European **Rev**iew for **Med**ical and **Pharmacol**ogical **Sci**ences 2004; 8: 69-77

Fluconazole resistance in *Candida albicans*: a review of mechanisms

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Abstract. – **Antifungal agents have greatly contributed to the improvement of public health. Nevertheless, antifungal resistant pathogens have increased during the past decade, becoming a serious concern. *Candida albicans* has been the most extensively studied pathogen in antifungal resistance because of their morbidity and mortality associated with infections in immunocompromised patients. This review describes the antifungal mechanims of the azole fluconazole widely used for the prophylaxis and treatment of candidal infections. The specific molecular pathways occurring in fluconazole-resistance of *C. albicans* and some issues about new antifungal agents are also discussed.**

Key Words:

Fluconazole, Candida albicans, Ergosterol, Antifungal resistance.

Introduction

In recent years, fungal infections have increased prevalently in immunocompromised hosts as a consequence of HIV infection, aggressive therapies for cancer, autoimmune disease and organ or tissue transplantion. *Candida albicans*, a commensal fungus of the oral cavity and gastrointestinal tract in humans, represents one the major causes of mucosal infection and systemic infection, which can be life threatening if not treated. Commonly used antifungal drugs inhibit membrane component sterol biosynthesis (azoles, allylamines and morpholines), directly interact with the cell membrane (polyenes) or target cell wall biosynthesis (echinocandins).

Resistance to azole antifungals was reported in the late 1980s in *C. albicans* after prolonged therapy with miconazole and ketoconazole.

Fluconazole is a bis-triazole discovered in the 1990s. This compound has been shown to possess potent antifungal activity against yeasts, dermatophytes and dimorfic fungi such as *C. immitis*, *H. capsulatum*, *B. dermatitidis*, *P. brasiliensis* and *S. schenckii*[1].

In spite of its widespread use in the medical community, many reports described the clinical failure of fluconazole therapy in individuals with HIV infection[2-4].

Recently, fluconazole-resistant *C. albicans* strains and intrinsically resistant *Candida* species such as *C. glabrata* and *C. krusei* are emerging in immunocompromised patients treated for therapy or prophylaxis[5-8].

These and other data have led to research on the molecular mechanisms operating to confer fluconazole resistance.

In this article we review the current knowledge on the principal resistance mechanisms to fluconazole (Table I). In addition, other potential explanations resulting from new experimental data about the above-mentioned mechanisms are discussed. The findings have lead to a new therapeutic approach in the prevention or control of *Candida* infections.

Fluconazole

Azoles are antifungal agents categorized into imidazoles and triazoles (Figure 1).

Imidazole compounds (miconazole, clotrimazole and ketoconazole) consist of a five-membered ring structure containing two nitrogen atoms with a complex side chain attached to one of the nitrogen atoms.

Table I. Overview of fluconazole resistance mechanisms in *C. albicans*.

Molecular basis of fluconazole resistance	*Final change accounting for resistance*	*References*
Modifications in the ERG11 gene by:		
Point mutations[a]	Reduced drug affinity for the target enzyme	[25-30] [33]
Overexpression	Increased ergosterol synthesis	[15-17] [19-21]
Alterations in other enzymes of the ergosterol biosynthetic pathway (e.g. C5,6-desaturase)	Production of various sterols supporting growth; cross-resistance to other azoles and AmB	[34-46]
Overexpresssion of CDRs and MDR genes encoding efflux pumps[b]	Reduced drug accumulation in the cell	[47-51]
Mechanisms to be defined	Variations in plasma membrane components	[56, 60]
	Altered cell wall proteins	[63, 64]

[a]Allelic differences elimination by gene conversion or mitotic recombination lead to identical mutations in two alleles; the resulting phenotype is significantly more resistant.
[b]CDRs genes are associated with cross-resistance to other azoles.

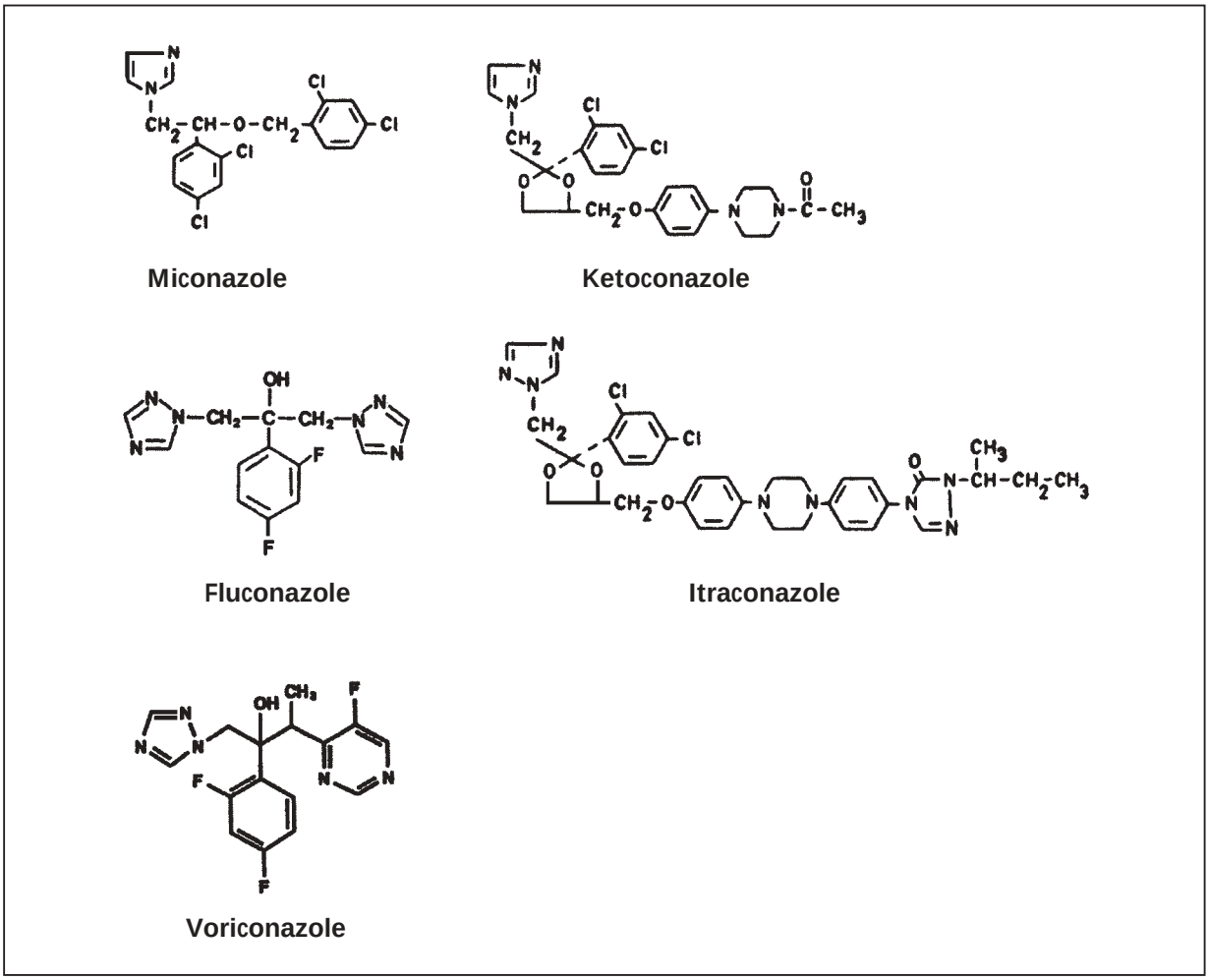

Figure 1. Chemical structures of azole antifungal agents.

Fluconazole and itraconazole are triazole compounds containing an additional nitrogen in the ring[9]. Other antifungals of new generation such as posaconazole, ravuconazole and voriconazole, also belong to triazoles.

The azole compounds inhibit the lanosterol demethylase enzyme (or 14α-sterol demethylase); this enzyme converts lanosterol to ergosterol removing the 14α-methyl group from lanosterol. The 14α-sterol demethylase is a cytochrome P450-dependent enzyme (P450-Erg11p or Cyp51p) which contains a heme moiety in its active site. The azoles bind to the heme iron through an unhindered nitrogen, thus inhibiting the enzymatic reaction. In addition, a second nitrogen of the azoles interacts directly with the apoprotein of lanosterol-demethylase. It is thought that the affinity of different azoles for the enzyme is also determined by the position of this second nitrogen[10-12].

Ergosterol is the predominant sterol in fungal plasma membranes; it is important for membrane integrity and for the activity of many membrane-bound enzymes.

The inhibition of 14α-sterol demethylase leads to the accumulation of 14α-methylated sterols, resulting in a defective cell membrane with decreased availability of ergosterol and altered permeabilty of the fungal cell.

Azoles also inhibit mammalian cytocrome P450 enzymes which convert lanosterol to cholesterol. However, the azoles used in therapeutic concentrations demonstrate greater affinity for fungal P-450 demethylase than for the mammalian enzyme. Fluconazole appears to be free of adverse effects on steroid hormone production[1] and it is available in both intravenous and oral formulations. Because of the low toxicity and ready distribution into aqueous body fluids such as cerebrospinal fluid (CSF), fluconazole has been used in the treatment of both superficial and systemic fungal infections.

Fluconazole displayed less toxicity than amphotericin B (polyene antifungal agent discovered in the early 1950s), a favourable pharmacokinetic profile (metabolic stability, water solubility) and availability as an oral and parenteral formulation. These factors have contributed to its therapeutic use in both normal and immunocompromised hosts.

Adverse effects associated with fluconazole therapy such as nausea, headache, skin rash, abdominal pain, vomiting and diarrhoea, hepatotoxicity, have rarely been reported.

Prophylactic administration of fluconazole has been reserved for selected patients considered to be at high risk of candidemia[13]. In particular, invasive fungal infections have become increasingly prevalent in individuals with impaired immune defenses including neutropenic patients, HIV-infected patients and transplant recipients. The agreement for the fluconazole-prophylaxis is still controversial[14]. However, there is a general consensus that resistant strains are related to drug exposure.

Fluconazole is fungistatic; this makes it clear that host factors contribute to the outcome of antifungal therapy.

ERG11 and Other ERG Genes

In all fungal species, ERG11 (also described as ERG16, CYP 51A1) is the gene encoding ERG11p or lanosterol 14α-demethylase, an essential enzyme for ergosterol synthesis. Resistance to azole antifungal drugs has been associated with ERG11 gene overexpression and/or point mutations and also alterations in the ergosterol biosynthetic pathway. Overexpression of ERG11 causes an increased copy number of the enzyme lanosterol 14α-demethylase and results in increased ergosterol synthesis which overwhelms the capacity of the antifungal drug. The effect of ERG11 gene overexpression on antifungal susceptibility has been described by several studies in *C. albicans*[15-17] and also in *C. glabrata* and *C. dubliniensis* clinical isolates[4,18,19].

Enhanced expression of the ERG11 gene in *C. albicans* as a consequence of azoles exposure was observed in matched sets of clinical isolates from the same strain[20,21]. In vitro azole-dependent ERG11 upregulation was demonstrated in additional *Candida* species such as *C. tropicalis*, *C. glabrata* and *C. krusei*[22].

Recently, in an analysis of unmatched sets of clinical isolates it was found that resistance did not correlate with overexpression of ERG11[23]. Moreover, it has been reported that depletion of the ERG11 gene in *C.*

glabrata results in the accumulation of 4,14-demethylzimosterol, which did not cause defective growth of fungal cells in vitro and in vivo[24].

Fluconazole and other azoles resistance has also been associated with point mutations of the ERG11 gene[25-27]; these mutations result in conformational changes that reduce effective binding between azoles and their target.

Several investigators found sequence differences of the ERG11 gene in fluconazole-resistant *C. albicans* and in *S. cerevisiae* transformants[28-30]. A list of different aminoacid exchanges has been provided by different studies that could simply reflect allelic variations[31]. In fluconazole-resistant *C. albicans* isolates frequently observed nucleotide changes were concerned with two aminoacids located near the heme binding site (R467K [arginine 467 replaced by lysine] and G464S [glycine 464 replaced by serine]); this probably resulted in structural or functional alterations reducing fluconazole affinity in Erg11p.

The correlation between decreased susceptibility to azole drugs and nucleotide changes in the ERG11 sequence was not always observed[32].

Recently, other nucleotide substitutions in ERG11 gene were identified (K143R [lysine 143 replaced by arginine], E266D [glutamic acid 266 replaced by aspartic acid], V404L [valine 404 replaced by leucine], V488I [valine 488 replaced by isoleucine]) in three *C. albicans* isolates[33]; these mutations were associated with the fluconazole resistance phenotype. As suggested by investigators, a single aminoacid change, not interacting with the active site of ERG11p, was unrelated to drug resistance. Moreover, mesh membrane structure developments were observed in the endoplasmic reticula of resistant cells[33].

Several molecular and genetic studies have described other ERG genes involved in the complex ergosterol biosynthesis as alternative pathways, which were more or less correlated to fluconazole exposure: ERG1, ERG2, ERG3, ERG4, ERG5, ERG6, ERG7, ERG9, ERG10, ERG13, ERG19, ERG24, ERG25, ERG26[34-41].

In *C. albicans*, increased expression of ERG1 gene encoding squalene epoxidase and of ERG2 gene encoding C8-sterol isomerase was associated with fluconazole resistance[22,34,35]. In contrast, other studies found that the ERG1 gene was repressed in resistant isolates[42].

The ERG3 gene encoding C5,6-desaturase was observed first in *S. cerevisiae*[43]. Defective sterol C5,6-desaturase was attributed as the cause of fluconazole resistance in *C. albicans* clinical isolates from AIDS patients[44]. Such isolates accumulated ergosterol precursors including ergosta-7-enol and ergosta-7,22-dienol. The molecular mechanisms associated with ERG3 defects are still unclear[45,46].

Expression of Two Major Efflux Pumps

Efflux pumps belong to two different classes: the ATP-Binding Cassette (ABC) transporters and the Major Facilitators Superfamily (MFS).

The ABC transporters are energy-dependent by ATP hydrolysis; the MFS transporters operate through a proton gradient. Two ABC transporters genes, CDR1 and CDR2 (Candida Drug Resistance), as well as that encoding a major facilitator, CaMDR1 (Candida albicans Multidrug Resistance), have been shown to be overexpressed[47-51] in *C. albicans* azole-resistant isolates. CaMDR1 is specific for fluconazole resistance but not for other azoles[48]. Upregulation of these efflux pumps reduces the effective concentrations of fluconazole in the fungal cell and is correlated to azole resistance in *C. albicans*. Genetic deletion of the CDR1 gene resulted in hypersusceptibility to azole drugs[52], whereas CDR2 gene disruption did not cause hypersusceptibility to these agents. The latter gene is closely related to CDR1 and disruption of CDR1 and CDR2 resulted in increased hypersusceptibility to azole antifungals[49].

MDR1 (previously named BEN^r, associated with benomyl resistance in *S. cerevisiae*) gene deletion in resistant strains of *C. albicans* does not result in increased susceptibility to azoles[52].

Some experiments have found that increased mRNA levels of CDR probe (CDR1 through CDR4) correlated with increased resistance to fluconazole, ketoconazole and

itraconazole[37]. This resistance, however, arose rapidly after fluconazole exposure and was transient. In fact, susceptibility resulted in azole-free media and also in vivo after the drug was no longer administered to the patient[53].

To date, the molecular mechanisms involving the efflux pumps (CDR genes and CaMDR) have not yet been elucidated. Recently, it has been shown that Cdr1p and Cdr2p (proteins encoded by CDR1 and CDR2 genes) in *C. albicans* act as phospholipids translocators eliciting in-to-out transbilayer phospholipid movement of plasma membrane. It is interesting that fluconazole could inhibit this transbilayer movement[54]. Moreover, Camdr1p showed no detectable exchange activity[55].

Recent results show that in vitro acquired resistance to fluconazole of *C. albicans* strains was associated with variation in membrane lipid fluidity and asymmetry[56].

Microarrays technology used to examine differences in gene expression identified new genes associated or not with drug resistance in *C. albicans*. Several of these genes were coordinately regulated with both CDR genes and CaMDR1, whereas others appeared not to be coordinately regulated with known resistance genes[35,36]. These data suggest that the efflux pumps may be regulated by combined expression of several genes. Analysis of these differentially regulated genes requires further investigation and opens up the possibility of finding new targets for antifungal therapeutics.

Other Changes in Fluconazole Resistance

Recently, antifungal resistance results in biofilm-associated infections[57-59]. Efflux pumps do not appear to contribute to fluconazole resistance in *C. albicans* at late (intermediate and mature) stages in biofilm formation[60], but solely in the early-phase. On the contrary, changes in sterol profile were expressed by resistant phenotypes at intermediate and mature phases. Therefore, phase-specific mechanisms are suggested to be operative in antifungal resistance of biofilm cells[60].

Some of the *C. albicans* cell wall glycoproteins have been found to be highly immunogenic and differently modulated according to fungal growth[61,62].

In vitro studies on the cell wall of fluconazole-susceptible and -resistant *C. albicans* strains detected altered distribution of cell wall glucan-associated proteins[63]. These results suggest that fluconazole treatment could have an effect on fungal cell wall metabolism and structure[63,64], and these effects may be stably incorporated into the cell wall upon acquisition of resistance[63].

The asexual and diploid nature of *C. albicans*[65,66] complicates the characterization of gene expression in antifungal drug resistance. Several studies investigating changes in chromosome copy number, loss (or not) of heterozygosity, gene disruption at definite loci and other genetic strategies have been linked to fluconazole resistance[67-70]. These studies show that other factors may contribute to fluconazole resistance development. However, a detailed analysis of these and other promising findings goes beyond our purposes.

Different Targets and New Therapeutic Approaches

Several studies aimed at identifying new regulatory patterns and new antifungal treatments are currently being undertaken.

Recently, cyclic AMP (cAMP) signaling pathway and modulation of the susceptibility to antifungal azoles have been examined. *C. albicans* mutants in the genes encoding the proteins responsible for cAMP synthesis showed pronounced hypersusceptibility to fluconazole and other sterol biosynthesis inhibitors[71]. The addition of cAMP conferred partial-to-complete reversal of this hypersusceptibility. These data suggest that antifungal susceptibility could be modulated by adenylate cyclase inhibitors.

The immunosuppressants Cyclosporine A (CsA) and tacrolimus hydrate (FK506, a 23-member macrolide) are promising candidates for antifungal therapy, due to their synergistic fungicidal effect in combination with azoles and non-azole antifungal agents[72,73]. Cyclosporine has several cellular targets including cell membrane, multidrug efflux

transporters and the cyclophilin-calmodulin-calcineurin pathway. The mechanism of this fungicidal synergism is unknown and was recently reported not to be involved with multidrug efflux transporters[74].

The use of immunoadjuvants added to antifungal drugs could also be another therapeutic approach[75].

Other agents currently being developed, are cell wall biosynthesis inhibitors. Moreover, novel antifungals with a broad spectrum of susceptibility are being proposed to circumvent cross-resistance within the fungal species[76,77].

In conclusion, in this review we have summarized the mechanisms of resistance to fluconazole in *C. albicans.* Excellent reviews, which the reader is referred to, have been published concerning this matter[77-83]. Our aim was to analyze (with ease) what is known about molecular factors involved and/or correlated to antifungal resistance in this organism. The resistant phenotype appears to result from different mechanisms not always arising. However it is possible that other resistance mechanisms are unknown. It could be interesting to investigate the cause of this variability and, if it exists, the specific step responsible for fluconazole resistance. Yet, a combination of different, not only antifungal, drugs could be a promising therapeutic strategy.

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Acknowledgments

We thank Emanuele Rodolà for his excellent technical assistance. This work was supported by MIUR, 60% and progetto FIRB n° RBNE01P4B5-006 and MINSAN, ICS 120.5/RF00.121.