Clinical relevance of mechanisms of antifungal drug resistance in yeasts

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A limited number of antifungal agents including azoles, polyenes, pyrimidine analogues are used today to combat infections caused by yeast pathogens. While clinical factors can contribute to failures to antifungal treatments, yeast pathogens exposed to these agents can still limit their action either because they are intrinsically resistant or because they acquire specific resistance mechanisms. Microbiological methods are available to measure the susceptibility of yeast pathogens against the existing antifungal agents and to distinguish between antifungal susceptible and antifungal resistant organisms. This distinction can ideally predict the success or failure of a treatment in clinical situations and is available only for a limited number of antifungal agents, i.e. the azole antifungals fluconazole and itraconazole and the pyrimidine analogue 5-fluorocytosine. Cases of antifungal resistance have been reported for almost all classes of antifungal agents, but they have been mainly documented for the pyrimidine analogue 5-fluorocytosine and azole antifungals mainly in *Candida* species and less frequently in *Cryptococcus* species. This review summarizes the current knowledge on the different mechanisms of resistance to these agents in these yeast pathogens.

Key words: **Antifungal drug resistance. *Candida*. *Cryptococcus*.**

Importancia clínica de los mecanismos de resistencia a los antifúngicos en levaduras

En la actualidad se están empleando un número limitado de agentes antifúngicos, inluyendo azoles, polienos y análogos de pirimidinas, para combatir las infecciones causadas por levaduras patógenas. Ciertos factores clínicos pueden contribuir al fracaso del tratamiento antifúngico; por otra parte, las levaduras expuestas a estos agentes pueden limitar la actividad de los mismos, por ser intrínsecamente resistentes o por adquirir mecanismos de resistencia específicos. Se dispone de métodos microbiológicos para medir la sensibilidad de las levaduras patógenas a los antifúngicos disponibles, y para distinguir entre organismos sensibles y resistentes a los mismos. Esta distinción, de forma ideal, debiera predecir el éxito o el fracaso del tratamiento desde un punto de vista clínico, pero está disponible sólo para un número limitado de antifúnficos: los azoles, fluconazol e itraconazol, y el análogo de la pirimidina 5-fluorocitosina. Se han publicado casos de resistencia a los antifúngicos para casi todas las clases disponibles de estos agentes, pero sobre todo se han documentado en relación con la 5-fluorocitosina y los azoles en especies de *Candida* y, con menos frecuencia, en especies de *Cryptococcus*. En esta revisión se resume el conocimiento actual de los diferentes mecanismos de resistencia a los antifúngicos en levaduras patógenas.

Palabras clave: **Resistencia a antifúngicos. *Candida*. *Cryptococcus*.**

Introduction

Fungal infections caused by yeast pathogens remains quite common in immuno-compromised hosts, especially in HIV-infected individuals, or in patients given immunosuppressive or broad-spectrum antibiotics. *Candida* spp. represent the major group of yeast species recovered from these infected individuals, however other yeast species such as *Cryptococcus neoformans* might also be isolated. Not only are a restricted number of antifungal agents available to treat these infections, but also resistance to antifungal treatments can occur. Table 1 summarizes the activity of known antifungal agents in several yeast species and includes antifungals in the late stage of development. Resistance to antifungal treatments can develop on the basis of clinical and microbiological factors. A persistent infection despite treatment with an antifungal drug at maximal dosage may be described as clinically resistant to the therapeutic agent. However, the infecting organism may show normal susceptibility to the agent *in vitro*[1]. Clinical resistance to treatment may result from microbial resistance to an agent, but it may also be the result of complex interactions between an antimicrobial agent and an infecting microbe in a human host. Microbiological resistance can be defined as a shift (i.e. a decrease) in antifungal drug susceptibility that can be measured *in vitro* by appropriate laboratory methods. Resistance to specific antifungal drugs can be intrinsic in some yeast pathogens, but can be also acquired either in a transient or permanent manner. The distinction between a susceptible and a resistant yeast or fungal isolate can be made when a threshold drug

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TABLE 1. **Activities of current and emerging antifungal agents against *Candida* species and *Cryptococcus neoformans***

Antifungal agent	MIC_{90}[a] (μg/ml)						Reference
	C. albicans	*C. glabrata*	*C. krusei*	*C. parapsilosis*	*C. tropicalis*	*C. neoformans*	
Amphotericin B (AmB)	0.5	1	0.5	0.5	0.5	1	11, 34
5-fluorocytosine (5-FC)	4	0.5	16-32	1	4	16	11
Azoles							
Fluconazole	1	64	64	2.0	2.0	16	
Itraconazole	0.25	4.0	2.0	0.5	0.5	1	35, 36
Voriconazole	0.06	2.0	1.0	0.12	0.25	0.25	34, 37, 38
Posaconazole	0.06	4.0	0.5	0.12	0.25	< 0.015	
Ravuconazole	0.03	4.0	0.5	0.12	0.25	0.25	
Cyclic lipopeptides:							
Caspofungin (MK-0991)	0.5	0.5	1	0.5	1	32	39
Micafungin (FK-463)	0.0156	0.156	0.125	1	0.033	> 64	40
V-Echinocandin (LY-303366)	0.125	0.5	0.25	4	0.125	> 16	41-44

[a]MIC_{90} is defined as the MIC value to which 90% of a study population belongs.

susceptibility value (i.e. the breakpoint MIC, for *M*inimal *I*nhibitory *C*oncentration) is reached. In medical practice, breakpoint values could ideally predict the success or the failure of an antifungal treatment. However, experiences accumulated with different antifungals showed that this association cannot be obviously applied[1]. Table 2 gives breakpoints values for the main categories of antifungal agents. In table 2, an intermediate notion is given, the DDS MIC (for *D*ose-*D*ependent *S*usceptible), and indicates that the drug dosage is important when a yeast possessing a DDS MIC value is isolated. The breakpoint MIC values of a given fungal pathogen for a specific drug is less relevant for the microbiologist or the molecular biologist, since only a modest shift of antifungal drug susceptibility measured by increase in MIC values can be the consequence of one or several cellular alterations linked to modifications of the genetic material. This review will summarize the present situation of antifungal resistance in yeast pathogens and will detail the current understanding of these mechanisms when engaged in clinical situations.

Antifungal drugs in current use: mode of action and resistance

Polyenes

Polyenes belong to a class of natural antifungal compounds discovered in the early 1950s. One of the most successful polyene derivative, amphotericin B (AmB), is produced by *Streptomyces nodosus.* AmB can form soluble salts in both basic and acidic environments, is not orally nor intramuscularly absorbed and is virtually insoluble in water. The primary mode of action of AmB is to bind ergosterol in the membrane bilayer of susceptible organisms. This interaction is thought to result in the production of aqueous pores consisting of polyenes molecules linked to the membrane sterols. This configuration gives rise to a pore-like structure, leakage of vital cytoplasmic components (mono- or divalent cations) and death of the organism. AmB has a strong fungicidal effect on most important yeast pathogens. Time-kill curves have been reported in several studies and showed that AmB induces a 3- to 4 log decrease in viable counts in a time span of 2 to 4 hours at supra-MIC concentrations. AmB MICs are dependent on several factors and among them the composition of the testing medium is important. Rex et al[2] recommend the use of a special broth medium (AM3) to determine AmB MICs in *Candida* species. Presently, a standard protocol using AM3 medium has been recommended by the NCCLS in the protocol M-27A. Recently, Peron et al[3] evaluated an agar diffusion method using E-test with RPMI or AM3 as media in order to discriminate AmB-resistant from AmB-susceptible *Candida* isolates. AmB MIC_{90} values of various *Candida* species including *C. albicans*, *C. glabrata*, *C. parapsilosis* or *C. tropicalis* ranged from 0.25 to 1 μg/ml. AmB fungicidal concentrations are usually 0.5 to 2 times the MIC in *Candida* species. Microbiological resistance to AmB can be intrinsic or acquired. Intrinsic resistance to AmB is common for some *C. lusitaniae*[4] and for *Trichosporon* species[5], while acquired resistance during antifungal treatments with AmB is still rarely reported among yeast isolates. Some *C. lusitaniae* isolates are also able to operate *in vitro* rapid switches to AmB resistance when exposed to the drug. Acquired resistance to AmB is often associated with alteration of membrane lipids and especially sterols. Recently, clinical *C. albicans* isolates resistant to AmB were described lacking ergosterol and accumulating other sterols (3β-ergosta-7,22-dienol and 3β-ergosta-8-enol) typical for a defect in the sterol $\Delta^{5,6}$ desaturase system[6]. Such a defect is known in *S. cerevisiae* harboring a defect of the $\Delta^{5,6}$ desaturase gene *ERG3*. A defect in Δ^{8-7} isomerase in a clinical *C. neoformans* isolate from an AIDS patient was linked also with AmB resistance[7]. A decrease in the content of cell membrane-associated ergosterol can also cause AmB resistance, since AmB requires the presence of ergosterol to damage fungal cells.

TABLE 2. **NCCLS interpretive breakpoints against *Candida albicans* (in μg/ml)**

Antifungal agent	Susceptible	Dose-dependent susceptible (DDS)	Resistant
Fluconazole	8	16-32	64
Itraconazole	0.125	0.25-0.5	1
5-Fluorocytosine	4		32

Different investigators supported this possibility by demonstrating that i) development of inducible resistance (induced by an adaptation mechanism) in a strain of *C. albicans* was accompanied by a decrease in the ergosterol content of the cells and that ii) clinical polyene-resistant *C. albicans* isolates obtained from neutropenic patients had a 74 to 85% decrease in their ergosterol content[8]. Another mechanism accounting for the resistance of yeast to AmB is thought to be mediated by increased catalase activity, which can contribute to diminish oxidative damage caused by this agent[9].

5-Fluorocytosine (5-FC)

5-FC belongs to the class of pyrimidine analogues and was developed in the 1950s as a potential antineoplastic agent. Abandoned as anti-cancer drug due to its lack of activity against tumors, it showed however a good *in vitro* and *in vivo* antifungal activity. Because it is highly water-soluble, it can be administered by oral and i.v. routes[10]. 5-FC is taken up by fungal cells by a cytosine permease and is deaminated by a cytosine deaminase to 5-fluorouracil (5-FU), a potent antimetabolite. 5-FU can be converted to a nucleoside triphosphate and, when incorporated into RNA, causes miscoding. In addition, 5-FU can be converted to a deoxynucleoside, which inhibits thymidilate synthase and thereby DNA synthesis. 5-FC shows little toxicity in mammalian cells, since cytosine deaminase is absent or poorly active in these cells. 5-FU is however a potent anti-cancer agent but is impermeable to fungal cells. The conversion of 5-FC to 5-FU is possible by intestinal bacteria and therefore 5-FC can show toxicity in oral formulations. 5-FC is fungicidal in susceptible yeasts and fungi. A high variability in 5-FC MICs is observed in *Candida* species and *C. neoformans*, because of the occurrence of intrinsic resistance. MIC_{90} of 5-FC are in the range of 0.5 to 4 μg/ml for *Candida* species including *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata* or for *C. neoformans*[11]. 5-FC is not usually administered as a single agent because of rapid development of resistance. It is therefore used mainly in combination with other agents and particularly with AmB. *In vitro* data regarding the combination of both drugs against *Candida* species and *C. neoformans* are numerous and are contradictory, showing antagonistic, indifferent or synergistic effects[12]. 5-FC is also an antifungal agent against which resistance can be intrinsic or acquired. Resistance may occur due to the deficiency or lack of enzymes implicated in the metabolism of 5-FC or may be due to the deregulation of the pyrimidine biosynthetic pathway, in which products can compete with the fluorinated metabolites of 5-FC. Detailed investigations on the molecular mechanisms of resistance to 5-FC have shown that intrinsic resistance to 5-FC in fungi can be due to a defect in the cytosine permease (as observed in *C. glabrata* but not in *C. albicans* and *C. neoformans*), while acquired resistance results from a failure to metabolize 5-FC to 5-FUTP and 5-Fd-UMP or from the loss of feedback control of pyrimidine biosynthesis.

Azoles

Azole antifungal agents discovered in the late 1960s are synthetic compounds belonging to the largest group of antifungal agents. Azole antifungal agents used in medicine are categorized into N-1 substituted imidazoles (ketoconazole, miconazole, clotrimazole) and triazoles (fluconazole, itraconazole). The new generation of azole antifungals under development (posaconazole, ravuconazole, voriconazole) belong also to triazoles.

Azoles have a cytochrome P450 as a common cellular target in yeast or fungi (see fig. 1). This cytochrome P450, now referred to as Erg11p, is the product of the *ERG11* gene. The unhindered nitrogen of the imidazole or triazole ring of azole antifungal agents binds to the heme iron of Erg11p as a sixth ligand, thus inhibiting the enzymatic reaction. The affinity of imidazole and triazole derivatives is not only dependent on this interaction, but is also determined by the N-1 substituent, which is actually responsible for the high affinity of azole antifungal agents to their target. Each of these agents has distinct pharmacokinetics and their antifungal efficacy are quite different between yeast and fungal species of medical relevance. Azole antifungals have a broad spectrum of activity. They are active against *Candida* species, *C. neoformans* and dimorphic fungi. Some azole derivatives are however more active than others in different cases. For example fluconazole is relatively inactive against *C. krusei*, as opposed to itraconazole. Azole antifungals are only fungistatic against most yeast species, with the exception of *C. neoformans*. Itraconazole is effective in the treatment of superficial candidiasis (vaginal candidiasis or oropharyngeal candidiasis) and some fluconazole-resistant superficial candidiasis in AIDS patients. Against *Candida* infections, fluconazole has demonstrated the broadest clinical efficacy for mucosal candidiasis (both vaginal and oropharyngeal) as well as chronic mucocutaneous candidiasis. Fluconazole is also recommended as a first choice in the treatment of invasive *Candida* infections in non-neutropenic patients such as solid organ transplant patients, surgical and ICU patients or those with urinary tract infections due to susceptible *Candida* spp.[13]. Even in neutropenic patients, candidemia can be successfully treated with fluconazole, as long as the patients are stable and the infection is not due to *Candida* species less susceptible to fluconazole (for example *C. glabrata*) or intrinsically resistant to this agent (for example *C. krusei*). High doses of fluconazole (600-800 mg/day) have been shown to be safe and afford a better response rate than lower doses in surgical patients with *Candida* deep seated infections.

Reports on resistance to azole antifungal agents have been rare until the late 1980s. The first cases of resistance were reported in *C. albicans* after prolonged therapy with miconazole and ketoconazole. Following the use of fluconazole for a wide variety of clinical settings, antifungal resistance to this agent has been more frequently reported[14]. There are several mechanisms by which yeasts can become resistant to azole antifungal agents. These mechanisms are illustrated in fig. 1.

Resistance by altered drug transport

Failure to accumulate azole antifungals has been identified as a cause of azole resistance in several post-treatment clinical yeast isolates. These isolates include yeast species such as *C. albicans*, *C. glabrata*, *C. krusei*, *C. dubliniensis* or *C. neoformans*[15]. In

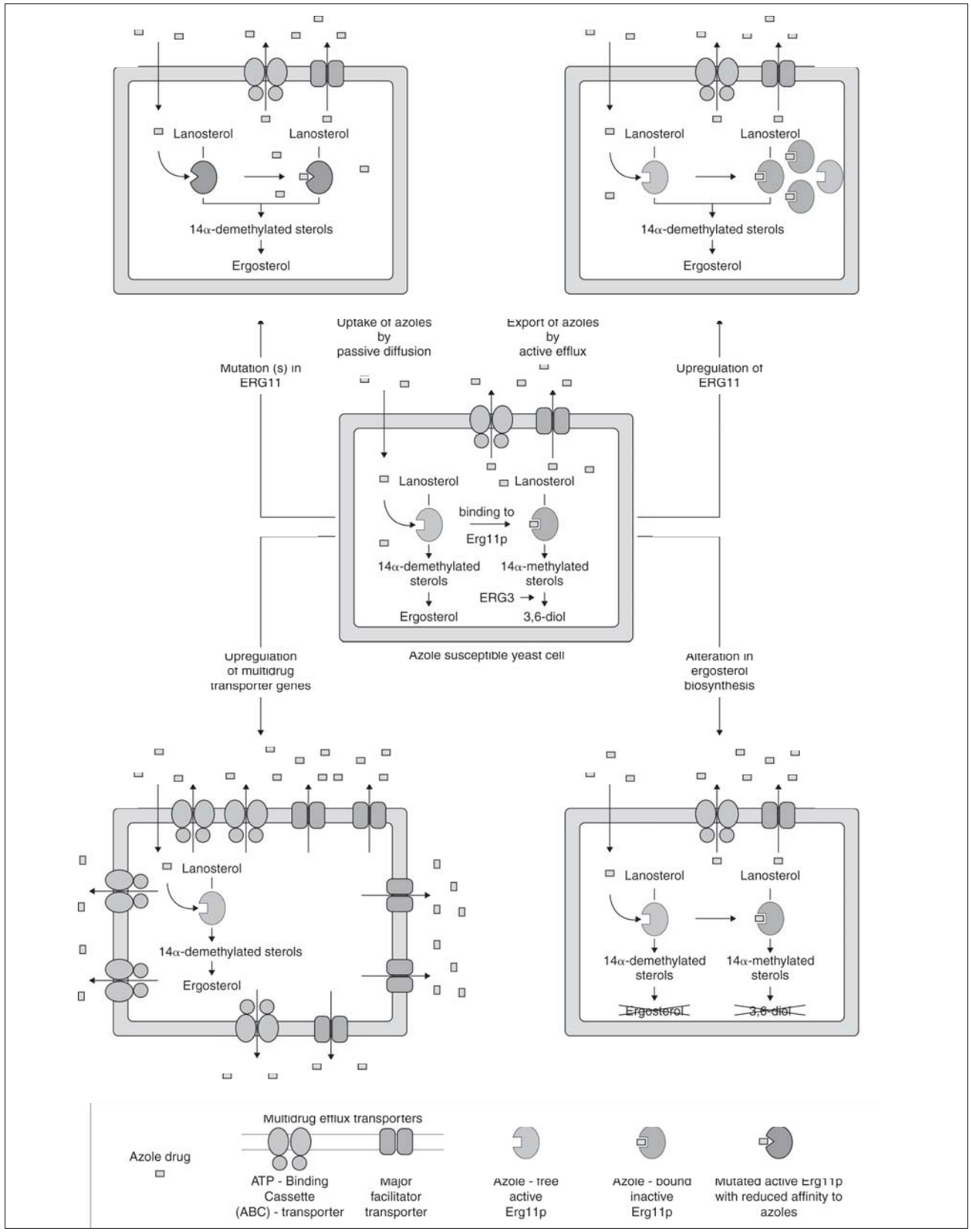

Figure 1. Schematic view of the four mains resistance mechanisms to azole antifungals in yeast pathogens. Erg11p, the cellular target of azole antifungals, is responsible for the demethylation of lanosterol. 14α -demethlylated sterols serve as further substrates in the formation of ergosterol. When azole drugs bind Erg11p, lanosterol demethylation is blocked and sterol metabolites remains methylated at the position 14α. The toxic metabolite 3,6-diol (14α-methylergosta-8,24(28)-dien-3β,6α-diol) is formed from the action of ERG3 on 14α-methylfecosterol. Details on specific resistance mechanisms are given in

azole-resistant *C. albicans* isolates from AIDS patients with oropharyngeal candidiasis (OPC), multidrug efflux transporters of the *A*TP-*b*inding *c*assette (ABC) superfamily and of the class of *M*ajor *F*acilitators (MF) have been reported to be responsible for the low level of accumulation of azole antifungal agents. Two genes for these transporters, the ABC-transporter gene *CDR1* and the MF gene *BEN*^{r} (also named *MDR1*) were shown to be upregulated in resistant isolates[16]. The upregulation of both transporters in azole-resistant *C. albicans* species has now been confirmed by several laboratories. The upregulation of ABC-transporter genes functionally similar to *CDR1* was also further evidenced in non-*C. albicans* species. Thus, in *C. glabrata* the *CgCDR1* and *PDH1* genes and, in *C. dubliniensis*, the *CdCDR1* and *CdMDR1* genes were shown to be upregulated in azole-resistant isolates[15].

Other multidrug efflux transporter genes of both classes exist in *C. albicans* and some of them were cloned recently. Among them, only the ABC-transporter gene *CDR2* is upregulated in *C. albicans* isolates cross-resistant to azole derivatives to the levels reached by *CDR1*[17]. In most cases, azole resistance acquired in clinical situations by multidrug transporters in yeast pathogens is maintained over a high number of generations *in vitro* without drug selection. Azole resistance can be however a reversible phenomenon. Marr and collaborators[18] obtained *C. albicans* isolates developing azole resistance from bone marrow transplant patients under fluconazole treatment. Increase in fluconazole MIC was coupled with upregulation of *CDR1* but was decreased with a paralleled decrease in *CDR1* expression in drug-free subculture. Azole-susceptible isolates from this type of patients, when exposed *in vitro* to fluconazole, developed reversible azole resistance by the same *CDR1* upregulation mechanism. Interestingly, only a portion of individually exposed colonies were rendered less susceptible to fluconazole, thus indicating that hetero-resistance, which was already described in azole-exposed *C. neoformans* isolates, could occur in specific *C. albicans* isolates[19]. Another interesting acquisition of azole resistance in a clinical context by multidrug transporter upregulation has been given by *C. glabrata*: this yeast could convert to azole resistance by loss of mitochondrial DNA. The phenomenon, also called HFAR (for *H*igh *F*requency *A*zole *R*esistance) because it occurred *in vitro* at high frequencies, was coupled with upregulation of *CgCDR1* and the novel ABC-transporter gene *CgCDR2*[20]. It is not clear at this moment how multidrug transporter genes are regulated in yeast pathogens. This particular topic is the focus of intensive investigations in several laboratories.

One of the implications of the involvement of multidrug efflux transporters in resistance to azole antifungals is that these transporters have the ability to mediate cross-resistance to unrelated antifungals or metabolic inhibitors. In order to determine whether or not a given substance is a potential substrate for multidrug efflux transporters, different approaches have been taken. One consists of functional expression of the *C. albicans* multidrug efflux transporters in the baker's yeast *S. cerevisiae* carrying a deletion of the *PDR5* gene[17,21]. Depending on the acquisition of resistance of *S. cerevisiae* mutants expressing these specific transporters against a given compound, the substance can be considered as a potential substrate for the expressed multidrug transporter. Potential substrates for the multidrug efflux transporters encoded by *CDR1* and *CDR2* included almost all azole antifungals of medical importance (fluconazole, itraconazole, posaconazole, ravuconazole) and other antifungal agents such as terbinafine and amorolfine. For the multidrug transporter encoded by *MDR1*, fluconazole was the only relevant substrate among azole antifungals. Several antifungal agents could not be assigned as substrates (AmB, 5-fluorocytosine). Since not only azole antifungals but other antifungals such as also terbinafine and amorolfine can be taken simultaneously as substrates by several multidrug efflux transporters, multidrug efflux transporter genes, when overexpressed in yeast clinical isolates, have the potential of mediating cross-resistance to different antifungal agents. Several data suggest that the upregulation of *MDR1* is responsible for the specific resistance to fluconazole in *Candida* isolates and is consistent with the observation that *MDR1* overexpression in *S. cerevisiae* was only conferring resistance to fluconazole[17].

Resistance to azole antifungals involving alterations of the cellular target

Alterations in the affinity of azole derivatives to Erg11p is another important mechanism of resistance which has been described in different post-treatment yeast species, namely *C. albicans* and recently in *C. neoformans*[23]. Affinity alterations are thought to be due to mutations in the gene encoding Erg11p (*ERG11*) which, by conformational changes, can affect the binding of azole derivatives. When comparing *ERG11* sequences from matched pairs of azole-susceptible and azole-resistant *C. albicans* isolates, several laboratories have described nucleotide substitutions in *ERG11* alleles from azole-resistant *C. albicans* isolates resulting in amino acid changes. A total of 83 amino acids substitutions have been reported by these studies[15]. This illustrates the high allelic variability for *ERG11*, which has still few equivalents in other genes in lower eukaryotes. Functional expression of PCR-amplified *ERG11* alleles in *S. cerevisiae* followed by azole susceptibility assays have also been performed as a convenient alternative to the first assay to reveal mutations coupled with the development of azole resistance[24]. While some *ERG11* alleles contain a single mutation responsible for azole resistance, other *ERG11* alleles were found to contain several mutations with potential additive effects. Upregulation of *ERG11* has been mentioned as a possible cause of azole resistance in few cases in *C. albicans* and *C. glabrata* clinical isolates. Upregulation of *ERG11* does not exceed a factor of 3 to 5 in azole-resistant isolates when compared to *ERG11* expression in related azole-susceptible strains[16]. Upregulation of *ERG11* can be achieved in principle by deregulating gene transcription or by gene amplification. This last possibility has been demonstrated in a *C. glabrata* isolate resistant to azole derivatives[25]. Upregulation of *ERG11* can also be obtained by exposure of *C. albicans* to ergosterol biosynthesis inhibitors, especially to azole antifungal agents. Exposure of *C. albicans* to these type of drugs affects the expression of

other *ERG* genes, as was recently confirmed by genome-wide expression studies performed in *C. albicans*[26].

Azole resistance mechanisms involving alterations in the ergosterol biosynthetic pathway

Analysis of the sterol composition of azole-resistant yeasts has provided several hypotheses on specific alterations of enzymes involved in the complex ergosterol biosynthetic pathway. Accumulation of ergosta-7,22-dienol-3β -ol was observed in two separate azole-resistant *C. albicans* clinical isolates, which is a feature consistent with an absence of sterol $\Delta^{5,6}$ desaturase activity encoded by *ERG3*[6]. Interestingly, azole resistance in these two cases was coupled with resistance to AmB, which was expected because of the absence of ergosterol in these cells. Some controversy still exists on the role of *ERG3* in development of azole resistance. The role of *ERG3* in azole resistance originates also from the observation that treatment of a normal yeast cell with azoles inhibits Erg11p and thus results in accumulation of 14α-methylated sterols and 14α-methylergosta-8,24(28)-dien-3β,6α-diol. Formation of this later sterol metabolite is thought to be catalyzed by the *ERG3* gene product (the sterol $\Delta^{5,6}$ desaturase) and thus inactivation of this gene suppresses toxicity and causes azole resistance (see fig. 1). This specific mechanism of resistance to azole derivatives seems to mimic azole resistance obtained in laboratory conditions in *S. cerevisiae* by mutations of the *ERG3* gene. Loss of function mutations in *ERG3* alleles from the known *C.albicans* azole-resistant Darlington strain were characterized recently[27]. Unfortunately, the effect of these mutations on azole resistance were masked by other azole resistance mechanisms in this strain[27].

Azole resistance mechanism and their combination in clinical isolates

In some studies investigating resistance mechanisms to azoles in clinical isolates, it was possible to recover sequential isolates from patients treated with these compounds showing a stepwise increase in azole resistance, as measured by susceptibility testing. The stepwise increase in azole resistance was indicating that different resistance mechanisms could operate and, through their sequential addition, explain the increase in azole MIC values. Several examples have been reported documenting 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, resulting for example in MIC values for fluconazole exceeding 64 μg/ml[28]. Alterations of the target enzymes by several distinct single or multiple mutations and upregulation of multidrug transporters from two different families gives a large flexibility for 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 that there are only a 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 large populations of azole-resistant isolates has been investigated in only a few studies. Perea et al[28] showed that 85% of azole-resistant isolates were upregulating multidrug transporter genes and that 65% were containing *ERG11* mutations linked to azole resistance. Overall 75% of the azole-resistant isolates were combining resistance mechanisms. These numbers matched our own data including the isolates of 18 HIV^+ patients in which azole-resistant isolates could be recovered: 82% of these isolates showed upregulation of multidrug transporter genes; 63% contained *ERG11* mutations linked to azole resistance; 50% showed combination of resistance mechanisms (D. Sanglard, unpublished). 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 approximately in twice as many azole-resistant isolates than is observed for isolates with *MDR1* upregulation.

Combination of resistance mechanisms is not always linked with high levels of resistance. In *C. glabrata* azole-resistant isolates, a single resistance mechanism (i.e. upregulation of the *CgCDR1* ABC-transporter gene) is responsible for acquisition of 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 of fluconazole MIC values near to those obtained in the parental azole-susceptible isolate[29].

Alternative mechanisms of azole resistance

Besides the resistance mechanisms described above, alternative pathways for the acquisition of azole resistance can be used by yeast and fungi. One interesting alternative for development of azole resistance uses the ability of fungal pathogens to form 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[30]. As reported in *C. albicans*, the expression of genes involved in azole resistance (i.e. multidrug transporter genes) can also be altered in biofilms and may participate to the relatively high azole resistance measured in the cell population of these dense structures[30]. The clinical relevance of biofilm formation and its coupled resistance to the action of antifungal agents is still under debate. There are at least two situations where biofilms can form *in vivo*: when cells grow as multilayers on mucosal surfaces (as seen in oropharyngeal candidiasis) or on synthetic surfaces of catheters. Resistance to antifungal agents by biofilm formation is therefore limited to specific clinical presentations.

Current situation of resistance to antifungal drugs

Antifungal resistance over the last 10 to 15 years has been seen with triazole antifungals (fluconazole, itraconazole) in relation to oropharyngeal *Candida* infections associated with HIV. However, with the introduction of highly active antiretroviral therapy (HAART) for HIV infection, the oral *Candida* problem has decreased and azole-resistant isolates from AIDS patients are now rarely isolated. The extensive use of azole antifungals during this period, either for treatments or prophilaxis of fungal diseases, could have been a favorable ground for the emergence of yeast species intrinsically resistant, such as *C. krusei* or *C. glabrata*. Available prospective data from oral and vaginal samples from more than 1220 women between 1993 and 1995 published by Sobel et al[32] showed however little shift in the spectrum of species with *C. albicans* accounting for 87% of isolates at the start of the study, 84% after one year and 83% after two years. The data for HIV-negative patients showed a similar (82-87%) prevalence of *C. albicans*. Therefore, azole usage has little effect on the prevalence of *Candida* species originating from mucosal surfaces towards non-*C. albicans* species with intrinsic resistance. Large surveillance studies performed in North America and in Europe have looked at the problem of antifungal resistance in disseminated infections. In a recent review[33] discussing this issue, the main conclusion was that for *C. albicans*, which is the main cause of candidemia, no significant shift in fluconazole or itraconazole MICs has been yet measured. Some population shift towards intrinsically resistant non-*C. albicans* species has been reported in specific institutions and the compilation of data presented in Sanglard and Odds[33] support a correlation of *C. glabrata* and *C. krusei* prevalence with the introduction of fluconazole therapy.

Conclusions

Studies on resistance mechanisms to antifungal agents have delivered the many different resources utilized by simple microorganisms to circumvent the effect of growth inhibitory substances. Several basic biological processes have emerged from these studies and will continue to be investigated and can be used for the purpose of new antifungal drug screening. One of the promising fields of investigation is the dissection of the pathways controlling 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 ideally should be non-existent or maintained to the minimum to minimize the use of compound extrusion as a potential resistance mechanism. Physicians faced with the treatment of fungal diseases have to take into account that yeast pathogens have versatile tools for raising resistance mechanisms: this phenomenon has been seen with azole resistance in AIDS patients before the introduction of antiretroviral therapy. New antifungal agents with improved properties (voriconazole) or with new mode of actions (candins) are now becoming available and offer attractive alternatives in the treatments of these diseases.

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ANEXO 1. **Clinical relevance of mechanisms of antifungal drug resistance in yeasts**

1. **La anfotericina B es un antifúngico incluido dentro del grupo de:**
   a) Equinocandina.
   b) Análogos de pirimidina.
   c) Polienos.
   d) Alilaminas.
   e) Ninguna de las anteriores es cierta.

2. **Indique cuál de las siguientes especies presenta resistencia intrínseca a anfotericina B:**
   a) Candida albicans.
   b) Cryptococcus neoformans.
   c) Candida glabrata.
   d) Candida torpicalis.
   e) Candida lusitaniae.

3. **La diana celular de los azoles es:**
   a) El citocromo P-450.
   b) Los enzimas de la síntesis de ADN.
   c) La ARN polimerasa.
   d) El ADN mitocondrial.
   e) La betaglucano sintetasa.

4. **La resistencia a los azoles en levaduras está causada por:**
   a) Eliminación activa.
   b) Síntesis alterada del ergosterol.
   c) Alteración de Erg11p.
   d) Las respuestas a), b) y c) son correctas.
   e) Ninguna de las respuestas anteriores es correcta.

5. **El gen *CDR1* de las levaduras codifica:**
   a) Una bomba de eliminación de la familia de facilitadores mayores.
   b) Un transportador de la familia ABC *(ATP-binding cassette)*.
   c) El citocromo P-450.
   d) Una desaturasa de esterol.
   e) Una sintetasa de ergosterol.

6. **El fenotipo de alta resistencia a azoles HFRA ("high frequency azole resistance") ha sido descrito en:**
   a) Candida albicans.
   b) Candida glabrata.
   c) Cryptococcus neoformans.
   d) Saccharomyces cerevisiae.
   e) Trichosporon spp.

7. **El incremento de la actividad controlada por el gen *ERG11* puede ocurrir por:**
   a) Duplicación génica.
   b) Regulación de la transcripción del gen.
   c) Inserción de una secuencia de inserción por delante del promotor del gen.
   d) Aumento de la actividad específica de las topoisomerasas tipo II de las levaduras.
   e) Las respuestas a) y b) son correctas.

8. **Las biocapas formadas por *Candida albicans*:**
   a) Son más sensibles a la anfotericina B que las células planctónicas.
   b) Son más resistentes a la anfotericina B que las células planctónicas.
   c) Son más sensibles a los azoles B que las células planctónicas.
   d) Son más resistentes a los azoles B que las células planctónicas.
   e) Las respuestas b) y d) son correctas.

9. **La resistencia de alto nivel a los azoles en levaduras aisladas de muestras clínicas se debe habitualmente a:**
   a) Combinación de varios mecanismos de resistencia.
   b) Regulación transcripcional de transportadores de la familia ABC *(ATP-binding cassette)*.
   c) Mutaciones puntuales en *ERG11*.
   d) Pérdida de proteínas formadoras de canales por donde penetran los azoles.
   e) Delección de los genes que controlan la síntesis de ergosterol.

10. **La principal causa del descenso de cepas de *Candida albicans* resistentes a fluconazol en paciente con candidiasis orofaríngea infectados con el virus de la inmunodeficiencia humana es:**
    a) El tratamiento masivo de los pacientes con anfotericina B.
    b) El tratamiento de los pacientes con nuevos azoles.
    c) Aparición de cepas más virulentas con menor capacidad de resistencia.
    d) Tratamiento antirretroviral efectivo que ha disminuido la frecuencia de candidiasis orofaríngea.
    e) Todas las respuestas anteriores son correctas.

Nota. Las respuestas de las preguntas están en la página 479.