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Amino acid substitutions of 14-α demethylase (ERG11) in fluconazole resistant *Cryptococcus neoformans* clinical isolates

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Background: C ryptococcal meningitis or meningoencephalitis is the most frequent manifestation of infections due to *Cryptococcus neoformans*. Treatment of patients with AIDS can be complicated by severe immunosuppression. The very high relapse rate after standard regimens of amphotericin B has led to the use of lifelong chronic suppressive therapy. Though the current recommendation for maintenance treatment is oral fluconazole, its long-term usage for fungal infections has been documented in several studies as contri buting to azole resistance. Paugam *et* al. and Friese *et* al. have reported cases of recurrent cryptococcosis during chronic suppressive therapy. Sequential isolates from these patients were found to have increased levels of fluconazole MICs.

Mechanisms of azole resistance in yeasts already described include altered affinity of the 14-α lanosterol demethylase (ERG11) to azole drugs due to target site mutation or its overexpression, and decreased accumulation of drugs due to enhanced energy-dependent drug efflux. The biochemical basis of fluconazole resistance in *C. neoformans* has been explored, especially regarding the reduction of azole cellular content and the altered activity of ERG11. Changes in azole affinity for ERG11 have already been related to low-level fluconazole resistance in *C. neoformans* isolates. In addition, the decreased affinity of ERG11 for azole derivatives, due to mutations that increase levels of MICs of fluconazole, has been described in sequential clinical isolates of *Candida albicans*.

To determine whether this mechanism could also be implicated in *C. neoformans* azole resistance, we compared the *ERG11* genomic sequence in five sequential isolates re c ove red from recurrent episodes of cryptococcal meningitis. Isolates 1–4 were fluconazole-susceptible and the fifth had a MIC of 16 μg/ml. PCR amplification and sequencing of the gene encoding 14-α lanosterol demethylase showed a point mutation responsible for the amino acid substitution G484S in the resistant strain only.

Aim: In order to detect any point mutations in this gene associated with amino acid substitutions of 14-α lanosterol demethylase as a mechanism of azole resistance, we studied the sequence of gene ERG11 in clinical fluconazole-resistant *C. neofor - mans* isolates.

Methodology: In order to select *C. neoformans* (CN) isolates with MICs ≥ 16 mg/l to fluconazole (FCZ), we evaluated the in vitro susceptibility against fluconazole of 200 isolates of from HIV patients with cryptococcal meningitis and stored in the culture collection of the Mycology Department, INEI, ANLIS: Dr. Malbrán.

CN strains from 25 patients were selected to study the gene ERG11. CN ATCC 90112 and 2 strains previously reported with and without an amino acid substitution G484S in the 14-α lanosterol demethylase were used as control strains (CN-1, without mutation and CN-5, with the ERG11 point mutation).

Results: Fragments of 2,147 bp containing the full sequence of ERG11 gene of all the isolates selected were obtained by amplification of DNA with a specific set of primers. From all the isolates tested, only CN strains isolated from two patients showed a single point mutation at the 1855 nucleotide of the ERG11 gene. The strains with this mutation correspond to Patient 3 (3rd Episode) and Patient 25 (2nd and 3rd Episodes): this point mutation was previously described and results in the substitution of amino acid glycine 484 for serine (G484S) in the deduced protein sequence of 14-α lanosterol demethylase. The G484 is a residue that forms part of the conserved hemo-binding domain and is conserved in all citochrome P450 ERG11/Cyp51 of yeasts and filamentous fungi. In all cases, FCZ MICs of these mutated isolates were ≥ 16 mg/l as the CN-5 control strain with the same point ERG11 mutation (1). In Patient 3 this mutation was absent in the previous isolate obtained during the 2nd episode (P-3), showing a FCZ MIC of 4 mg/l. Unfortunately, in case of patient 25, the only strains available at our laboratory and at the Hospital were those from episode number 2 and 3. In both, the mutation was detected, and they were highly resistant to FCZ (MIC of 32 mg/l and 128 mg/l, respectively). To verify that this point mutation was not due to errors introduced by the PCR amplification, the ERG11 gene from these mutated isolates was newly amplified and sequenced a second time and the point mutations confirmed.

Discussion: The results showed that strains of *C. neoformans* isolated from only 2 of the 25 patients studied had a point mutation. Surprisingly, both had the same amino acid substitution in the 14-α lanosterol demethylase (ERG11) enzyme (G484S), which coincides with the one previously reported for *C. albicans.* The authors remarked that most of these substitutions were present in enzyme domains highly conserved across yeasts and filamentous fungi. Four amino acid substitutions (D116K, K128T, E266D, and G464S) were more frequent, but only G464S was observed in resistant strains. In the *C. neoformans* isolates from these two patients it was also clear that the high MIC of FCZ was associated with the G484S amino acid substitution. However, due to the low frequency of mutation, the involvement of another concomitant molecular mechanism of resistance is quite possible. ❖