Available online at www.sciencedirect.com
ScienceDirect
Diagnostic Microbiology and Infectious Disease 66 (2010) 373–384
DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE
www.elsevier.com/locate/diagmicrobio

Mycology

Screening for amino acid substitutions in the *Candida albicans* Erg11 protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature

Florent Morio[a,b], Cedric Loge[a], Bernard Besse[c], Christophe Hennequin[d], Patrice Le Pape[a,b,*]

[a]*Université de Nantes, Nantes Atlantique Universités, Département de Parasitologie et Mycologie Médicale, EA 1155—IICiMed, Faculté de Pharmacie, 1 rue Gaston Veil, 44035 Nantes, France*
[b]*CHU Nantes, Laboratoire de Parasitologie-Mycologie, Institut de Biologie, 5 allée de l'île Gloriette, 44000 Nantes, France*
[c]*CHU Nantes, Laboratoire de Virologie, Institut de Biologie, 5 allée de l'île Gloriette, 44000 Nantes, France*
[d]*Assistance Publique des Hôpitaux de Paris, Hôpital Saint-Antoine, Laboratoire de Parasitologie-Mycologie, 184 rue du Faubourg St Antoine, 75012 Paris, France*

Received 30 August 2009; accepted 12 November 2009

Abstract

For several years, azole antifungal drugs have been a treatment option for potentially life-threatening *Candida* infections. However, azole resistance can occur through various mechanisms such as alterations in *ERG11*, encoding lanosterol 14α-demethylase (CYP51). In this study, we investigated the antifungal susceptibility to fluconazole, itraconazole, and voriconazole of 73 clinical isolates of *Candida albicans*. Screening for amino acid substitutions in Erg11 was performed on each of the 73 isolates. Twenty isolates displayed a marked decrease in azole susceptibility. Amino acid substitutions were detected in more than two-thirds of the strains. In all, 23 distinct substitutions were identified. Four have not been described previously, among which N136Y and Y447H are suspected to be involved in azole resistance. We suggest that the high genetic polymorphism of *ERG11* must be considered in the rationale design of new azole compounds targeting lanosterol 14α-demethylase. A review of all Erg11 amino acid polymorphisms described to date is given.

Keywords: *Candida albicans*; *ERG11*; Azole drugs; Antifungal resistance; Amino acid substitutions

1. Introduction

Candida albicans is responsible for a wide spectrum of clinical infections in humans, ranging from mucosal infections such as vaginitis or oropharyngeal candidiasis to potentially life-threatening systemic infections such as candidemia. Because of their safety profile and high therapeutic index, azole antifungal drugs have been used to treat *Candida* infections for many years, as first-line therapy, antifungal prophylaxis, or empirical or preemptive treatment. However, as a consequence of long-term exposure to azole drugs, resistance can arise. To date, at least 4 distinct mechanisms have been shown to confer azole resistance in *C. albicans*: i) reduced intracellular accumulation of azoles due to the overexpression of genes encoding efflux transporters belonging to the Adenosine-5'-triphosphate (ATP)-binding cassette superfamily (*CaCDR1* and *CaCDR2*) or major facilitator superfamily (*CaMDR1*); ii) genetic alterations in the *ERG11* gene encoding lanosterol 14α-demethylase (CA-CYP51), the primary target of azoles; iii) overexpression of the *ERG11* gene; and iv) alterations in the ergosterol biosynthetic pathway (Sanglard and Odds, 2002). Importantly, these mechanisms are often combined in clinical isolates (Cernicka and Subik, 2006; Chau et al., 2004; Coste et al., 2007; Franz et al., 1998; Goldman et al., 2004). Recently, Selmecki et al. (2006) demonstrated that azole resistance could also result from the formation of an

* Corresponding author. Tel.: +33-2-40-41-28-66; fax: +33-2-40-41-28-66.
E-mail address: patrice.le-pape@univ-nantes.fr (P. Le Pape).

0732-8893/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.diagmicrobio.2009.11.006

Table 1
Review of all Erg11 amino acid substitutions described to date in clinical isolates of *C. albicans*

Amino acid substitutions in Erg11	Hot spot	References	Notes
Y33C		Cernicka and Subik, 2006	Described in combination with other amino acid changes in an FLZ-, ITZ-, and VOR-resistant isolate
F14L		Sanglard and Bille, 2002	
V19A		Sanglard and Bille, 2002	
L35S		Sanglard and Bille, 2002	
Y39C		Cernicka and Subik, 2006	Described in combination with other amino acid changes in an FLZ-, ITZ-, and VOR-resistant isolate
R44G		Sanglard and Bille, 2002	
P49R/T		Cernicka and Subik, 2006	Described in combination with other amino acid changes in an FLZ-, ITZ-, and VOR-resistant isolate
W54Stop		Cernicka and Subik, 2006	Described in combination with other amino acid changes in an FLZ-, ITZ-, and VOR-resistant isolate
W57K		Sanglard and Bille, 2002	
A61V[a,b]		Chau et al., 2004	Associated with resistance. Responsible for a 4-fold increase in FLZ MIC. Residue suspected to interact with POS and ITZ binding
F72L		Favre et al., 1999; Marichal et al., 1999	Described in azole-susceptible isolates
Y79C		Goldman et al., 2004	Described in combination with T199I in an FLZ-resistant isolate
D81G		Long et al., 2002	
F83Y		Jiang et al., 2006	
K90E		Sanglard and Bille, 2002	
K99T		Goldman et al., 2004	Described in combination with amino acid changes involved in azole resistance in an isolate with a moderately high FLZ MIC (8 μg/mL)
G100E		Sanglard and Bille, 2002	
F105L	I	Loffler et al., 1997	Described in azole-resistant strains as a single mutation or in combination with amino acid changes involved in azole resistance
A107T	I	Chau et al., 2004	Described as a single mutation in sequential isolates displaying a reduced susceptibility to FLZ
K108E	I	Wang et al., 2009	Described in combination with Y132H and S405F in an FLZ- and VOR-resistant isolate
S110P	I	Sanglard and Bille, 2002	
A114S	I	Jiang et al., 2006; Xu et al., 2008	Described as a single mutation in FLZ resistant isolates
A114V	I	Sanglard and Bille, 2002	
D116E[b]	I	Chau et al., 2004; Favre et al., 1999; Marichal et al., 1999; Perea et al., 2001; Sanglard et al., 1998; White et al., 2002; Xu et al., 2008	Not associated with resistance. Described in azole-susceptible and azole-resistant isolates
K119L	I	Cernicka and Subik, 2006	Described in an FLZ-, ITZ-, and VOR-resistant isolate
K119N	I	Xu et al., 2008	Described in an azole-susceptible isolate
F126L[b]	I	Favre et al., 1999; Perea et al., 2001	Described in azole-resistant isolates. Responsible for an increase in FLZ MIC when combined with K143R, E266D, S405F, or V437I
K128T[b]	I	Chau et al., 2004; Loffler et al., 1997; Marichal et al., 1999; Perea et al., 2001; Sanglard et al., 1998; White et al., 2002; Xu et al., 2008	Not associated with resistance Described in azole-susceptible and azole-resistant isolates

Table 1 (*continued*)

Amino acid substitutions in Erg11	Hot spot	References	Notes
G129A[a,b]	I	Sanglard et al., 1998	Described in an FLZ- and ITZ-resistant isolate. G129A alone is not sufficient to increase FLZ MIC. Cumulative effect with G464S
V130I	I	Goldman et al., 2004	Described in an FLZ-resistant isolate
Y132F[b]	I	Chau et al., 2004; Goldman et al., 2004; Perea et al., 2001	Associated with resistance. Significantly increase FLZ MIC
Y132H[a,b,c]	I	Chau et al., 2004; Favre et al., 1999; Kakeya et al., 2000; Marichal et al., 1999; Sanglard et al., 1998; Xu et al., 2008	Associated with resistance. Responsible for a 4-fold increase in FLZ MIC. Cumulative effect with S405F and R467K
N136Y	I	This article	Described in combination with Y132H in a single isolate with reduced azole susceptibility
R138K	I	Sanglard and Bille, 2002	
M140T	I	Sanglard and Bille, 2002	
M140R	I	Xu et al., 2008	Described in a azole-susceptible isolate
K143E[b]	I	Favre et al., 1999; Goldman et al., 2004	Described in combination T229 and P503L in isolates with reduced azole susceptibility. K143E significantly increase FLZ MIC when combined with T229A
K143R[b]	I	Chau et al., 2004; Goldman et al., 2004; Lee et al., 2004; Manavathu et al., 1999; Perea et al., 2001; White et al., 2002	Associated with resistance. Responsible for a 64-fold increase in FLZ MIC
F145L	I	Chau et al., 2004; Goldman et al., 2004	Described in azole-susceptible and azole-resistant isolates
K147R	I	Loffler et al., 1997	Described in an azole-susceptible isolate
A149V	I	Marichal et al., 1999	Described in FLZ- and ITZ-resistant isolates
D153E	I	Marichal et al., 1999	Described in an azole-susceptible isolate
R157K	I	Sanglard and Bille, 2002	
V159I	I	This article; Sanglard and Bille, 2002	Described in combination with D116E and K128T in azole-susceptible isolates
K161N	I	Xu et al., 2008	Described in an azole-susceptible isolate
R163T	I	Xu et al., 2008	Described in an azole-susceptible isolate
E165Y	I	Marichal et al., 1999	Described in an azole-susceptible isolate
E165K	I	Xu et al., 2008	Described in an azole-susceptible isolate
S175G		Sanglard and Bille, 2002	
T199I		Goldman et al., 2004	Described in combination with Y79C in an azole-resistant isolate
S203F		Sanglard and Bille, 2002	
F205L		Sanglard and Bille, 2002	
Y221H		This article	Described in an single-azole susceptible isolate
D225Y		Xu et al., 2008	Described in an azole-susceptible isolate
D225H		Xu et al., 2008	Described in an azole-susceptible isolate
T229A[b]		Favre et al., 1999; Perea et al., 2001	Described in azole-resistant isolates. Responsible for a significative increase in FLZ MIC when combined with F449S or K143E
P230L		Li et al., 2004; Xiao et al., 2004	In combination with other amino acid changes, P230L confers increase resistance to POS and ITZ. P230 is predicted to be in close contact with POS side chain
N237Y		Sanglard and Bille, 2002	
I253V		Goldman et al., 2004	Described as a single mutation in an azole-resistant isolate
Y257H[b]		Chau et al., 2004; Xiao et al., 2004; Xu et al., 2008	Only described in azole-resistant isolates. Combination with G464S significantly increases FLZ MIC
R265G		Goldman et al., 2004	Described in an azole-susceptible isolate
E266D	II	Chau et al., 2004; Favre et al., 1999; Goldman et al., 2004; Loffler et al., 1997; Marichal et al., 1999; White et al., 2002; Xu et al., 2008	Not associated with resistance. Described in azole-susceptible and azole-resistant isolates
E266Q[b]	II	Sanglard et al., 1998	Not associated with resistance. Described in azole-susceptible isolates
R267H	II	Manavathu et al., 1999	Described in combination with K128T, K143R, E266D, and D278E in 2 FLZ resistant isolates
K274H	II	Sanglard and Bille, 2002	
L276S	II	This article	Described in a single azole-susceptible isolate
D278N[b]	II	Li et al., 2004	Described in combination with D116E, K128T, Y132H, and G464S in sequential azole-resistant isolates

(continued on next page)

Table 1 (*continued*)

Amino acid substitutions in Erg11	Hot spot	References	Notes
D278E	II	Manavathu et al., 1999	Described in combination with D116E, K128T, K143R, E266D, and R267H in 2 FLZ-resistant isolates
S279F	II	Marichal et al., 1999	Described in combination with Y132H and G465S in an FLZ- and ITZ-resistant isolate
H283D	II	Goldman et al., 2004	Described as a single mutation in an isolate with reduced azole susceptibility to FLZ
H283R	II	Chau et al., 2004	Described in combination with Y132H and G464S in an FLZ-, ITZ-, and VOR-resistant isolate
K287R	II	Loffler et al., 1997, Manavathu et al., 1999	Described only in azole-resistant isolates
D294G		Long et al., 2002	
A298T		Sanglard and Bille, 2002	
A298V		Sanglard and Bille, 2002	
G303D		Goldman et al., 2004	Described in an azole-susceptible isolate
I304N		Sanglard and Bille, 2002	
I304T		Sanglard and Bille, 2002	
L305P		Goldman et al., 2004	Described in an azole-susceptible isolate
G307S[b]		Chau et al., 2004; Goldman et al., 2004; Perea et al., 2001	Probably involved in azole resistance. Mutation only described in azole-resistant isolates. Combination with D116E and G450E or Y257H and G464S confers azole resistance
H310R		Sanglard and Bille, 2002	
H334C		Sanglard and Bille, 2002	
K342R		Goldman et al., 2004	Described in an azole-susceptible isolate
K344R		Sanglard and Bille, 2002	
P360S		Sanglard and Bille, 2002	
I366T		Sanglard and Bille, 2002	
H373L		Sanglard and Bille, 2002	
M374V		Long et al., 2002	
P375Q		Xu et al., 2008	Described in an azole-susceptible isolate
L376V		Sanglard and Bille, 2002	
F380L		Sanglard and Bille, 2002	
F380S		Goldman et al., 2004	Described as a single mutation in an FLZ-resistant isolate
R381I		Xu et al., 2008	Described in an azole-susceptible isolate
P386L		Long et al., 2002	
E391G		Wang et al., 1999	
K398N		Sanglard and Bille, 2002	
H400R		Long et al., 2002	
V404L		Maebashi et al., 2003	Described in an FLZ-resistant isolate
V404I		Lee et al., 2004	Described in an isolate with reduced azole susceptibility to FLZ
S405F[a,b,c]	III	Chau et al., 2004; Favre et al., 1999; Perea et al., 2001; Sanglard et al., 1998	Associated with resistance. Responsible for a 4-fold increase in FLZ MIC. Cumulative effect with Y132H
S405P	III	Sanglard and Bille, 2002	
G421D	III	Sanglard and Bille, 2002	
R426K	III	Sanglard and Bille, 2002	
A432C	III	Sanglard and Bille, 2002	
V437I[b]	III	Favre et al., 1999; Goldman et al., 2004; Lee et al., 2004; Perea et al., 2001; Sanglard et al., 1998; White et al., 2002	Not associated with resistance. Described in azole-susceptible and azole-resistant isolates
V439L	III	Sanglard and Bille, 2002	
N440K	III	Wang et al., 2005	
S442F	III	Sanglard and Bille, 2002	
G443E	III	Sanglard and Bille, 2002	
D446G	III	Sanglard and Bille, 2002	
D446N[b]	III	Perea et al., 2001	Described in an FLZ-resistant isolate. Combination with V437I has been associated with a significant increase in FLZ MIC
Y447G	III	Sanglard and Bille, 2002	
Y447H	III	This article	Described in combination with G307S in a single isolate with a reduced azole susceptibility to FLZ and ITZ
G448E	III	Loffler et al., 1997	Described in an FLZ- and ITZ-resistant isolate
G448R	III	White et al., 2002	Described in an FLZ- and ITZ-resistant isolate

Table 1 (*continued*)

Amino acid substitutions in Erg11	Hot spot	References	Notes
G448V[b]	III	Chau et al., 2004	Described in combination with Y132H in 2 FLZ-, ITZ-, and VOR-resistant isolates. Responsible for a 64-fold increase in FLZ MIC when combined with Y132H
F449L	III	Favre et al., 1999	Described in combination with D116E and Y132H in an FLZ- and ITZ-resistant isolate
F449S[b]	III	Chau et al., 2004; Perea et al., 2001	Associated with resistance. Responsible for a significant increase in FLZ MIC
F449V	III	Lee et al., 2004	Described as a single mutation in an isolate with reduced azole susceptibility to FLZ
F449Y	III	Xu et al., 2008	Described as a single mutation in an FLZ-resistant isolate
G450E[b]	III	Chau et al., 2004; Favre et al., 1999; Goldman et al., 2004; Loffler et al., 1997, Perea et al., 2001	Described only in azole-resistant isolates. Combination with D116E or Y132H increase FLZ MIC
G450R	III	Sanglard and Bille, 2002	
G450V	III	Wang et al., 2009	Described in combination with G307S in an FLZ-resistant isolate
V452A	III	Chau et al., 2004; Marichal et al., 1999	Described only in azole-resistant isolates
V456I	III	This article; Sanglard and Bille, 2002	Described in combination with E266D, G464S, and V488I in a single FLZ-resistant isolate
Y460H	III	Sanglard and Bille, 2002	
G464S[a,b,c]	III	Chau et al., 2004; Franz et al., 1998; Li et al., 2004; Loffler et al., 1997; Marichal et al., 1999; Perea et al., 2001; Sanglard et al., 1998	Associated with resistance. Responsible for a 64-fold increase in FLZ MIC. Cumulative effect with G129A and R467K
G465S	III	Marichal et al., 1999	Described in combination with Y132H and S279F in an isolate with reduced azole susceptibility to FLZ and ITZ
R467I[b]	III	Chau et al., 2004	Described in combination with G464S in an FLZ- and VOR-resistant isolate. Responsible for an 8-fold increase in FLZ MIC when combined with K128T and G464S
R467K[a,b,c]	III	Lamb et al., 2000; Lee et al., 2004; Sanglard et al., 1998; White, 1997	Associated with resistance. Responsible for a 4-fold increase in FLZ MIC. Cumulative effect with G464S. Reduce CYP51 affinity for FLZ
H468Y	III	Sanglard and Bille, 2002	
I471T[a]	III	Kakeya et al., 2000; Xu et al., 2008	Associated with resistance. Cumulative effect with Y132H Mutation only described in azole-resistant isolates
I471V	III	Sanglard and Bille, 2002	
Q474K	III	Long et al., 2002; Xu et al., 2008	Described as a single mutation in an FLZ-resistant isolate
L480S	III	Sanglard and Bille, 2002	
T486P	III	Cernicka and Subik, 2006	Described in combination with other amino acid changes in an FLZ-, ITZ-, and VOR-resistant isolate
F487L	III	Wang et al., 1999	
V488I	III	Chau et al., 2004; Franz et al., 1998; Goldman et al., 2004; Loffler et al., 1997, Xu et al., 2008	Not associated with resistance. Described in azole-susceptible and azole-resistant isolates
V488G	III	Wang et al., 1999	
L491V		Cernicka and Subik, 2006	Described in combination with other amino acid changes in an FLZ-, ITZ-, and VOR-resistant isolate
R492G		Sanglard and Bille, 2002	
T494A		Cernicka and Subik, 2006	Described in combination with other amino acid changes in an FLZ-, ITZ-, and VOR-resistant isolate
T494I		Sanglard and Bille, 2002	
T494P		Wang et al., 1999	
P503L		Goldman et al., 2004	Described in combination with K143E in an FLZ-resistant isolate
D504G		Wang et al., 1999	
V509M		Chau et al., 2004; Lee et al., 2004	Described only in azole-resistant isolates
T513P		Sanglard and Bille, 2002	
W520G		Sanglard and Bille, 2002	
W520R		Sanglard and Bille, 2002	
E521D		Sanglard and Bille, 2002	

For each amino acid change, notes regarding the putative involvement in azole resistance and references are given. The new mutations identified in this study are underlined. Amino acid changes depicted in boldface have been clearly associated with azole resistance using the following in vitro experiments: a) increased MIC after heterologous gene expression in *Saccharomyces cerevisiae* by site-directed mutagenesis, b) or functional expression of *C. albicans* PCR-amplified *ERG11*, c) or decreased affinity of lanosterol 14α-demethylase for azole. The location of the 3 hot spot regions (I, II, and III) is indicated. FLZ = fluconazole; ITZ = itraconazole; VOR = voriconazole; POS = posaconazole.

isochromosome harboring *ERG11* and *TAC1* genes (encoding a transcription factor involved in *CDR1* and *CDR2* upregulation) through segmental aneuploidy.

To our knowledge, more than 140 different amino acid substitutions have been reported to date in Erg11 of clinical isolates of *C. albicans* (reviewed in Table 1). This high genetic polymorphism suggests that lanosterol demethylase is highly permissive to structural changes. Interestingly, most of these substitutions, instead of being randomly dispersed, are clustered into 3 hot spot regions ranging from amino acids 105 to 165, 266 to 287, and 405 to 488 (Marichal et al., 1999). Several lines of evidence indicate that these amino acid changes do not contribute equally to azole resistance. Whereas some substitutions such as K143R, S405F, G464S, R467K, or I471T have been recovered exclusively from azole-resistant strains and their involvement in azole resistance has been confirmed using in vitro experiments (heterologous gene expression, affinity between azoles and CA-CYP51), others such as E266D or V488I probably do not contribute to azole resistance because they are found in both azole-resistant and azole-susceptible strains (Chau et al., 2004; Kakeya et al., 2000; Lamb et al., 2000; Loffler et al., 1997; Sanglard et al., 1998). Finally, T315A, Y118A, Y118F, and Y118T have been clearly associated with resistance but have not yet been detected in clinical isolates (Chen et al., 2007; Lamb et al., 1997). Such findings clearly show that further study of azole-susceptible and azole-resistant strains is required for a better understanding of azole resistance mechanisms. In addition, there is now increasing evidence that mapping of all Erg11 amino acid changes involved in azole resistance onto the active site and channels of the 3-dimensional modeled structure of CA-CYP51 could help in the design of new azole antifungals with potent activity against resistant strains (Chen et al., 2007; Fukuoka et al., 2003; Rupp et al., 2005; Sheng et al., 2004, 2009; Xiao et al., 2004).

During the course of an ongoing project in our laboratory, aimed at the design and synthesis of new azole antifungal drugs through a modeling approach, Erg11 amino acid substitutions were screened in a large number of azole-susceptible and azole-resistant clinical isolates of *C. albicans* (Giraud et al., 2008; Lebouvier et al., 2007; Pagniez et al., 2002). All of the amino acid changes occurring in Erg11 are reviewed with reference to the literature.

2. Materials and methods

2.1. C. albicans strains

A collection of 73 clinical isolates of *C. albicans* from the Mycobank of the Laboratory of Parasitology and Medical Mycology, Nantes University Hospital, France, was investigated. Most of the strains (n = 62) were isolated during routine laboratory procedures from different hospitals. The remaining isolates were provided by the French National Reference Center for Mycoses and Antifungals (n = 11; Institut Pasteur, Paris, France). All strains were identified using either the VITEK2 system® or chromogenic medium *Candida* ID2® (bioMérieux, Marcy-l'Etoile, France). These strains, selected for their antifungal susceptibility profile (azole-susceptible as well as azole-resistant strains), represented a large variety of clinical samples. Eighteen isolates were recovered from blood cultures (Table 2).

2.2. Antifungal drug susceptibility testing

Antifungal susceptibility to fluconazole, itraconazole, and voriconazole was determined for each isolate using the broth microdilution reference method as recommended by the Clinical and Laboratory Standards Institute (CLSI) document M27-A2 (National Committee for Clinical Laboratory Standards, 2002). *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as controls. Fluconazole was purchased from Sigma (Saint Quentin Fallavier, France), itraconazole from Janssen-Cilag (Neuss, Germany), and voriconazole from Mycobiotics (Université de Nantes, France). MIC that is the lowest drug concentration that resulted in 50% growth inhibition relative to the growth in the control well was determined visually after 48 h of incubation at 35 °C. MIC values for fluconazole, itraconazole, and voriconazole were compared with the CLSI interpretative guidelines on antifungal susceptibility testing. Briefly, MICs ≤8 μg/mL were considered as susceptible (S), 16 to 32 μg/mL as susceptible dose dependent (SDD), and ≥64 μg/mL as resistant (R) for fluconazole; MICs ≤0.125 μg/mL as susceptible, 0.25 to 0.5 μg/mL as SDD, and ≥1 μg/mL as resistant for itraconazole; and MICs ≤1 μg/mL as susceptible, 2 μg/mL as SDD, and ≥4 μg/mL as resistant for voriconazole.

2.3 ERG11 gene amplification and sequencing

Amplification was performed directly from colonies grown on Sabouraud's agar plates without prior DNA extraction. Briefly, a single colony was gently removed with a micropipette tip and transferred directly to a polymerase chain reaction (PCR) tube. The complete *ERG11* open reading frame (1451 bp) was amplified with a PTC-100 thermocycler (MJ Research, Waltham, MA) using the primers described previously: ERG11ORF-F—GAAAGGGAATTCAATCG and ERG11ORF-R—TGTTAATCCAACTAAGTAAC (Lee et al., 2004). Reaction mixtures contained 1 μmol/L of each primer, 10 μL of 5× buffer, 2 mmol/L of $MgCl_2$, 0.2 mmol/L of each deoxyribonucleoside triphosphate, 0.1 U of GoTaq® Flexi DNA polymerase (Promega, Madison, WI), and sterile water up to a final volume of 50 μL. Amplification parameters were as follows: initial denaturation at 96 °C for 3 min followed by 35 cycles of annealing at 56 °C for 1 min, elongation at 72 °C for 2 min, and denaturation at 95 °C for 1 min. PCR products were purified and sequencing was performed using a BigDye terminator sequencing kit on a ABI PrismR 3130 genetic analyzer (Applied Biosystems, Foster City, CA) using additional primers (Lee et al., 2004).

Nucleotide sequences were assembled using Seqscape Navigator software (Applied Biosystems). For each strain, the entire *ERG11* open reading frame sequence was compared with a previously described *ERG11* sequence (accession number X13296) obtained from a fluconazole-susceptible strain (Lai and Kirsch, 1989). Silent mutations were not considered. Each substitution leading to an amino acid change was inspected visually for allelic dosage (homozygosity or heterozygosity). Each of the new amino acid change was checked by a second round of amplification and sequencing.

2.4. Nucleotide sequence accession number

The *ERG11* sequences of the strains displaying new amino acid substitutions have been submitted to GenBank database under accession numbers EU885932 to EU885938.

3. Results

3.1. Antifungal susceptibility testing

The results of in vitro susceptibility testing for the 73 clinical isolates of *C. albicans* are shown in Table 2. According to the CLSI breakpoints, most of the isolates were susceptible to fluconazole, itraconazole, and voriconazole (n = 53, 73%). Twenty isolates (27%) exhibited a marked decrease in azole susceptibility, including 9 that were resistant to at least 1 of the 3 antifungal drugs. More precisely, 15 isolates had reduced susceptibility to fluconazole (7 SDD and 8 R), 18 to itraconazole (12 SDD and 6 R), and 6 to voriconazole (1 SDD and 5 R). Cross-reduced azole susceptibility was observed for 6 isolates. Most of these isolates were recovered from the mouth or respiratory tract. All but 1 of the 18 bloodstream isolates were azole susceptible.

3.2. Analysis of Erg11 amino acid substitutions

Amplification of the complete *ERG11* gene and screening for amino acid substitutions was performed for each of the 73 clinical isolates (Table 2). As expected from previous studies, a large number of silent polymorphisms were identified (data not shown). Only 13 isolates had no amino acid substitution. All were azole susceptible and 6 were bloodstream isolates. Interestingly, among the remaining 60 isolates carrying amino acid substitutions in Erg11 (40 azole-susceptible and 20 isolates with reduced azole susceptibility), the number of amino acid substitutions varied between the isolates and ranged from 1 (n = 13) to 4 substitutions per strain (n = 4). Most of the isolates had 2 amino acid changes (n = 35). In all, 23 distinct amino acid substitutions were identified including some which have previously been associated with resistance. Interestingly, the following 4 are new: N136Y, Y221H, L276S, and Y447H. Most of the substitutions (88/123, 72%) occurred in both *ERG11* alleles (i.e., homozygous). The 23 distinct amino acid changes identified in our study could be divided into 2 subsets. The first subset consisted of substitutions that appeared to be restricted to isolates displaying a reduced azole susceptibility phenotype. The following 12 substitutions fall into this category: Y132F, Y132H, K143R, F145L, G307S, S405F, G448E, G448V, G450E, V456I, and the new amino acid changes N136Y and Y447H. Of note, these 2 new substitutions were recovered from 2 isolates displaying reduced susceptibility to more than 1 antifungal (CAAL-61 and CAAL-70). Strikingly, CAAL-61, which contained Y447H, was resistant to fluconazole (MIC >64 μg/mL) and itraconazole (MIC >16 μg/mL) but remained highly susceptible to voriconazole (MIC = 0.125 μg/mL). The second subset concerned substitutions recovered from either azole-susceptible or reduced susceptibility isolates and included 11 amino acid changes: D116E, K128T, G129A, D153E, V159I, E266D, V437I, G464S, V488I, and the new substitutions Y221H and L276S. Y221H and L276S occurred in a single allele in 2 susceptible strains (CAAL-62 and CAAL-100, respectively). D116E, K128T, E266D, and V488I appeared in a large number of isolates (n = 29, 19, 18, and 17 isolates, respectively). D116E was frequently recovered in combination with K128T (n = 19 isolates), whereas E266D was recovered with V488I (n = 16). G129A occurred in a single allele in combination with D116E and E266D in a single azole-susceptible strain. Obviously, other mechanisms are required to explain azole resistance in some of our strains such as CAAL-90 harboring only this kind of amino acid substitutions.

Alignment of 15 different CYP51 amino acid sequences from various organisms, focusing on residues surrounding the 4 new amino acid changes (residues 136, 221, 276, and 447) as shown in Table 3, revealed that the 2 new amino acid changes with a potential involvement in azole resistance (N136Y and Y447H) affect highly conserved residues.

4. Discussion

Mutations in the *ERG11* gene sequence leading to amino acid substitutions represent one of the main mechanisms contributing to azole resistance in clinical isolates of *C. albicans*. To date, more than 140 distinct amino acid substitutions have been reported in the literature, but only a few of these have been clearly associated with azole resistance. In this study, our aim was to screen for Erg11 amino acid substitutions in a large collection of *C. albicans* clinical isolates displaying various levels of azole susceptibility. To provide as complete a picture as possible of the genetic alterations occurring in this gene, we chose to study the complete *ERG11* coding sequence instead of focusing on the 3 hot spot regions described previously (Marichal et al., 1999). For each isolate, the complete *ERG11* gene was amplified using a colony-PCR approach, a quick and easy method based on amplification of a target gene directly from a single colony without any prior DNA extraction step (Luo and Mitchell, 2002).

Table 2
Results of in vitro antifungal susceptibility testing and amino acid substitutions in Erg11 for the 73 clinical isolates of *C. albicans*

Strain	Site of isolation	MIC (μg/mL)			Amino acid change(s) in Erg11
		FLZ	ITZ	VOR	
CAAL-1	Mouth	16	0.031	0.125	K143R, V437I
CAAL-2	Respiratory tract	>64	>16	>8	D116E^{h}, D153E^{h}
CAAL-3	Blood	<0.125	<0.031	0.031	None
CAAL-4	Respiratory tract	0.125	<0.031	0.031	D116E, K128T
CAAL-5	Blood	<0.125	<0.031	<0.016	None
CAAL-6	Respiratory tract	0.125	<0.031	<0.016	D116E, G129A^{h}, E266D^{h}
CAAL-7	BAL	<0.125	<0.031	<0.016	D116E, K128T
CAAL-8	Blood	<0.125	<0.031	<0.016	D116E, K128T
CAAL-9	Blood	<0.125	<0.031	0.031	E266D, V488I
CAAL-10	Blood	<0.125	<0.031	<0.016	E266D, V488I
CAAL-11	Blood	<0.125	<0.031	<0.016	D116E, K128T
CAAL-12	Blood	<0.125	<0.031	<0.016	None
CAAL-13	Blood	<0.125	<0.031	<0.016	E266D, V488I
CAAL-14	ND	0.125	<0.031	<0.016	D116E, K128T
CAAL-15	Blood	<0.125	<0.031	<0.016	D116Eh, K128T^{h}, V159I^{h}
CAAL-17	Blood	<0.125	<0.031	<0.016	None
CAAL-18	Gastric fluid	<0.125	<0.031	<0.016	V437I
CAAL-19	Preservation fluid	<0.125	<0.031	<0.016	D116E, K128T
CAAL-20	Blood	<0.125	<0.031	<0.016	None
CAAL-21	Gastric fluid	<0.125	<0.031	<0.016	E266D, V488I^{h}
CAAL-22	Gastric fluid	0.5	0.031	0.031	G464S
CAAL-23	Urine	<0.125	<0.031	<0.016	D116E
CAAL-24	Mouth	0.5	<0.031	0.016	G464S
CAAL-28	ND	64	0.125	0.5	S405F
CAAL-29	Blood	<0.125	<0.031	<0.016	D116E^{h}, K128T^{h}
CAAL-30	ND	<0.125	<0.031	<0.016	D116E
CAAL-31	Gastric fluid	0.25	<0.031	0.031	G464S
CAAL-32	Respiratory tract	<0.125	<0.031	<0.016	D116E, K128T
CAAL-33	Urine	0.125	<0.031	<0.016	None
CAAL-34	Gastric fluid	<0.125	<0.031	<0.016	None
CAAL-35	Urine	<0.125	<0.031	<0.016	E266D, V488I^{h}
CAAL-37	Respiratory tract	>64	0.25	0.25	E266D, G464S, V456I^{h}, V488I
CAAL-38	Gastric fluid	<0.125	<0.031	<0.016	D116E, K128T
CAAL-39	Blood	<0.125	<0.031	<0.016	None
CAAL-40	Blood	<0.125	<0.031	<0.016	D116E, K128T
CAAL-41	Respiratory tract	<0.125	<0.031	<0.016	D116E, E266D
CAAL-42	Blood	<0.125	0.031	<0.016	E266D, V488I^{h}
CAAL-43	Respiratory tract	<0.125	<0.031	<0.016	E266D, V488I^{h}
CAAL-44	Gastric fluid	<0.125	<0.031	<0.016	E266D, V488I^{h}
CAAL-45	Gastric fluid	<0.125	<0.031	<0.016	E266D, V488I
CAAL-58	Blood	0.125	0.031	0.031	D116E, E266D
CAAL-60	Urine	<0.125	<0.031	<0.016	D116E, K128T
CAAL-61	Mouth	>64	>16	0.125	G307S, **Y447H**
CAAL-62	Intra-abdominal	0.125	<0.031	<0.016	D116E^{h}, K128T^{h}, **Y221H**^{h}
CAAL-63	Mouth	<0.125	<0.031	<0.016	None
CAAL-64	Intra-abdominal	0.5	0.125	0.063	E266D, V488I^{h}
CAAL-65	Mouth	32	0.5	0.5	F145L
CAAL-66	Mouth	8	0.5	0.125	D116E, K128T, G448E
CAAL-67	ND	32	0.25	0.25	G450E
CAAL-68	ND	8	0.5	1	E266D, V437I, G450E
CAAL-69	Urine	<0.125	<0.031	<0.016	None
CAAL-70	Mouth	16	0.5	2	Y132H, **N136Y**
CAAL-71	Mouth	32	1	4	Y132H, G450E
CAAL-72	Mouth	<0.125	0.031	<0.016	D116E, E266D
CAAL-73	Mouth	<0.125	<0.031	<0.016	D116E, E266D
CAAL-74	Mouth	>64	0.25	0.125	Y132F, E266D, G448V, V488I
CAAL-75	Intra-abdominal	>64	1	4	D116E, Y132H, K143R
CAAL-76	Blood	>64	>16	>8	D116E^{h}, K128T^{h}
CAAL-79	Blood	<0.125	<0.031	<0.016	D116E^{h}, K128T^{h}
CAAL-82	ND	4	0.25	0.5	Y132H^{h}, E266D, G450E, V488I
CAAL-90	ND	>64	>16	>8	E266D, V488I^{h}

Table 2 (*continued*)

Strain	Site of isolation	MIC (μg/mL)			Amino acid change(s) in Erg11
		FLZ	ITZ	VOR	
CAAL-91	ND	16	0.5	0.25	K143R
CAAL-92	ND	32	0.5	1	G464S
CAAL-93	ND	0.125	<0.031	<0.016	E266D, V488I
CAAL-94	ND	0.125	<0.031	0.016	D116E^{h}, K128T^{h}, E266D^{h}, V488I^{h}
CAAL-95	ND	0.125	<0.031	0.016	None
CAAL-96	ND	0.25	<0.031	<0.016	D153E
CAAL-97	ND	0.12	0.25	0.015	D116E, K128T
CAAL-98	ND	1	<0.03	0.125	None
CAAL-99	ND	4	0.125	0.25	None
CAAL-100	ND	1	0.06	0.125	E266D, **L276S**^{h}, V488I^{h}
CAAL-101	ND	0.5	0.03	0.06	D116E^{h}, K128T^{h}, V159I^{h}
CAAL-102	ND	0.25	0.25	0.015	D116E

ND = not defined; FLZ = fluconazole; ITZ = itraconazole; VOR = voriconazole; BAL = bronchoalveolar lavage; h = heterozygous (i.e., mutation in a single allele). The new substitutions are shown in bold.

Our data clearly show that point mutations leading to amino acid changes are a frequent event in *ERG11* observed not only in azole-resistant strains but also in azole-susceptible ones. Indeed, only 13 (18%) of the 73 isolates displayed the wild-type sequence (i.e., no amino acid change), including 6 bloodstream isolates. In all, of the 23 distinct amino acid substitutions identified, 19 have been reported previously. Thus, 4 substitutions described in this study are new (N136Y, Y221H, L276S, and Y447H). Except for Y221H, all substitutions were in the 3 hot spot regions described previously (Marichal et al., 1999). In agreement with a previous study, we report a high frequency of D116E, K128T, and E266D (White et al., 2002). Moreover, a large number of clinical strains exhibited the same pattern of amino acid substitutions (D116E and K128T; 19/73, 26%).

Although all isolates displaying a phenotype of reduced azole susceptibility (20/20, 100%) had genetic alterations in Erg11, amino acid polymorphisms were also identified in susceptible isolates (40/53, 75%). This means that the presence or absence of amino acid polymorphism is usually not sufficient to predict azole susceptibility. Finally, allelic dosage (homozygosity versus heterozygosity) must also be taken into consideration in this diploid species (Jones et al., 2004). In this way, the finding that 11 of the 23 amino acid changes (D116E, K128T, G129A, D153E, V159I, E266D, V437I, G464S, V488I, and the 2 new Y221H and L276S) recorded here were found in both azole-susceptible and azole-resistant strains strongly suggests that these substitutions are probably not associated with resistance. However, this is largely supported by previous studies for D116E,

Table 3
Alignment of 15 CYP51 amino acid sequences from various organisms

Organism	132						139	218						224	273						279	444						450
C. albicans	D	C	P	N	S	R	L	A	Q	L	Y	S	D	L	N	R	D	L	I	D	S	E	V	D	Y	G	F	G
C. dubliniensis	D	C	P	N	S	R	L	A	Q	L	Y	S	D	L	S	R	D	L	I	D	S	E	V	D	Y	G	F	G
C. tropicalis	D	C	P	N	S	R	L	A	Q	L	Y	A	D	L	K	R	D	L	I	D	S	T	V	D	Y	G	F	G
C. glabrata	D	C	P	N	H	R	L	A	Y	L	Y	S	D	L	N	R	D	L	I	D	E	E	V	D	Y	G	F	G
C. krusei	D	C	P	N	W	K	L	A	E	M	Y	S	D	L	N	E	D	L	V	D	A	T	V	D	Y	G	F	G
A. fumigatus	D	C	P	N	S	K	L	A	D	L	Y	H	D	L	-	S	D	M	I	W	N	V	V	D	Y	G	Y	G
P. italicum	D	C	P	N	S	K	L	A	D	L	F	H	D	L	G	T	D	M	I	S	N	T	V	D	Y	G	Y	G
S. cerevisiae	D	C	P	N	S	R	L	A	Y	L	Y	S	D	L	D	R	D	L	I	D	S	E	V	D	Y	G	F	G
S. pombe	D	I	P	N	H	V	F	A	D	L	Y	H	D	L	G	T	D	M	I	W	T	Q	I	D	Y	G	Y	G
U. maydis	D	V	P	N	A	V	F	A	Q	L	Y	H	D	L	E	N	D	M	I	A	A	K	Q	D	F	G	F	G
A. thaliana	D	V	D	Y	S	V	R	S	A	L	F	H	D	L	E	N	D	M	L	Q	C	-	-	S	P	G	R	E
D. discoideum	D	S	E	T	E	I	M	A	D	L	Y	H	E	L	V	D	D	V	L	Y	T	-	-	D	V	-	-	-
M. tuberculosis	D	A	S	P	E	R	R	A	K	L	Y	H	E	L	D	R	D	M	L	D	V	-	-	E	Q	P	R	Q
R. norvegicus	D	V	P	N	A	V	F	A	Q	L	Y	A	D	L	A	E	D	I	L	Q	T	-	-	L	Q	-	-	D
H. sapiens	D	V	P	N	P	V	F	A	Q	L	Y	A	D	L	I	D	D	I	L	Q	T	-	-	L	Q	-	-	D

Only residues surrounding the 4 new amino acid substitutions N136Y, Y221H, L276S, and Y447H are shown. The alignment was generated using the ClustalW program. Amino acid numbering is taken from the *C. albicans* gene sequence. Arrow heads indicate new amino acid changes. "-" is indicative of a gap in the protein sequence. Amino acids conserved across fungi are underlined in gray and amino acids conserved across all 15 CYP51 sequences are underlined in black. The following accession numbers have been used: *C. albicans*, X13296; *Candida dubliniensis*, AY034876; *Candida tropicalis*, M23673; *Candida glabrata*, S75389; *Candida krusei*, S75391; *S. cerevisiae*, M18109; *Aspergillus fumigatus*, AF338659; *Schizosaccharomyces pombe*, Q09736; *Ustilago maydis*, Z48164; *Penicillium italicum*, Z49750; *Homo sapiens*, D55653; *Rattus norvegicus*, D55681, *M. tuberculosis*, P0A512; *Arabidopsis thaliana*, AB014459; *Dictyostelium discoideum*, XP_001134568.

K128T, D153E, E266D, V437I, and V488I; the case of G129A and G464S needs to be discussed in light of our results (Chau et al., 2004; Favre et al., 1999; Franz et al., 1998; Li et al., 2004; Marichal et al., 1999; Park and Perlin, 2005; Perea et al., 2001; White et al., 2002; Xu et al., 2008). According to a previous report, G129A alone is not sufficient to confer azole resistance but has been shown to be responsible for a 16-fold increase in fluconazole MIC when combined with G464S (Sanglard et al., 1998). Here, the recovery of G129A in an azole-susceptible strain supports the hypothesis that G129A must be associated with specific substitutions to contribute to azole resistance in *C. albicans*. Regarding G464S, previously associated with resistance by various methods (Chau et al., 2004; Sanglard et al., 1998), no robust hypothesis can be proposed to explain why this amino acid change occurred here in 3 highly azole-susceptible isolates. Similar discrepancies have been also reported in the literature for Y132H (Bellamine et al., 2004; Sanglard et al., 1998). Because the new substitutions Y221H and L276S were recovered from azole-susceptible isolates, they are unlikely to be associated with resistance. V159I, a substitution that was rarely described in the literature, occurred here in azole-susceptible isolates (CAAL-15 and CAAL-101).

As suggested previously, amino acid substitutions that appear to be restricted to reduced susceptibility isolates could be useful as predictive markers of azole resistance as well as for the rationale design of new azole antifungal drugs using homology models of CA-CYP51 constructed based on the X-ray crystal structure of *Mycobacterium tuberculosis* CYP51 (MT-CYP51) (Fukuoka et al., 2003; Macchiarulo et al., 2002; Park and Perlin, 2005; Podust et al., 2001; Xiao et al., 2004). Twelve substitutions recovered in our study fit this criterion because they are restricted to reduced susceptibility isolates: Y132F, Y132H, K143R, F145L, G307S, S405F, G448E, G448V, G450E, V456I, and the 2 new substitutions N136Y and Y447H. Except for F145L, previously reported in susceptible as well as resistant strains, and V456I reported by Sanglard and Bille (2002) without information relative to the susceptibility of the corresponding strains, these findings are in agreement with previous reports. The contribution of some of the amino acid changes (Y132F, K143R, G307F, and S405F) to azole resistance has been confirmed using in vitro experiments (Chau et al., 2004; Kakeya et al., 2000; Lee et al., 2004; Loffler et al., 1997; Perea et al., 2001; Sanglard et al., 1998; White et al., 2002). Hence, our data support the involvement of Y132F, Y132H, K143R, G307S, S405F, G448E, G448V, and G450E in azole resistance and their potential use as predictive markers of azole resistance.

The 2 new amino acid substitutions N136Y and Y447H were only recovered from isolates with a reduced azole susceptibility phenotype and are therefore likely to be involved in azole resistance. N136Y occurred in both alleles in a single isolate with reduced susceptibility to fluconazole, itraconazole, and voriconazole. Complementary investigations revealed that this isolate also had a moderately high MIC to posaconazole (E-test, MIC = 0.5 μg/mL). According to the secondary structure of CA-CYP51, residue 136 is close to the C helix, a region that is in close proximity to the heme and ligand binding site (Xiao et al., 2004). In the MT-CYP51/fluconazole crystal structure, the open BC loop and C helix exhibited high thermal motion, suggesting significant changes in protein conformation. Being localized in the region of the mouth of channel 1, this residue could interfere with azole entry or its binding to the active site if the BC loop adopts a closed conformation (Podust et al., 2001). Moreover, the finding that this residue is conserved between fungal CYP51s suggests that it may play an important role in CYP51s tridimensional conformation. Because N136Y occurred simultaneously with Y132H, more studies are now warranted to determine whether the resistant phenotype is the result of Y132H alone or is due to an additive effect with Y132H. Y447H was recovered from a single isolate displaying resistance to both fluconazole and itraconazole but that remained susceptible to voriconazole simultaneously with G307S, a substitution that could be associated with azole resistance according to Perea et al. (2001). Y447H is close to the C terminus part of CA-CYP51 near the L helix and cysteine residue 470 and would therefore be too far away to interact directly with azole binding (Xiao et al., 2004). However, this residue that appears to be conserved among *Candida* species is part of a large insertion located from residues 439 to 457 that was not modeled in detail in this report (Xiao et al., 2004).

Obviously, the reduced susceptibility phenotype observed in some of our strains could not be explained by only studying the genetic alterations in *ERG11*. This again shows that several azole resistance mechanisms are frequently combined in clinical strains of *C. albicans* (Cernicka and Subik, 2006; Chau et al., 2004; Coste et al., 2007; Franz et al., 1998; Goldman et al., 2004).

To conclude, our study highlights the high diversity and frequency of amino acid substitutions in Erg11, the primary target of azole antifungal drugs. This genetic polymorphism should be taken into consideration in the rationale design of new antifungal compounds targeting lanosterol 14α-demethylase with potent activity against resistant strains. Further experiments will provide answers regarding the contribution of the new amino acid substitutions to azole resistance.

Acknowledgments

The authors are grateful to Jean-Yves Leberre, Philippe Rispail, Yvon Sterkers, Marie-Denise Linas, Claire Lacroix, and the French National Reference Center for Mycoses and Antifungals (Institut Pasteur, Paris, France) for providing some of the strains used in this study. The authors thank Olivier Barre for technical assistance.

References

Bellamine A, Lepesheva G, Waterman MR (2004) Fluconazole binding and sterol demethylation in three CYP51 isoforms indicate differences in active site topology. *J Lipid Res* 45:2000–2007.

Cernicka J, Subik J (2006) Resistance mechanisms in fluconazole-resistant *Candida albicans* isolates from vaginal candidiasis. *Int J Antimicrob Agents* 27:403–408.

Chau AS, Mendrick CA, Sabatelli FJ, Loebenberg D, McNicholas PM (2004) Application of real-time quantitative PCR to molecular analysis of *Candida albicans* strains exhibiting reduced susceptibility to azoles. *Antimicrob Agents Chemother* 48:2124–2131.

Chen SH, Sheng CQ, Xu XH, Jiang YY, Zhang WN, He C (2007) Identification of Y118 amino acid residue in *Candida albicans* sterol 14alpha-demethylase associated with the enzyme activity and selective antifungal activity of azole analogues. *Biol Pharm Bull* 30:1246–1253.

Coste A, Selmecki A, Forche A, Diogo D, Bougnoux ME, d'Enfert C, Berman J, Sanglard D (2007) Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. *Eukaryot Cell* 6: 1889–1904.

Favre B, Didmon M, Ryder NS (1999) Multiple amino acid substitutions in lanosterol 14alpha-demethylase contribute to azole resistance in *Candida albicans*. *Microbiology* 145(Pt 10):2715–2725.

Franz R, Kelly SL, Lamb DC, Kelly DE, Ruhnke M, Morschhauser J (1998) Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. *Antimicrob Agents Chemother* 42:3065–3072.

Fukuoka T, Johnston DA, Winslow CA, de Groot MJ, Burt C, Hitchcock CA, Filler SG (2003) Genetic basis for differential activities of fluconazole and voriconazole against *Candida krusei*. *Antimicrob Agents Chemother* 47:1213–1219.

Giraud F, Loge C, Pagniez F, Crepin D, Le Pape P, Le Borgne M (2008) Design, synthesis, and evaluation of 1-(*N*-benzylamino)-2-phenyl-3-(1*H*-1,2,4-triazol-1-yl)propan-2-ols as antifungal agents. *Bioorg Med Chem Lett* 18:1820–1824.

Goldman GH, da Silva Ferreira ME, dos Reis Marques E, Savoldi M, Perlin D, Park S, Godoy Martinez PC, Goldman MH, Colombo AL (2004) Evaluation of fluconazole resistance mechanisms in *Candida albicans* clinical isolates from HIV-infected patients in Brazil. *Diagn Microbiol Infect Dis* 50:25–32.

Jiang W, Tan S, Jiang G (2006) Synergistic effect of terbinafine combined with fluconazole or itraconazole on stable fluconazole-resistant *Candida albicans* induced by fluconazole in vitro. *Chin J Microbiol Immunol* 26: 360–364.

Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB, Newport G, Thorstenson YR, Agabian N, Magee PT, Davis RW, Scherer S (2004) The diploid genome sequence of *Candida albicans*. *Proc Natl Acad Sci USA* 101:7329–7334.

Kakeya H, Miyazaki Y, Miyazaki H, Nyswaner K, Grimberg B, Bennett JE (2000) Genetic analysis of azole resistance in the Darlington strain of *Candida albicans*. *Antimicrob Agents Chemother* 44:2985–2990.

Lai MH, Kirsch DR (1989) Nucleotide sequence of cytochrome P450 L1A1 (lanosterol 14 alpha-demethylase) from *Candida albicans*. *Nucleic Acids Res* 17:804.

Lamb DC, Kelly DE, Schunck WH, Shyadehi AZ, Akhtar M, Lowe DJ, Baldwin BC, Kelly SL (1997) The mutation T315A in *Candida albicans* sterol 14alpha-demethylase causes reduced enzyme activity and fluconazole resistance through reduced affinity. *J Biol Chem* 272: 5682–5688.

Lamb DC, Kelly DE, White TC, Kelly SL (2000) The R467K amino acid substitution in *Candida albicans* sterol 14alpha-demethylase causes drug resistance through reduced affinity. *Antimicrob Agents Chemother* 44:63–67.

Lebouvier N, Pagniez F, Duflos M, Le Pape P, Na YM, Le Baut G, Le Borgne M (2007) Synthesis and antifungal activities of new fluconazole analogues with azaheterocycle moiety. *Bioorg Med Chem Lett* 17: 3686–3689.

Lee MK, Williams LE, Warnock DW, Arthington-Skaggs BA (2004) Drug resistance genes and trailing growth in *Candida albicans* isolates. *J Antimicrob Chemother* 53:217–224.

Li X, Brown N, Chau AS, Lopez-Ribot JL, Ruesga MT, Quindos G, Mendrick CA, Hare RS, Loebenberg D, DiDomenico B, McNicholas PM (2004) Changes in susceptibility to posaconazole in clinical isolates of *Candida albicans*. *J Antimicrob Chemother* 53:74–80.

Loffler J, Kelly SL, Hebart H, Schumacher U, Lass-Florl C, Einsele H (1997) Molecular analysis of cyp51 from fluconazole-resistant *Candida albicans* strains. *FEMS Microbiol Lett* 151:263–268.

Long F, Zhang Y, Lan H (2002) The point mutation of cytochrome P-450 lanosterol 14-demethylase *ERG11* gene in fluconazole-resistant *Candida albicans*. *Chin J Infect Dis* 20:211–214.

Luo G, Mitchell TG (2002) Rapid identification of pathogenic fungi directly from cultures by using multiplex PCR. *J Clin Microbiol* 40: 2860–2865.

Macchiarulo A, Costantino G, Fringuelli D, Vecchiarelli A, Schiaffella F, Fringuelli R (2002) 1,4-Benzothiazine and 1,4-benzoxazine imidazole derivatives with antifungal activity: a docking study. *Bioorg Med Chem* 10:3415–3423.

Maebashi K, Kudoh M, Nishiyama Y, Makimura K, Kamai Y, Uchida K, Yamaguchi H (2003) Proliferation of intracellular structure corresponding to reduced affinity of fluconazole for cytochrome P-450 in two low-susceptibility strains of *Candida albicans* isolated from a Japanese AIDS patient. *Microbiol Immunol* 47:117–124.

Manavathu EK, Kallakuri S, Arganoza MT, Vazquez JA (1999) Amino acid variations of cytochrome P-450 lanosterol 14 alpha-demethylase (CYP51A1) from fluconazole resistant clinical isolates of *Candida albicans*. *Rev Iberoam Micol* 16:198–203.

Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FC, Odds FC, Bossche HV (1999) Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. *Microbiology* 145(Pt 10):2701–2713.

National Committee for Clinical Laboratory & Standards (NCCLS) (2002) Reference method for broth dilution antifungal susceptibility testing of yeast—second edition: approved standard M27-A2. Wayne, PA: NCCLS.

Pagniez F, Le Borgne M, Marchand P, Na YM, Le Baut G, Robert-Piessard S, Le Pape P (2002) In vitro activity of a new antifungal azolyl-substituted indole against *Aspergillus fumigatus*. *J Enzyme Inhib Med Chem* 17:425–429.

Park S, Perlin DS (2005) Establishing surrogate markers for fluconazole resistance in *Candida albicans*. *Microb Drug Resist* 11:232–238.

Perea S, Lopez-Ribot JL, Kirkpatrick WR, McAtee RK, Santillan RA, Martinez M, Calabrese D, Sanglard D, Patterson TF (2001) Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob Agents Chemother* 45:2676–2684.

Podust LM, Poulos TL, Waterman MR (2001) Crystal structure of cytochrome P450 14alpha-sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. *Proc Natl Acad Sci USA* 98:3068–3073.

Rupp B, Raub S, Marian C, Holtje HD (2005) Molecular design of two sterol 14alpha-demethylase homology models and their interactions with the azole antifungals ketoconazole and bifonazole. *J Comput Aided Mol Des* 19:149–163.

Sanglard D, Bille J (2002) Action of and resistance to antifungal agents. In: *Candida and candidiasis*. Calderone RA, Ed. Washington DC: American Society For Microbiology, pp. 370.

Sanglard D, Odds FC (2002) Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect Dis* 2:73–85.

Sanglard D, Ischer F, Koymans L, Bille J (1998) Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob Agents Chemother* 42: 241–253.

Selmecki A, Forche A, Berman J (2006) Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science* 313:367–370.

Sheng C, Zhang W, Zhang M, Song Y, Ji H, Zhu J, Yao J, Yu J, Yang S, Zhou Y, Zhu J, Lu J (2004) Homology modeling of lanosterol 14alpha-demethylase of *Candida albicans* and *Aspergillus fumigatus* and insights into the enzyme-substrate interactions. *J Biomol Struct Dyn* 22:91–99.

Sheng C, Chen S, Ji H, Dong G, Che X, Wang W, Miao Z, Yao J, Lu J, Guo W, Zhang W (2009) Evolutionary trace analysis of CYP51 family: implication for site-directed mutagenesis and novel antifungal drug design. *J Mol Model* 16:279–284.

Wang H, Kong F, Sorrell T, Wang B, Mcnicholas P, Pantarat N, Ellis D, Xiao M, Widmer F, Chen SC (2009) Rapid detection of *ERG11* gene mutations in clinical *Candida albicans* isolates with reduced susceptibility to fluconazole by rolling circle amplification and DNA sequencing. *BMC Microbiol* 14:167 (Epub ahead of print).

Wang WL, Wang DL, Li RY (1999) A study of the resistant mechanisms of *Candida albicans* to azole antifungal agents. *Chin J Derm Venereol* 13: 360–364.

Wang YB, Wang H, Guo HY, Zhao YZ, Luo SQ (2005) Analysis of *ERG11* gene mutation in *Candida albicans*. *Di Yi Jun Yi Da Xue Xue Bao* 25: 1390–1393.

White TC (1997) The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14-demethylase in *Candida albicans*. *Antimicrob Agents Chemother* 41: 1488–1494.

White TC, Holleman S, Dy F, Mirels LF, Stevens DA (2002) Resistance mechanisms in clinical isolates of *Candida albicans*. *Antimicrob Agents Chemother* 46:1704–1713.

Xiao L, Madison V, Chau AS, Loebenberg D, Palermo RE, McNicholas PM (2004) Three-dimensional models of wild-type and mutated forms of cytochrome P450 14alpha-sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into posaconazole binding. *Antimicrob Agents Chemother* 48:568–574.

Xu Y, Chen L, Li C (2008) Susceptibility of clinical isolates of *Candida* species to fluconazole and detection of *Candida albicans* ERG11 mutations. *J Antimicrob Chemother* 61:798–804.