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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* mutations[3] were included in this study. MICs of itraconazole, voriconazole and posaconazole were previously determined.[3] 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

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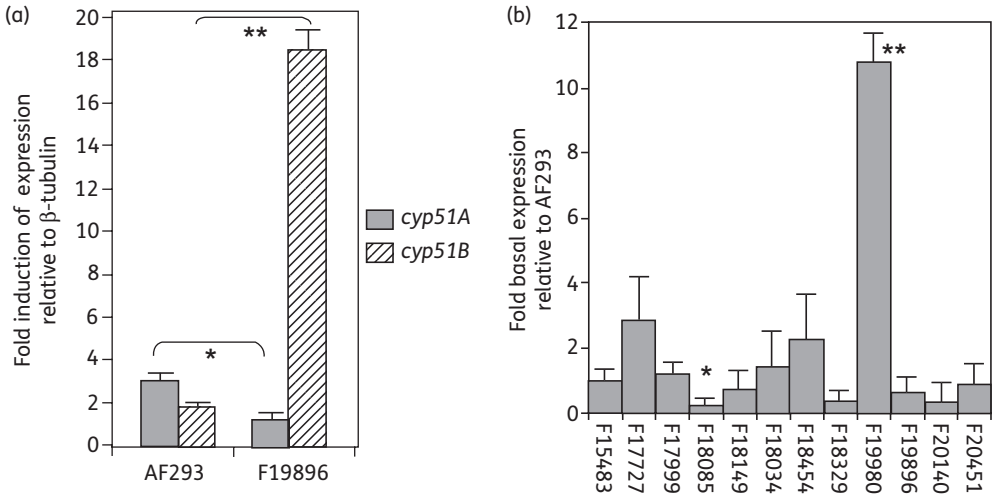

Figure 1. Basal and azole-induced expression of *cyp51A* and *cyp51B* in non-*cyp51A* mutant isolates of *A. fumigatus*. (a) Induction of *cyp51A* and *cyp51B* genes 4 h after exposure to 1 mg/L itraconazole. (b) Uninduced expression levels for *cyp51B* in 12 clinical isolates shown to be azole resistant relative to expression in a clinical azole-susceptible isolate, AF293. $^{*}P<0.05$; $^{**}P<0.01$.

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 β-tubulin as comparator. Primers used were cyp51BqPCR-For (5′-AGCAGAAGAAGTTCGTCAAATAC), cyp51BqPCR-Rev (5′-TCGAAGACGCCCTTGTG), cyp51AqPCR-For (5′-TGCAGAGAAAAGTATGGCGA) and cyp51AqPCR-Rev (5′-CGCATTGACATCCTTGAGC) at 10 nM.

Experimental design and data analysis were carried out according to current best practice.[9] Basal expression levels were estimated as described previously[10] 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

C. B. M. has received grant support from the Fungal Research Trust and Pfizer, travel grants from Astellas and has been paid for talks on behalf of Pfizer. D. W. D. holds founder shares in F2G Ltd and Myconostica Ltd, both University of Manchester spinoff companies, and has received grant support from F2G as well as the Fungal Research Trust, the Wellcome Trust, Moulton Trust, the Medical Research Council, Chronic Granulomatous Disease Research Trust, National Institute of Allergy and Infectious Diseases, the National Institute of Health Research, the European Union, AstraZeneca and Basilea. He continues to act as an advisor/consultant to F2G and Myconostica as well as other companies over the last 5 years, including Basilea, Vicuron (now Pfizer), Pfizer, Schering-Plough, Nektar, Daiichi, Astellas, Gilead and York Pharma. He has been paid for talks on behalf of Schering, Astellas, Merck, Dainippon and Pfizer. P. B. has received grant support from the Fungal Research Trust, the National Institute of Health Research, the European Union and AstraZeneca. A. B.: none to declare.

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