AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

MECHANISMS OF RESISTANCE

In Vitro Exposure to Increasing Micafungin Concentrations Easily Promotes Echinocandin Resistance in *Candida glabrata* Isolates

María Ángeles Bordallo-Cardona,[a,b] Pilar Escribano,[a,b] Elia Gómez G. de la Pedrosa,[d,e] Laura Judith Marcos-Zambrano,[a,b] Rafael Cantón,[d,e] Emilio Bouza,[a,b,c,f] Jesús Guinea[a,b,c,f]

Clinical Microbiology and Infectious Diseases, Hospital General Universitario Gregorio Marañón, Madrid, Spain[a]; Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain[b]; CIBER Enfermedades Respiratorias-CIBERES (CB06/06/0058), Madrid, Spain[c]; Clinical Microbiology, Hospital Universitario Ramón y Cajal and Instituto Ramón y Cajal de Investigación Biomédica, Madrid, Spain[d]; Red Española de Investigación en Patología Infecciosa (REIPI), Madrid, Spain[e]; Medicine Department, School of Medicine, Universidad Complutense de Madrid, Madrid, Spain[f]

ABSTRACT We assessed the *in vitro* susceptibility of five echinocandin-susceptible *Candida glabrata* isolates after exposure to micafungin. The direct exposure to plates at different micafungin concentrations resulted in the inhibition of growth at 0.062 μg/ml. The progressive exposure was performed on plates using 0.031 μg/ml of micafungin and sequential propagation on plates containing the next 2-fold concentration; the MICs of micafungin and anidulafungin increased sequentially, and all the isolates became echinocandin resistant, showing *fks2* mutations.

KEYWORDS echinocandin, micafungin, *fks* mutation, *Candida glabrata*, FKS

Received 15 July 2016 **Returned for modification** 28 October 2016 **Accepted** 12 November 2016

Accepted manuscript posted online 21 November 2016

Citation Bordallo-Cardona MA, Escribano P, de la Pedrosa EGG, Marcos-Zambrano LJ, Cantón R, Bouza E, Guinea J. 2017. *In vitro* exposure to increasing micafungin concentrations easily promotes echinocandin resistance in *Candida glabrata* isolates. Antimicrob Agents Chemother 61:e01542-16. https://doi.org/10.1128/AAC.01542-16.

Address correspondence to Pilar Escribano, pilar.escribano.martos@gmail.com, or Jesús Guinea, jguineaortega@yahoo.es.

Echinocandins are currently recommended as the first-line treatment for invasive candidiasis (1–3). Although the resistance to echinocandins reported in Spain remains low (4), emerging *Candida glabrata* echinocandin-resistant isolates have been reported elsewhere (5, 6). We hypothesized that, as in the case of *Aspergillus fumigatus* (7), the *in vitro* exposure to increasing concentrations of echinocandins could promote the generation of mutations conferring resistance.

(This study was partially presented at the 26th European Congress of Clinical Microbiology and Infectious Diseases in Amsterdam, The Netherlands, 2016 [8].)

C. glabrata isolates from five patients with candidemia admitted to Ramón y Cajal Hospital (Madrid) were initially tested for antifungal susceptibility to micafungin (Astellas Pharma, Inc., Tokyo, Japan), anidulafungin, and fluconazole (Pfizer Pharmaceutical Group, New York, NY, USA) according to the EUCAST EDef 7.2 microdilution procedure ($MIC_{initial}$) (9–12).

The isolates were grown on chromogenic agar plates and incubated at 35°C for 24 h to check for purity. A loopful of cultured isolates was suspended in 10 ml of yeast extract-peptone-dextrose (YPD) broth (Becton Dickinson, Madrid, Spain) and incubated at 30°C overnight with vigorous shaking (150 to 160 × *g*) in an orbital shaker. Yeast cells were collected by centrifugation (3,000 × *g* for 5 min), and the pellet was resuspended in phosphate-buffered saline (PBS) (Oxoid, Hampshire, UK) and centrifuged again for washing. Washed suspensions were adjusted to 2×10^9 to 4×10^9 (mean, $2.94 \pm 0.89 \times 10^9$) CFU/ml using a Neubauer chamber and stroked on micafungin-containing plates under two different sets of conditions (direct exposure and progressive exposure). Sabouraud dextrose agar plates were prepared using eight different micafungin concentrations (0.015, 0.031, 0.062, 0.125, 0.25, 0.5, 1, and 2 μg/ml) for the exposure

TABLE 1 Susceptibilities of *C. glabrata* isolates to micafungin and anidulafungin before and after progressive exposure to micafungin

	MIC (μg/ml)			
	Micafungin		Anidulafungin	
Exposure[a]	Range	Geometric mean	Range	Geometric mean
Initial	0.015	0.015	0.062	0.062
Progressive				
Subsequent	0.015–2	0.18[b]	0.031–4	0.56[b]
Final	0.062–4	1.15[c]	1–4	2.30[c]

[a]Initial, before progressive exposure to micafungin; Subsequent and Final, after exposure to micafungin.
[b]Data represent results of comparisons between the geometric means of the initial MIC and the subsequent MIC, for which significant differences were determined ($P < 0.05$).
[c]Data represent results of comparisons between the geometric means of the initial MIC and the final MIC, for which significant differences were determined ($P < 0.05$).

experiments, all of which were set up in duplicate to ensure reproducibility. In the direct micafungin exposure experiments, adjusted inocula (100 μl) were directly transferred to plates containing the eight different micafungin concentrations and the plates were incubated at 35°C and visually inspected for growth after 24 h. The MICs of micafungin and anidulafungin against the isolates growing on the plates containing the highest micafungin concentration were determined. In the progressive micafungin exposure experiments, adjusted inocula (100 μl) were stroked on plates containing micafungin at 0.031 μg/ml and the plates were incubated at 35°C for 24 h. If growth was observed, a loopful of cultured isolates was spread on the plate with a concentration of micafungin that was 2-fold greater. These steps were repeated up to the concentration of 2 μg/ml of micafungin. Each sequential suspension was used to study the MIC of micafungin and anidulafungin at each subsequent propagation step ($MIC_{subsequent}$) and final propagation step (MIC_{final}), and echinocandin and fluconazole MICs were determined using the plates containing 2 μg/ml of micafungin with the aid of EUCAST. Geometric means of $MIC_{initial}$, $MIC_{subsequent}$, and MIC_{final} of micafungin and anidulafungin were compared using the Wilcoxon signed-rank test (PASW Statistics 18.0; SPSS Inc., Chicago, IL). The comparisons were considered statistically significant with a *P* value of <0.05.

fks1 and *fks2* genes from the isolates used to study the $MIC_{initial}$, $MIC_{subsequent}$, and MIC_{final} were amplified (13, 14). The stability of phenotypic and genotypic resistance was studied.

The isolates were genetically unrelated, fluconazole intermediate, echinocandin susceptible ($MIC_{initial}$), and wild type (Table 1). The direct micafungin exposure allowed all isolates to grow on plates containing micafungin at concentrations up to 0.031 μg/ml, but the echinocandin MICs studied in these isolates were identical to $MIC_{initial}$. The exposure to progressively increasing concentrations of micafungin allowed isolates to grow on all micafungin-containing plates. Both $MIC_{subsequent}$ and MIC_{final} were significantly higher than the $MIC_{initial}$ (Table 1). Overall, a trend toward higher echinocandin MICs was observed with increasing micafungin concentrations in the plates but with stable MICs of fluconazole. All isolates grown on plates with micafungin at 0.062 μg/ml or 0.125 μg/ml became resistant to anidulafungin and/or micafungin, but two relevant observations were made (Table 2). First, at 0.125 μg/ml, four isolates were micafungin and anidulafungin resistant and *fks2* mutations were found; the remaining isolate (CG3) was resistant only to anidulafungin but had the wild-type *fks2* gene. However, the MIC of anidulafungin against the CG3 isolate rose progressively and a deletion at F658 in *fks2* was found when the isolate was grown on 0.25 μg/ml micafungin plates, although micafungin resistance was detected only in the last plate. Second, the CG1 isolate was anidulafungin and micafungin resistant on plates containing micafungin at 0.062 μg/ml, and two mutations (D666Y and S663P) in the *fks2* gene were found; however, only the S663P substitution was found when isolates were grown

TABLE 2 Antifungal activity of micafungin and anidulafungin against the five isolates before and after progressive micafungin exposure[a]

Exposure	Result(s) for indicated isolate									
	CG1		CG2		CG3		CG4		CG5	
	EUCAST MIC (μg/ml) (MYC/AND)	*fks2* mutation	EUCAST MIC (μg/ml) (MYC/AND)	*fks2* mutation	EUCAST MIC (μg/ml) (MYC/AND)	*fks2* mutation	EUCAST MIC (μg/ml) (MYC/AND)	*fks2* mutation	EUCAST MIC (μg/ml) (MYC/AND)	*fks2* mutation
Initial	0.015/0.062	WT	0.015/0.062	WT	0.015/0.062	WT	0.015/0.062	WT	0.015/0.062	WT
Subsequent (MYC concn in plates, μg/ml)										
0.031	0.015/0.031	WT	0.015/0.062	WT	0.015/0.062	WT	0.015/0.031	WT	0.015/0.031	WT
0.062	**0.5/2**	**S663P D666Y**	0.015/0.062	WT	0.015/**0.125**	WT	0.015/0.062	WT	0.015/0.062	WT
0.125	**1/2**	**S663P**	**0.125/0.5**	**S663Y**	0.031/**0.25**	WT	**2/2**	**Del F658**	**2/2**	**Del F658**
0.25	**1/2**	**S663P**	**0.125/1**	**S663Y**	0.031/**0.5**	**Del F658**	**2/2**	**Del F658**	**2/2**	**Del F658**
0.5	**2/2**	**S663P**	**0.25/2**	**S663Y**	0.031/**1**	**Del F658**	**2/2**	**Del F658**	**2/2**	**Del F658**
1	**2/4**	**S663P**	**0.5/2**	**S663Y**	0.031/**1**	**Del F658**	**2/2**	**Del F658**	**2/4**	**Del F658**
2	**4/4**	**S663P**	**1/2**	**S663Y**	**0.062/1**	**Del F658**	**2/2**	**Del F658**	**4/4**	**Del F658**
Subsequent geometric mean of MICs	0.74/1.34		0.12/0.55		0.03/0.37		0.49/0.67		0.55/0.82	

[a]MYC, micafungin; AND, anidulafungin; Del, deletion. Boldface characters represent MICs above the clinical breakpoints and FKS point mutations.

on the plates with the subsequent concentration of micafungin. The *fks* substitutions and the phenotypic resistance were stable and reproducible after several propagations on micafungin-free agar plates. Genotyping showed that isolates were identical before and after micafungin progressive exposure, thus excluding any potential contamination of the isolates during the propagation steps.

The emergence of echinocandin resistance could be caused by the predisposition of this pathogen to easily acquire mutations in response to drug pressure due to its haploid nature and to alterations caused by mismatched repair genes (15). The five isolates studied became echinocandin resistant when grown even on plates containing low concentrations of micafungin. Micafungin penetrates slightly in the peritoneal fluid; the reported peritoneal fluid/plasma ratio (area under the concentration-time curve from 0 to 24 h [AUC_{0-24}]) is 0.3 (16). Shields et al. hypothesized that the abdomen of patients with previous exposure to echinocandins can be a hidden reservoir for mutant-resistant *C. glabrata* isolates (6). This suggests that the exposure to low echinocandin concentrations may promote the generation of mutant isolates that may potentially cause invasive infections. In our *in vitro* study, resistance was obtained at concentrations close to the MIC.

No resistant isolates were obtained with direct exposure. On the other hand, mutations were found in the progressive exposure experiment with the same isolates, suggesting that increasing micafungin concentrations may be effective in terms of selecting and enriching underrepresented mutant populations. In this sense, the CG1 isolate illustrates the phenomenon of coexistence of several populations, as two mutations were detected when the isolate was grown on plates containing low micafungin concentrations. The D666Y mutation confers weak resistance, while the S663P mutation confers strong resistance (17). However, only the S663P mutation was found when the isolate was grown on plates containing higher micafungin concentrations. CG3 became resistant to anidulafungin, and this isolate also showed micafungin resistance in the last step of progressive exposure. Some isolates can be resistant to anidulafungin and susceptible to micafungin (18). This supports the use of anidulafungin as a surrogate marker to predict echinocandin resistance. Furthermore, the deletion at position F658 does not correlate with a predictable pattern of susceptibility to candins.

Echinocandin resistance in *C. glabrata* has been associated with a loss of fitness in isolates with mutations at S663P in the *fks2* gene (17). The fitness cost for the isolates carrying a mutation could explain the relatively low spread of resistant isolates reported to date. Further studies should be done on this topic.

This study had limitations. Despite of the low number of isolates analyzed, this proof of concept would be enhanced by the inclusion of a large number of clinical isolates. This was an *in vitro* study, and its impact in clinical practice is unknown, although our observations help improve understanding of the previous clinical reports on the presence of *C. glabrata* mutant isolates sourced from anatomical sites with low echinocandin concentrations. In conclusion, we found that a progressive exposure to increasing concentrations of micafungin can easily promote resistance to echinocandins in *C. glabrata* clinical isolates.

ACKNOWLEDGMENTS

This study was supported by grants PI14/00740 and MSI15/00115 from the Fondo de Investigación Sanitaria (FIS) (Instituto de Salud Carlos III; Plan Nacional de I+D+I 2013-2016) and grant CM-SANTANDER (GR3/2014; group 920200) and by the Spanish Network for Research in Infectious Diseases (REIPI RD12/0015) and was cofinanced by the European Regional Development Fund (FEDER) (A way of making Europe). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

M.A.B.-C. is a recipient of a predoctoral grant from the Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain (grant number II-Predoc-2016-IISGM). P.E.

(CPI15/00115) and J.G. (CPII15/00006) are recipients of a Miguel Servet contract; L.J.M.-Z. is supported by a grant from FIS (PI14/00740).

J.G. has received funds for speaking at symposia organized on behalf of Astellas, Gilead, MSD, and United Medical; he has also received funds for research from the Fondo de Investigación Sanitaria, Gilead, and Scynexis.

R.C. has received funds for speaking at symposia organized on behalf of Astellas, Gilead, and MSD.

All other authors have nothing to declare.

REFERENCES

1. Pappas PG, Kauffman CA, Andes D, Benjamin DK, Jr, Calandra TF, Edwards JE, Jr, Filler SG, Fisher JF, Kullberg BJ, Ostrosky-Zeichner L, Reboli AC, Rex JH, Walsh TJ, Sobel JD. 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis 48:503–535. https://doi.org/10.1086/596757.
2. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, Zaoutis TE, Sobel JD. 2016. Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis 62:e1–e50.
3. Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, Meersseman W, Akova M, Arendrup MC, Arikan-Akdagli S, Bille J, Castagnola E, Cuenca-Estrella M, Donnelly JP, Groll AH, Herbrecht R, Hope WW, Jensen HE, Lass-Florl C, Petrikkos G, Richardson MD, Roilides E, Verweij PE, Viscoli C, Ullmann AJ. 2012. ESCMID guideline for the diagnosis and management of *Candida* diseases 2012: non-neutropenic adult patients. Clin Microbiol Infect 18(Suppl 7):S19–S37.
4. Guinea J, Zaragoza O, Escribano P, Martin-Mazuelos E, Peman J, Sanchez-Reus F, Cuenca-Estrella M. 2014. Molecular identification and antifungal susceptibility of yeast isolates causing fungemia collected in a population-based study in Spain in 2010 and 2011. Antimicrob Agents Chemother 58:1529–1537. https://doi.org/10.1128/AAC.02155-13.
5. Alexander BD, Johnson MD, Pfeiffer CD, Jimenez-Ortigosa C, Catania J, Booker R, Castanheira M, Messer SA, Perlin DS, Pfaller MA. 2013. Increasing echinocandin resistance in *Candida glabrata*: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. Clin Infect Dis 56:1724–1732. https://doi.org/10.1093/cid/cit136.
6. Shields RK, Nguyen MH, Press EG, Clancy CJ. 2014. Abdominal candidiasis is a hidden reservoir of echinocandin resistance. Antimicrob Agents Chemother 58:7601–7605. https://doi.org/10.1128/AAC.04134-14.
7. Escribano P, Recio S, Pelaez T, Gonzalez-Rivera M, Bouza E, Guinea J. 2012. In vitro acquisition of secondary azole resistance in *Aspergillus fumigatus* isolates after prolonged exposure to itraconazole: presence of heteroresistant populations. Antimicrob Agents Chemother 56:174–178. https://doi.org/10.1128/AAC.00301-11.
8. Bordallo-Cardona MA, Escribano P, de la Pedrosa EGG, Marcos-Zambrano LJ, Cantón R, Bouza E, Guinea J. 2016. Abstr 26th European Congress of Clinical Microbiology and Infectious Diseases, Amsterdam, The Netherlands, poster P1603.
9. Arendrup MC, Cuenca-Estrella M, Lass-Florl C, Hope W. 2012. EUCAST technical note on the EUCAST definitive document EDef 7.2: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). Clin Microbiol Infect 18:E246–E247. https://doi.org/10.1111/j.1469-0691.2012.03880.x.
10. European Committee on Antimicrobial Susceptibility Testing. 2013. Micafungin and *Candida* spp.: rationale for the clinical breakpoints, version 1.0. http://www.eucast.org.
11. European Committee on Antimicrobial Susceptibility Testing. 2013. Anidulafungin: rationale for the clinical breakpoints, version 2.0. http://www.eucast.org.
12. European Committee on Antimicrobial Susceptibility Testing. 2013. Fluconazole: rationale for the clinical breakpoints, version 2.0. http://www.eucast.org.
13. Thompson GR, III, Wiederhold NP, Vallor AC, Villareal NC, Lewis JS, II, Patterson TF. 2008. Development of caspofungin resistance following prolonged therapy for invasive candidiasis secondary to *Candida glabrata* infection. Antimicrob Agents Chemother 52:3783–3785. https://doi.org/10.1128/AAC.00473-08.
14. Zimbeck AJ, Iqbal N, Ahlquist AM, Farley MM, Harrison LH, Chiller T, Lockhart SR. 2010. FKS mutations and elevated echinocandin MIC values among *Candida glabrata* isolates from U.S. population-based surveillance. Antimicrob Agents Chemother 54:5042–5047. https://doi.org/10.1128/AAC.00836-10.
15. Healey KR, Zhao Y, Perez WB, Lockhart SR, Sobel JD, Farmakiotis D, Kontoyiannis DP, Sanglard D, Taj-Aldeen SJ, Alexander BD, Jimenez-Ortigosa C, Shor E, Perlin DS. 2016. Prevalent mutator genotype identified in fungal pathogen *Candida glabrata* promotes multi-drug resistance. Nat Commun 7:11128. https://doi.org/10.1038/ncomms11128.
16. Grau S, Luque S, Campillo N, Samso E, Rodriguez U, Garcia-Bernedo CA, Salas E, Sharma R, Hope WW, Roberts JA. 2015. Plasma and peritoneal fluid population pharmacokinetics of micafungin in post-surgical patients with severe peritonitis. J Antimicrob Chemother 70:2854–2861. https://doi.org/10.1093/jac/dkv173.
17. Arendrup MC, Perlin DS. 2014. Echinocandin resistance: an emerging clinical problem? Curr Opin Infect Dis 27:484–492. https://doi.org/10.1097/QCO.0000000000000111.
18. Arendrup MC, Perlin DS, Jensen RH, Howard SJ, Goodwin J, Hope W. 2012. Differential in vivo activities of anidulafungin, caspofungin, and micafungin against *Candida glabrata* isolates with and without FKS resistance mutations. Antimicrob Agents Chemother 56:2435–2442. https://doi.org/10.1128/AAC.06369-11.