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BRIEF REPORTS

Amino acid substitution in *Cryptococcus neoformans* lanosterol 14-α-demethylase involved in fluconazole resistance in clinical isolates

María E. Bosco-Borgeat*, Mariana Mazza, Constanza G. Taverna, Susana Córdoba, Omar A. Murisengo, Walter Vivot, Graciela Davel

Departamento Micología, Instituto Nacional de Enfermedades Infecciosas ''Dr. Carlos G. Malbrán'', Ciudad Autónoma de Buenos Aires, Argentina

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Abstract The molecular basis of fluconazole resistance in *Cryptococcus neoformans* has been poorly studied. A common azole resistance mechanism in *Candida* species is the acquisition of point mutations in the *ERG11* gene encoding the enzyme lanosterol 14-α-demethylase, target of the azole class of drugs. In *C. neoformans* only two mutations were described in this gene. In order to evaluate other mutations that could be implicated in fluconazole resistance in *C. neoformans* we studied the genomic sequence of the *ERG11* gene in 11 clinical isolates with minimal inhibitory concentration (MIC) values to fluconazole of ≥16 μg/ml. The sequencing revealed the G1855A mutation in 3 isolates, resulting in the enzyme amino acid substitution G484S. These strains were isolated from two fluconazole-treated patients. This mutation would not intervene in the susceptibility to itraconazole and voriconazole.

PALABRAS CLAVE
Cryptococcus neoformans;
Resistencia al fluconazol;
Gen *ERG11*;
Sustitución aminoacídica;
Mutación

Sustitución aminoacídica en la enzima lanosterol 14 α-demetilasa de *Cryptococcus neoformans* involucrada en la resistencia al fluconazol de aislamientos clínicos

Resumen Las bases moleculares de la resistencia al fluconazol en *Cryptococcus neoformans* han sido poco estudiadas. Un mecanismo de resistencia a los azoles en *Candida albicans* es la adquisición de mutaciones puntuales en el gen *ERG11*, que codifica la enzima lanosterol 14 α-demetilasa, blanco de las drogas azólicas. En *C. neoformans* solo 2 mutaciones en este gen han sido descriptas. Con el objetivo de estudiar otras mutaciones que podrían estar implicadas en la resistencia al fluconazol en *C. neoformans*, realizamos la secuenciación del gen *ERG11*

* Corresponding author.
E-mail address: ebosco@anlis.gov.ar (M.E. Bosco-Borgeat).

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de 11 aislamientos clínicos con valores de concentración inhibitoria mínima (CIM) de fluconazol ≥16 μg/ml. En 3 aislamientos, la secuenciación reveló la mutación G1855A, que da como resultado la sustitución aminoacídica G484S. Estos aislamientos fueron recuperados de 2 pacientes tratados con fluconazol. Esta mutación no intervendría en la sensibilidad al itraconazol y al voriconazol.

Cryptococcosis is a life-threatening infection caused by the encapsulated basidiomycetous yeast *Cryptococcus neoformans* that affects mainly immunocompromised patients, especially those suffering from AIDS[4]. The most common manifestation is cryptococcal meningitis, which is fatal unless treated. *C. neoformans* is found worldwide and is responsible for approximately one million cases/year which result in over 600 000 deaths annually[7].

Fluconazole (FLC), a triazole antifungal drug, is the drug of choice for consolidation and maintenance therapy due to its efficacy, excellent central nervous system penetration and minor toxic effects[9].

Due to the use of FLC in long-term therapies, there is concern about the emergence of antifungal resistance in *C. neoformans*[3]. Several authors have associated *in vitro* resistance with treatment failure and infection relapse[1,3].

The molecular basis of resistance to azole antifungals has been poorly studied in *C. neoformans*.

One resistance mechanism proposed is the duplication of chromosome 1 and consequently of two of its resident genes: *ERG11*, which encodes for the FLC target enzyme lanosterol 14-α-demethylase, and *AFR1*, which encodes for an ABC transporter[14]. It has been demonstrated that upregulation of the *AFR1* gene is involved in the resistance to FLC in this yeast[11].

A common FLC resistance mechanism in *Candida* species is the acquisition of point mutations in the *ERG11* gene resulting in an altered target with reduced affinity for or inability to bind azoles[8]. Only two mutations in this gene have been associated with resistance to FLC in *C. neoformans*[10,13]. Furthermore, one of them caused resistance to both FLC and voriconazole (VRC) and increased susceptibility to itraconazole (ITC) and posaconazole (PSC); this mutation was identified in an isolate with an exceptionally high level of heteroresistance[13].

To elucidate if more mutations could be implicated in FLC resistance, we studied the *ERG11* genomic sequence of eleven clinical isolates from the Mycology Department Culture Collection (DMic) of Instituto Nacional de Enfermedades Infecciosas ''Dr. Carlos G. Malbrán'', Buenos Aires, Argentina. The research proposal does not involve experimentation on humans and non-clinical samples were used. Including yeast isolates are anonymous and belong to the Mycology Department Culture Collection. These isolates were selected for having high minimal inhibitory concentration (MIC) values to FLC (MIC values ≥16 μg/ml). One isolate with a lower MIC value was incorporated because it came from a patient who had presented an isolate with a high MIC value. The isolates and the patients' clinical data are described in Table 1. All the isolates included in this study were *C. neoformans* var *grubii* genotype VNI determined by PCR-RFLP of the *URA5* gene[6].

The minimal inhibitory concentration (MIC) was determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) E.Def 7.2 reference document[2]. Amphotericin B (AMB) and ITC (Sigma–Aldrich Quimica, Argentina); FLC and VRC (Pfizer S.A., Argentina); were the drugs tested and were provided as standard powders of known potency. The susceptibility tests were repeated from 4 to 10 times for each isolate.

DNA was extracted according to the method reported by Möller et al. To obtain the complete sequence of the *ERG11* gene, four PCRs were performed according to Rodero et al.[10]. PCR products were purified using the PureLink purification kit (Invitrogen) and were sequenced on both strands using the initial amplification primers with an automated DNA sequencer ABI Genetic Analyzer 3500 (Applied Biosystems, CA). Sequences were edited using the BioEdit Versión 7.0.0 (Tom May, North Carolina State University). All *ERG11* gene sequences were deposited in the GenBank database (Table 2).

The FLC susceptibility testing confirmed that the strains selected for this study presented high MIC values (≥16 μg/ml) (Table 2). All the isolates exhibited similar *in vitro* susceptibility patterns toward AMB, ITC and VRC as the FLC susceptible isolate included in this study and other isolates of our collection with FLC MIC values <16 μg/ml (data not shown). Only one isolate (patient B, isolate no. DMic 021206) exhibited higher MIC values to ITC and VRC (0.5 and 1 μg/ml respectively).

The Genbank accession numbers of the *ERG11* gene sequences obtained are listed in Table 2. Eight of the eleven isolates studied contained nucleotide variations compared to the wild type published sequences of *ERG11* (GenBank accession No. AY265353 and JQ044790). Only one of these nucleotide variations resulted in an amino acid substitution, the G1855A mutation producing substitution G484S. Two isolates recovered from the same episode of patient D and the isolate recovered from the third episode of patient A presented this mutation. We include in this study an isolate recovered from the initial episode of patient A, which did not contain this nucleotide variation and was susceptible to FLC. The other five isolates, obtained from three patients, contained different combinations of five nucleotide variations that did not result in any amino acid substitution: C233T present in an intron, and the silent nucleotide changes A1032G, C1659T, A1779G. Three isolates did not exhibit nucleotide variation compared to the published wild type sequence of the *ERG11* gene.

Table 1 Medical records and clinical source of the Isolates

Patient	Episode	Isolate no.	Clinical source	Clinical data
A	First episode	DMic 032018	CSF	- Cryptococcosis as hallmark of HIV infection. - AMB treatment, until a 580 mg cumulative dose. - The patient left the hospital without medical authorization. Two months later he was readmitted with a relapse.[a] - AMB treatment was restarted, until a 975 mg cumulative dose. - FLC treatment started (800 mg/day) with a favorable outcome.
	Third episode	DMic 031564	CSF	- Ten months after the first episode. - The patient died.
B	Second episode	DMic 021206	CSF	Unknown
C		DMic 031528	Blood	Unknown
D	First isolate	DMic 951594	CSF	Both isolates were recovered from different samples of the same episode. Prior to these isolates the patient had received AMB (1300 mg of cumulative dose) and over one month of treatment with FLC 800 mg/day.
	Second Isolate	DMic 961930	CSF	
E		DMic 042077	CSF	Unknown
F		DMic 073103	Unknown	Unknown
G		DMic 052504	CSF	Unknown
H	Third episode	DMic 031631	CSF	[a] First episode: treatment with AMB 50 mg/day. [a] Second episode: 4 months later. FLC 400 mg/day for one month. Then FLC 200 mg/day.
	Fifth episode	DMic 021146	CSF	Third episode: 13 months after the first episode. [a] Fourth episode: 21 months after the first episode. Treatment with FLC 1200 mg/day. Fifth episode: 34 months after the first episode. Treatment with AMB three times a week.
	Sixth episode	DMic 031862	CSF	Sixth episode: 42 months after the first episode. Treatment with AMB 50 mg/day was restarted. After that, the patient was treated for 1½ month with posaconazole. The patient left the hospital and was readmitted 1½ month later. The isolate from the sixth episode was recovered and the patient died.

CSF, cerebrospinal fluid; AMB, amphotericin B; FLC, fluconazole; PSC, Posaconazole.
[a] The isolates from these episodes are unavailable.

The study of specific *C. neoformans* physiological responses and the possible resistance mechanisms to drugs used in the treatment of cryptococcosis are important both to identify potential new treatments for the infection and to enhance the inhibitory effects of existing drugs.

The *ERG11* gene encodes the lanosterol 14-α-demethylase involved in ergosterol biosynthesis and the primary target for the azole class of antifungals. Several point mutations in this gene leading to different amino acid substitutions have been shown to decrease the target affinity for FLC resulting in drug resistance in *C. albicans*[5,8]. Two of them have been described and related to FLC resistance in *C. neoformans*: substitutions G484S and Y145F[10,13].

In this study, three clinical isolates with high MIC values presented the G484S substitution. These isolates were recovered from two patients who had had previous cryptococcosis episodes with a history of treatment with FLC. Moreover, we were able to study the isolate obtained from one of these patients' first episode, where cryptococcosis was the hallmark of HIV and the patient had not received any treatment. This initial isolate presented a lower MIC value and did not carry the amino acid substitution. These results reinforce the hypothesis that relates the G484S substitution to FLC resistance in *C. neoformans*. This relationship was proposed previously by Rodero et al. as a result of the study of a resistant isolate recovered from a patient suffering four episodes of relapse[10].

According to the 3-dimensional model of Lanosterol 14-α-demethylase from *C. neoformans*, the amino acid G484 is located in the heme environment into the active site of the enzyme[12]. It is proposed that this amino acid substitution

Table 2 Antifungal susceptibilities, nucleotide mutations in the *ERG11* gene and amino acid substitutions from *Cryptococcus neoformans* isolates

Patient	Episode	Isolate no.	MIC (μg/ml)[a]				Nucleotide mutations[b]	Amino acid substitution	Genbank accession no.
			FLC	ITC	VRC	AMB			
A	First episode	DMic 032018	4	0.13	0.06	0.5	–	–	KP294185
	Third episode	DMic 031564	32	0.03	0.13	0.25	G1855A	G484S	KP334107
B	Second episode	DMic 021206	64	0.5	1	0.25	–	–	KP419999
C		DMic 031528	16	0.03	0.06	0.13	–	–	KP420000
D	First isolate	DMic 951594	32	<0.015	0.25	0.5	G1855A	G484S	KP420001
	Second Isolate	DMic 961930	16	0.06	0.25	0.06	G1855A	G484S	KP420002
E		DMic 042077	16	<0.015	0.25	0.06	C233T A1032G A1779G	–	KP635002
F		DMic 073103	16	0.03	0.13	0.13	A1032GC1659TA1779G	–	KP635003
G		DMic 052504	16	0.03	0.25	0.5	–	–	KP635004
H	Third episode	DMic 031631	16	0.03	0.06	0.25	C233T A1032G A1779G	–	KP635005
	Fifth episode	DMic 021146	32	0.13	0.5	0.25	C233T A1032G A1779G	–	KP635006
	Sixth episode	DMic 031862	16	0.13	0.25	0.5	C233T A1032G A1779G	–	KP635007

MIC, minimal inhibitory concentration; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; AMB, amphotericin B.

[a] The values expressed represent the mode MIC values obtained for each isolate.

[b] The base numbers are with respect to the first ATG codon of the *ERG11* gene.

might decrease the flexibility required for binding with the substrate and the azole antifungal agents[12].

Mutation G1885A leading to amino acid G484S substitution was found independently in isolates from different patients and may represent a ''hot spot'' for the development of azole resistance; furthermore this substitution correlates with substitution G464S in *C. albicans* also proposed as a ''hot spot'' for that species[8].

The structure of VRC is very similar to FLC and in accordance with the three-dimensional models in *C. neoformans*, VRC might show higher affinity with the enzyme than FLC[12]. On the other hand ITC and PSC have very long side chains and might present the lowest interaction with the enzyme. With one exception, the isolates included in the present study exhibited low MIC values for VRC and ITC; moreover, we found no differences in the VRC and ITC MIC values between the isolates with the G484S substitution and others in our collection susceptible to FLC (data not shown), suggesting that the G484S substitution would not intervene in the enzyme interaction with ITC and VRC. In contrast, the other amino acid substitution, the Y145F, described in *C. neoformans*, afforded resistance to VRC but increased susceptibility to ITC and posaconazole[13].

We also found different combinations of five nucleotide variations that did not result in any amino acid substitution, which may indicate allelic differences present in the *ERG11* gene, and heterogeneity in the *C. neoformans* population. These allelic differences were also observed in *C. albicans ERG11* gene[8]. It is worthy of note that all the isolates included in this study were *C. neoformans* var. *grubii* genotype VNI in line with the worldwide distribution since this genotype is the most ubiquitous and prevalent and causes most of the cryptococcal infections[4,6].

Xu et al. proposed that mutation to FLC resistance in *C. neoformans* is a dynamic and heterogeneous process involving multiple simultaneous mechanisms[15]. Overexpression of efflux transporters and chromosome duplication may occur in the isolates without any amino acid substitution and may also be acting together with the G484S amino acid substitution. It remains to be determined how this mutation individually contributes to FLC resistance.

In summary, the results showed that FCZ resistance in *C. neoformans* may result from the presence of the G1855A point mutation in the *ERG11* gene responsible for the amino acid substitution G484S. This mutation would not change susceptibility to ITC and VRC.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

1. Aller AI, Martin-Mazuelos E, Lozano F, Gomez-Mateos J, Steele-Moore L, Holloway WJ, Gutiérrez MJ, Recio FJ, Espinel-Ingroff A. Correlation of fluconazole MICs with clinical outcome in cryptococcal infection. Antimicrob Agents Chemother. 2000;44:1544–8.
2. Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope W, the Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). Document E.DEF 7.2: Method for the determination of broth dilution of antifungal agents for fermentative yeasts, revised March, 2012 [On-Line]; 2012 http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/EUCAST_EDef_7_2_revision.pdf
3. Bicanic T, Harrison T, Niepieklo A, Dyakopu N, Meintjes G. Symptomatic relapse of HIV-associated cryptococcal meningitis after initial fluconazole monotherapy: the role of fluconazole resistance and immune reconstitution. Clin Infect Dis. 2006;43:1069–73.
4. Bovers M, Hagen F, Boekhout T. Diversity of the *Cryptococcus neoformans–Cryptococcus gattii* species complex. Rev Iberoam Micol. 2008;25:S4–12.
5. Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FC, Odds FC, Bossche HV. Contribution of mutations in the cytochrome P450 14-α-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. Microbiology. 1999;145:2701–13.
6. Meyer W, Castaneda A, Jackson S, Huynh M, Castaneda E. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg Infect Dis. 2003;9:189–95.
7. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS. 2009;23:525–30.
8. Perea S, Lopez-Ribot JL, Kirkpatrick WR, McAtee RK, Santillan RA, Martinez M, Calabrese D, Sanglard D, Patterson TF. 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. 2001;45:2676–84.
9. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, Harrison TS, Larsen RA, Lortholary O, Nguyen MH, Pappas PG, Powderly WG, Singh N, Sobel JD, Sorrell TC. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of America. Clin Infect Dis. 2010;50:291–322.
10. Rodero L, Mellado E, Rodriguez AC, Salve A, Guelfand L, Cahn P, Cuenca-Estrella M, Davel G, Rodriguez-Tudela JL. G484S amino acid substitution in lanosterol 14-α-demethylase (ERG11) is related to fluconazole resistance in a recurrent *Cryptococcus neoformans* clinical isolate. Antimicrob Agents Chemother. 2003;47:3653–6.
11. Sanguinetti M, Posteraro B, La Sorda M, Torelli R, Fiori B, Santangelo R, Delogu G, Fadda G. Role of AFR1, an ABC transporter-encoding gene, in the in vivo response to fluconazole and virulence of *Cryptococcus neoformans*. Infect Immun. 2006;74:1352–9.
12. Sheng C, Miao Z, Ji H, Yao J, Wang W, Che X, Dong G, Lü J, Guo W, Zhang W. Three-dimensional model of lanosterol

14-α-demethylase from *Cryptococcus neoformans*: active-site characterization and insights into azole binding. Antimicrob Agents Chemother. 2009;53:3487–95.
13. Sionov E, Chang YC, Garraffo HM, Dolan MA, Ghannoum MA, Kwon-Chung KJ. Identification of a *Cryptococcus neoformans* Cytochrome P450 Lanosterol 14-α-Demethylase (Erg11) Residue Critical for Differential Susceptibility between Fluconazole/Voriconazole and Itraconazole/Posaconazole. Antimicrob Agents Chemother. 2012;56:1162–9.
14. Sionov E, Lee H, Chang YC, Kwon-Chung KJ. *Cryptococcus neoformans* overcomes stress of azole drugs by formation of disomy in specific multiple chromosomes. PLoS Pathog. 2010;6:e1000848.
15. Xu J, Onyewu C, Yoell HJ, Ali RY, Vilgalys RJ, Mitchell TG. Dynamic and heterogeneous mutations to fluconazole resistance in *Cryptococcus neoformans*. Antimicrob Agents Chemother. 2001;45:420–7.