



# Linking calcium signaling and mitochondrial function in fungal drug resistance

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The host range for *Aspergillus fumigatus* is wide, including mammals, aves, and insecta (stonebrood). This is linked to the significant adaptability of this important fungal pathogen. It is thermotolerant, able to grow up to 70 °C, and astonishingly also remains viable down to –20 °C (1). It is microaerophilic and a halophile; forms extensive biofilms, a problem for antifungal eradication in patients; and has >20 secondary metabolite biosynthetic clusters, some of the products of which have immunosuppressive and cytotoxic properties, such as gliotoxin. *A. fumigatus* also produces a large number of extracellular enzymes, many of which are allergenic, making this organism the only common human pathogen to also cause allergic disease. Further, its pathogenic capacity includes human lungs, airways, paranasal sinuses, and keratin-rich toenails, all in those without overt immune deficits. In immunocompromised people, invasion of lungs, skin, or paranasal sinuses is progressive and fatal, unless detected rapidly and optimally treated. *A. fumigatus* produces more allergenic proteins ( $n = \sim 60$ ) than any other living organism yet found. These allergens are produced in situ (usually in the airways) in those with asthma or cystic fibrosis, greatly worsening the patient's condition, i.e., driving mild asthma to severe. All of these forms of aspergillosis are preferentially treated with oral triazole therapy, as the most efficacious and deliverable via intravenous and oral routes. With hundreds of thousands of people with life-threatening invasive aspergillosis and millions with chronic and allergic aspergillosis, our dependence on triazole therapy for better health is profound. Given the extraordinary range of biological attributes of *A. fumigatus*, it is no surprise that another adaptive mechanism of antifungal drug resistance has been described (2).

Resistance to azoles is increasing (3). In cases of invasive disease the majority of resistance is caused by mutation in the gene encoding the target enzyme, Cyp51A. Strains with one resistant allelic variant dominate in this setting and harbor both a tandem

repeat in the *cyp51A* promoter (TR34) and a secondary nonsynonymous mutation within its coding sequence (L98H). These strains are often pan-azole resistant and acquired from the environment (4). Importantly, however, recent work (5) has shown that the most significant burden of disease caused by *A. fumigatus* relates to its chronic forms of infection that last many months or years. Patients with these conditions receive long-term therapy with azole drugs and resistance usually occurs through non-target-mediated mechanisms arising from mutations acquired during growth of the fungus within the lung under azole treatment pressure (6). One theory to account for this discrepancy in resistance profiles is that the stress responses used by the fungus to survive in the host allow first tolerance of low azole levels and then, after mutation, frank resistance to the drug.

A considerable body of evidence concerning mechanisms of non-target-mediated azole resistance has accumulated in recent years (Table 1). The observed mechanisms include mitochondrial respiratory function, calcium signaling, cell wall processes, and efflux pumps—a similar resistance landscape to that observed in yeast fungi such as *Candida albicans*. Several of these mechanisms are related to environmental stress conditions such as hypoxia and oxidative damage that may also lead directly to drug resistance phenotypes.

In PNAS, Li et al. (2) from the Nanjing laboratory of Ling Lu, building on considerable previous literature, found a calcium-dependent mitochondrial mode of action of triazoles against *A. fumigatus* that connects several of the previously observed processes. Reduced cytosolic calcium allows the transcription factor CrzA to up-regulate several azole transporters (and chitin synthetases). Reduced calcium egress from the mitochondria (mediated by SNPs in Cox10), allows this imbalance in the cell to occur. Among the up-regulated genes are AtrF (which our laboratory described in 2002) (7), but not Cdr1B, the main triazole efflux transporter in patient isolates (8). Thus calcium signaling and mitochondrial functions such as those

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**Table 1. Non-target-based mechanisms of resistance in *A. fumigatus* and their link to clinical cases**

	Effect of loss of function on azole susceptibility (where described)	Associated with azole resistance in the clinic
Target driven		
Cyp51A (13)	Increased	Yes
Cyp51B (14)		Yes
Sterol/lipid biosynthesis		
Hmg1 (15)		Yes
CybE (16)	Increased	No*
OrmA (17)	Increased	None identified
Regulatory		
CBC (HapB/C/E) (10)	Decreased	Yes
HapX (10)	Decreased	None identified
SrbA (18)	Increased	None identified
AtrR (19)	Increased	None identified
Drug transport		
Cdr1B (8)	Increased	Yes
Calcium signaling		
PmrA (20)		None identified
cnaA (21)	Decreased	None identified
crzA (22)		None identified
Mitochondrial function		
NADH oxidoreductase 29.9 KD subunit (23, 24)	Decreased	Yes
Complex III (rip1) (25)		None identified
Cytochrome C (cycA) (25)		None identified
Dnm1 (26)	Decreased	None identified
Fis1 (26)	Decreased	None identified
Mdv1 (26)	Decreased	None identified
HorA (coenzyme Q biosynthesis) (27)	Decreased	None identified
Cox10 (heme O biosynthesis) (2, 28)	Decreased	Yes
Cox15 (hemeA biosynthesis) (2)	Decreased	None identified

\*Gain of function does not lead to azole resistance.

previously observed by others, including our laboratories and those of Cramer and Steinbach (Table 1), all appear to form part of the same global regulatory process, probably, as suggested by Cramer and coworkers (9), a consequence of adaptation to low oxygen levels with consequent reduction in mitochondrial respiration. This would require down-regulation of many cytoplasmic cellular processes possibly via the CCAAT binding-protein regulatory system (10). Interestingly the sterol pathway itself is a key player in oxygen sensing in *A. fumigatus* (10), controlled by the end product activation of the *srbA* transcription factor.

One implication of this work may be that azole action may not derive entirely from loss of membrane ergosterol or production of toxic sterol intermediates but may arise from disruption of important cellular stress responses required to limit cell self-harm during growth in a stressful environment.

In the clinical setting, samples from patients are often culture negative, despite the absence of antifungal therapy. This greatly hampers the clinical laboratory's ability to test susceptibility and detect resistance. While the yield can be increased by high-volume culture (11), many samples remain steadfastly negative. Strong PCR signals from these patients could reflect residual DNA in the airways, but are equally likely to represent "false negative" cultures. Newly introduced commercial diagnostic PCR assays include primers and probes to detect common CYP51A SNPs that confer resistance (12). Unfortunately no commercial assay will detect any of the non-target-based mechanisms of resistance. Furthermore, frequently negative cultures (conceivably because of a fitness cost related to resistance) currently prevent any real determination of the relative incidence of these less common mechanisms of resistance, especially as some mechanisms are probably transient and present only under triazole pressure.

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