High-level expression of cyp51B in azole-resistant clinical
Aspergillus fumigatus isolates

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Objectives: Resistance to azole antifungal drugs in Aspergillus fumigatus that is not mediated by target gene mutations is now common in some locations. The aim of this study was to investigate possible new mechanisms of resistance in non-target resistance.

Methods: Twelve azole-resistant A. fumigatus isolates previously shown not to carry mutations in cyp51A were tested to determine whether the alternative cyp51B gene was overexpressed.

Results: Of 12 isolates one showed overinduction of cyp51B after exposure to itraconazole and another showed high constitutive expression of cyp51B.

Conclusions: cyp51B overexpression is a possible azole resistance mechanism in A. fumigatus

Keywords: antifungal, fungi, drug resistance

Introduction

Aspergillus fumigatus is the most common serious mould infection in man. Few classes of therapeutics exist to treat fungal disease, and the recent description of resistance to the most commonly used azole class of compounds is of great concern.1–3 The study of azole resistance mechanisms in A. fumigatus has followed previous work in Candida albicans that has shown azole resistance to arise via mutation at the drug target lanosterol 14-α demethylase encoded by the ERG11 gene or through up-regulation of drug efflux pumps. However, genome sequencing and comparative genomics studies have shown that A. fumigatus is a more complex organism than C. albicans, with the potential for more elaborate resistance mechanisms; in particular, A. fumigatus possesses two parallel pathways to ergosterol synthesis and two copies of the target gene, the ERG11 orthologues cyp51A and cyp51B.4 Either gene is dispensable for growth, and cyp51A deletion mutants appear to be hypersusceptible to azoles, suggesting that cyp51B is both a viable enzyme intermediate in ergosterol biosynthesis and that the cyp51B protein is effectively targeted by azoles.5–7 Other filamentous fungi such as the plant pathogens Mycosphaerella graminicola and Fusarium graminearum possess multiple lanosterol 14-α demethylase paralogues and it is known that azole resistance in these organisms can result from mutation of the cyp51B orthologue rather than the cyp51A orthologues.8 Given that cyp51B can function as an effective cyp51A replacement in A. fumigatus, it is expected that mutation or overexpression of cyp51B could provide an effective mechanism for azole resistance. This has never been observed in a clinical azole-resistant isolate of A. fumigatus. Current molecular methods for the detection of azole resistance in A. fumigatus rely on accurate knowledge of possible resistance mechanisms, and previous work relying on the detection of mutations in cyp51A is not useful for monitoring the increasing incidence of non-cyp51A-mediated azole resistance. Here we present the observation that a clinical azole-resistant isolate of A. fumigatus that does not carry a cyp51A mutation or overexpress the cyp51A gene, displays high overinduction of cyp51B expression. A second non-cyp51A-mediated azole-resistant isolate displays a high basal cyp51B expression level.

Materials and methods

Twelve isolates of A. fumigatus previously shown to have no cyp51A mutations9 were included in this study. MICs of itraconazole, voriconazole and posaconazole were previously determined.4 In order to measure cyp51B expression, cultures of A. fumigatus were grown at 37°C for 16 h then split equally into six flasks containing 50 mL of Sabouraud broth. Itraconazole was added to three flasks to a final concentration...
of 1 mg/L, chosen as a concentration greater than the MIC for azole-susceptible strains, but that did not affect the minimum functional concentration at this timepoint. RNA was extracted from equal wet weights of mycelium and RT–PCR was performed on 100 ng of total RNA for each replicate, then analysed as previously described using triplicate technical replicates for each biological replicate with \( \beta \)-tubulin as comparator. Primers used were cyp51BqPCR-For (5\' -AGCAGAAGAAGTTCGTCAAATAC), cyp51BqPCR-Rev (5\' -TCGAAGACGCCCTTGTG), cyp51AqPCR-For (5\' -TGAAGACGCCCTTGTG), cyp51AqPCR-Rev (5\' -CGCATTGACATCCTTGAGC) and cyp51AqPCR-Rev (5\' -CGCATTGACATCCTTGAGC) at 10 nM.

Experimental design and data analysis were carried out according to current best practice. Basal expression levels were estimated as described previously in RNA from cultures that were not treated with azole, with the assumption that equivalent total RNA input and observed equal PCR efficiency would provide comparable \( C_t \) values.

Results

For 10 strains that exhibited azole resistance (itraconazole MIC >8 mg/L) without cyp51A mutation, the mean induction of cyp51B by azole was 2.5-fold with a standard deviation (SD) of 1.22. This was not significantly different from that observed in control isolates—AF293, AF210 and AF300—that were azole susceptible, where induction of 2.7-fold (SD 1.3) was observed. One itraconazole-resistant isolate, F19896, showed induction of 18.7-fold (SD 1.12) (Figure 1a). In the azole-susceptible clinical isolates, induction of cyp51A was observed to be 4.8-fold (SD 2.7), whereas in F19896, no induction of cyp51A was observed (Figure 1a). Basal levels of cyp51B were not found to differ significantly between resistant (\( n=10 \)) and susceptible (\( n=3 \)) isolates. However, in the case of F19980, the basal expression of cyp51B was found to be 10.8-fold higher than that observed in AF293 in the absence of azole (Figure 1b). Induction of cyp51B in this isolate by azole was 2.2-fold. Promoter sequences 1 kb upstream of the cyp51B gene were amplified and sequenced; however, no mutations were observed in this region, suggesting that the observed dysregulation may be the result of altered transcription factor function.

MICs of itraconazole, voriconazole and posaconazole were 8, 8 and 1 mg/L for F19896 and 8, 1 and 0.125 for F19880, respectively.

Discussion

The observation that cyp51B can be either overinduced or constitutively overexpressed in A. fumigatus isolates that are azole resistant, but lack mutations in the azole target cyp51A, is significant, as it provides a possible basis for drug resistance. cyp51B has been shown to be a functional biosynthetic enzyme that is inhibited by azoles, and hence it is likely to have the ability to play a role in azole resistance either through overexpression or through mutations in the gene that lead to a decreased interaction of the protein with azoles. The levels of constitutive expression and overinduction observed here are comparable to those seen in ERG11 or cyp51A overexpression azole-resistant isolates of either C. albicans or A. fumigatus. This is the first report of cyp51B overexpression in A. fumigatus; however, proof of the involvement of this mechanism in resistance will require demonstration by either gene knockout in a cyp51B overexpression isolate or by overexpression of cyp51B in an azole-susceptible isolate. The mechanisms of resistance in the remaining azole-resistant isolates remain obscure, but may involve up-regulation of efflux pumps in a manner analogous to C. albicans.

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Transparency declarations

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