the findings achieved by studies on resistance mechanisms. It is possible to test potential interactions existing between a candidate drug and a specific multidrug transporter, which should ideally be non-existent or maintained at the lowest level to minimize the use of compound extrusion as a potential resistance mechanism. Finally, genome-wide expression profiling will give the unique opportunity to obtain a comprehensive analysis of changes in gene expression in several clinical yeast isolates acquiring antifungal drug resistance over time. This, until now, has been limited to a restricted number of genes and has led to the description of a limited number of resistance mechanisms.

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