Rare modification in the ergosterol biosynthesis pathway leads to amphotericin B resistance in *Candida auris* clinical isolates

Milena Kordalewska,[a]# Kevin D. Guerrero,[a] Timothy D. Mikulski,[a] Tony N. Elias,[a] Rocio Garcia-Rubio,[a] Indira Berrio,[b,c] Dianne Gardam,[d] Christopher H. Heath,[d,e,f,g] Anuradha Chowdhary,[h] Nelesh P. Govender,[i,j,h] David S. Perlin[a]

[a] Center for Discovery and Innovation, Hackensack Meridian Health, Nutley, NJ, USA

[b] Medical and Experimental Mycology Group, Corporación para Investigaciones Biológicas (CIB), Medellín, Colombia

[c] Hospital general de Medellin "Luz Castro de Gutiérrez" ESE, Medellín, Colombia

[d] Department of Microbiology, PathWest Laboratory Medicine FSH Network, Fiona Stanley Hospital, Murdoch, Western Australia (WA), Australia

[e] Department of Infectious Diseases, Fiona Stanley Hospital, Murdoch, Australia

[f] Infectious Diseases, Royal Perth Hospital, Perth, WA, Australia

[g] Faculty of Health & Medical Sciences, University of Western Australia, Crawley, WA, Australia

[h] Medical Mycology Unit, Department of Microbiology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India

[i] Centre for Healthcare-Associated Infections, Antimicrobial Resistance and Mycoses, National Institute for Communicable Diseases, a Division of the National Health Laboratory Service, Sandringham 2131, South Africa

[j] School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Parktown 2193, South Africa

[h] MRC Centre for Medical Mycology, University of Exeter, Exeter, EX4 4PY, United Kingdom

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#Address correspondence to Milena Kordalewska, milena.kordalewska@hmh-cdi.org

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ABSTRACT

We determined amphotericin B (AmB) susceptibility and sequenced key genes of the ergosterol biosynthesis pathway implicated in AmB resistance (*ERG2*, *ERG3*, *ERG6*, *ERG11*) of 321 clinical isolates of *Candida auris*. In antifungal susceptibility testing, 19 (5.9%) isolates were categorized as AmB-resistant (MIC ≥2 mg/l). Only one AmB-resistant isolate presented a unique non-wild-type *ERG6* genotype that was confirmed to confer amphotericin B resistance (MIC >32 mg/l) when introduced into a susceptible strain (MIC = 0.5 mg/l).

Amphotericin B (AmB), a polyene antifungal drug, has a broad-spectrum activity against pathogenic fungi, including *Candida* spp. (1). Its mode of action is rather unusual, as it does not inhibit an enzyme. Instead, AmB binds to ergosterol (ERG) (2), an abundant sterol that regulates permeability and fluidity of the fungal cell membrane (3). In most *Candida* species, AmB resistance is rare in comparison to resistance to other antifungal drug classes (azoles and echinocandins) (4). Most often, AmB resistance stems from alterations in the sterol composition of the fungal cell membrane due to mutations in genes of the ERG biosynthesis pathway. Whole genome sequence (WGS) analysis of AmB-resistant clinical isolates revealed a transposon insertion in *ERG2* (C-8 sterol isomerase) of *C. albicans*, while a missense mutation in *ERG3* (C-5 sterol desaturase) and deletion of 170 nucleotides in *ERG11* (lanosterol 14α-demethylase) were detected in *C. tropicalis* (4). Moreover, a single AmB-resistant *C. albicans* isolate was reported to harbor a substitution in *ERG11* and a sequence repetition (10 duplicated amino acids) leading to loss of function of *ERG5* (C-22 sterol desaturase) (5). In *C. glabrata* clinical isolates, targeted gene sequencing revealed the presence of AmB resistance-conferring mutations in *ERG2* and *ERG6* (C-24 sterol methyltransferase) (6-8). Additionally, *in vitro* evolution experiments confirmed the role of *ERG6* mutations in AmB resistance in *C. albicans* (4).

Clinical isolates of *C. auris*, a recently emerged nosocomial pathogen, were reported to have a higher prevalence of AmB resistance (based on tentative breakpoints), e.g., 30% in the U.S. (9). In contrast to relatively well-studied mechanisms of azole and echinocandin resistance (10-16), information about *C. auris* response to AmB is scarce. So far, suggestions for an underlying mechanism of AmB resistance in *C. auris* have come from WGS analysis. The researchers did not find any loss-of-function mutations in genes previously implicated in AmB resistance in *C. albicans* but listed various genes with non-synonymous mutations which may potentially play a

role (17, 18). However, no follow-up studies (e.g., targeted gene sequencing, genetic engineering) were reported to confirm these observations.

The current antifungal armamentarium is extremely limited, with only three classes of systemic drugs widely available to treat *Candida* spp. infections. Antifungal treatment of *C. auris* infections is further complicated by several factors, including the scale of azole resistance (e.g. 90% of *C. auris* isolates in the U.S. are fluconazole-resistant) (9), emergence of multidrug resistance involving two or more drug classes (19, 20), and scarce availability of first-line therapy drugs, echinocandins, in resource-limited countries (21). Thus, a better understanding of the scale and molecular mechanism of AmB resistance in *C. auris* is urgently needed.

Here, we analyzed distribution of AmB minimal inhibitory concentration (MIC) values for 321 clinical isolates of *C. auris* representing five clades (I – South Asian, n=48; II – East Asian, n=6; III – South African, n=30; IV – South American, n=236; V – Iranian, n=1). Antifungal susceptibility testing (AFST) with AmB was performed with Etest gradient diffusion strips (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions. As recommended by the Centers for Disease Control and Prevention (CDC), MICs of 1.5 mg/l were rounded up to 2 mg/l. A tentative AmB MIC breakpoint of ≥2 mg/l, determined by the CDC on the basis of pharmacokinetic/pharmacodynamic study results (murine model of *C. auris* infection), was used to categorize isolates as resistant to AmB (9). A summary of AFST results and MIC values for individual isolates are presented in Table 1 and Supplementary Table 1, respectively. Three hundred and two isolates (94.1%) exhibited AmB MIC values <2 mg/l and were categorized as AmB-susceptible. A total of 19 isolates (5.9%), including 3 isolates of clade I, 1 isolate of clade III, and 15 isolates of clade IV, exhibited AmB MIC values ≥2 mg/l and therefore were categorized as AmB-resistant (Table 1). This rate is similar to the one reported recently from South Africa (6%) (16), but considerably lower than majority of the studies

conducted so far, where anywhere from 10% to 35% of isolates (30% of U.S. isolates according to the CDC) were reported as AmB-resistant (9, 22-24).

To decipher the molecular resistance mechanism in isolates exhibiting elevated MIC values (≥2 mg/l), we amplified and sequenced the following genes of ergosterol biosynthesis pathway (in the entire collection of clinical isolates): *ERG2* (C-8 sterol isomerase; B9J08_004943), *ERG3* (C-5 sterol desaturase; B9J08_003737), *ERG6* (sterol 24-C-methyltransferase; B9J08_005340), and *ERG11* (lanosterol 14-α-demethylase; B9J08_001448), which were previously implicated in AmB resistance in other *Candida* spp. Gene sequences of *C. auris* strain B8441 extracted from FungiDB (fungidb.org) served as a reference for primer design (Supplementary Material 2) and sequence analysis. Primers were synthesized by Integrated DNA Technologies (Coralville, IA, United States), and Sanger sequencing was performed by Genewiz (South Plainfield, NJ, United States). A summary of sequencing results and genotypes of individual isolates are presented in Table 1 and Supplementary Table 1, respectively. In 18 of 19 AmB-resistant isolates no mutations in *ERG2*, *ERG3*, *ERG6*, or *ERG11* were found that could explain elevated AmB values (Table 1). We noticed that amplification of *ERG6* from one South African isolate (SA18, clade III; AmB MIC = 6 mg/l) yielded a much shorter PCR product (Figure 1). Sequence analysis revealed that this isolate presented a unique non-wild-type *ERG6* genotype where 492 base pairs (from 52 to 543) were deleted (SA18's *ERG6* is 636 bp long in comparison to the wild-type's (WT's) 1128 bp), which corresponds to the deletion of 164 amino acids (from 18 to 181) and a shorter Erg6 (SA18's Erg6 is 211 amino acids long in comparison to the 375 AA of WT) (Figure 2). Representative sequences were deposited at GeneBank (NCBI) with the accession numbers OK564516 - OK564551 (*ERG2*), OK564552 - OK564587 *(ERG3*), OK564588 - OK564623 (*ERG6*), and OK564624 - OK564654 (*ERG11*). Accession numbers for individual isolates are presented in Supplementary Table 1.

To confirm that the SA18's *ERG6* variant confers AmB resistance, a WT *ERG6* gene in AmB-susceptible (MIC = 0.5 mg/l) isolate VPCI 717/P/14 (clade I) was replaced with an *ERG6* of SA18 fused with nourseothricin (NAT) resistance gene by using CRISPR/Cas9 system as described before (25), except that the cells were made electrocompetent with the Frozen-EZ yeast transformation kit (Zymo Research, Irvine, CA, USA). The transformants were selected on YPD plates containing 300 mg/l NAT. Correctness of transformation was validated by PCR and sequencing. All PCR conditions, reagents and primers used are listed in Supplementary Material 2. After that, the AmB MIC of the correct transformant (MKKG066) was determined by Etest. MIC >32 mg/l (no zone of inhibition around the Etest strip) was read, indicating that amphotericin B resistance was induced when a wild-type *ERG6* was replaced with *ERG6* of SA18 in a susceptible strain.

ERG6 encodes C-24 methyltransferase, which converts zymosterol to fecosterol in the ERG biosynthesis pathway. In *C. albicans*, a fragment between amino acids 127 and 135 of Erg6 is a highly conserved S-adenosylmethionine binding site (26). This fragment (amino acids 128-136 in Erg6 of *C. auris*) is not present in SA18's Erg6 due to the deletion of amino acids 18-181 (Figure 2). It was previously shown that lack of Erg6 activity leads to the accumulation of zymosterol, which can support fungal cell growth, but the absence of ergosterol in the cell membrane confers AmB resistance (6).

In conclusion, we found that mutations in key genes of ergosterol biosynthesis, which can be linked directly to AmB resistance, are extremely rare. Only 1 of 313 clinical isolates screened (0.3%) had an *ERG6* variant which induced AmB resistance in a WT strain. It is possible that mechanisms other than *ERG6* mutations may also contribute to reduced AmB susceptibility in *C. auris*, although this remains to be determined.

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Table 1. Results of AFST and gene sequencing performed for 321 *C. auris* isolates belonging to five geographic clades: I – South Asian (n=48); II – East Asian (n=6); III – South African (n=30); IV – South American (n=236); V – Iranian (n=1).

AmB – amphotericin B; MIC – minimal inhibitory concentration

* The actual range is 1.5-2 mg/l, but all AmB MIC values of 1.5 mg/l obtained by Etest were rounded up to 2, as recommended by the CDC.

** K177R/N335S/E343D – present in all isolates of clade IV

Clade	Number of isolates	AmB MIC range [mg/l]	Erg2	Erg3	Erg6	Erg11
I	45	0.19 - 1	WT	WT	WT	WT; Y132F; K143R
	3 (6.3%)	2*	WT	WT	WT	Y132F
II	6	0.094 - 0.5	D39E	WT	WT	WT
	0	≥2	-	-	-	-
III	29	0.19 - 0.38	D39E	WT	WT	V125A/F126L
	1 (3.3%)	**6**	**D39E**	**WT**	**deletion of AA 18-181**	**V125A/F126L**
IV	221	0.25 - 1	D39E	S58T	WT	WT**; E102K; Y132F; K143R; G459S; I466M; Y501H
	15 (6.4%)	2*	D39E	S58T	WT	WT**
V	1	0.19	WT	I31V/S37N/L63M	A287T	K177R/N335S/ E343D/T366I
	0	≥2	-	-	-	-

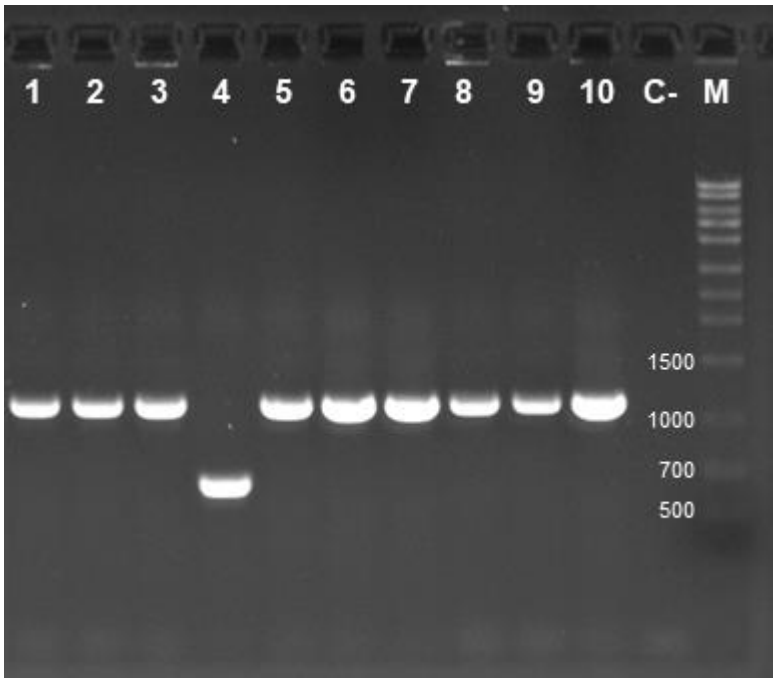

Figure 1. Results of PCR amplification of *ERG6* gene from *C. auris* clinical isolates SA15-SA24 (lanes 1-10).

C-, negative control; M: molecular size marker

Figure 2. Alignment of Erg6 sequence from *Candida albicans* and *C. auris*.