COMMENTARY

Linking calcium signaling and mitochondrial function in fungal drug resistance

Paul Bowyer[a], Michael J. Bromley[a], and David W. Denning[a,1]

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 gilotoxin. *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 = ∼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

[a]Manchester Fungal Infection Group (MFIG), Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, M13 9PL, United Kingdom

Author contributions: P.B., M.J.B., and D.W.D. wrote the paper.

The authors declare no competing interest.

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[1]To whom correspondence may be addressed. Email: ddenning@manchester.ac.uk.

First published January 3, 2020.

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
Cytochorome 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.

1 C. Paulussen *et al.*, Ecology of aspergillosis: Insights into the pathogenic potency of *Aspergillus fumigatus* and some other *Aspergillus* species. *Microb. Biotechnol.* **10**, 296–322 (2017).

2 Y. Li *et al.*, Mitochondrial dysfunctions trigger the calcium signaling-dependent fungal multidrug resistance. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 1711–1721 (2020).

3 A. Abdolrasouli *et al.*, Surveillance for azole-resistant *Aspergillus fumigatus* in a centralized diagnostic mycology service, London, United Kingdom, 1998-2017. *Front. Microbiol.* **9**, 2234 (2018).

4 J. W. van der Linden *et al.*, Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clin. Infect. Dis.* **57**, 513–520 (2013).

5 F. Bongomin, S. Gago, R. O. Oladele, D. W. Denning, Global and multi-national prevalence of fungal diseases-estimate precision. *J. Fungi (Basel)* **3**, E57 (2017).

6 S. J. Howard *et al.*, Frequency and evolution of Azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg. Infect. Dis.* **15**, 1068–1076 (2009).

7 J. W. Slaven *et al.*, Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate. *Fungal Genet. Biol.* **36**, 199–206 (2002).

8 M. G. Fraczek *et al.*, The cdr1B efflux transporter is associated with non-cyp51a-mediated itraconazole resistance in Aspergillus fumigatus. *J. Antimicrob. Chemother.* **68**, 1486–1496 (2013).
9 M. Blatzer *et al.*, SREBP coordinates iron and ergosterol homeostasis to mediate triazole drug and hypoxia responses in the human fungal pathogen *Aspergillus fumigatus*. *PLoS Genet.* **7**, e1002374 (2011).
10 F. Gsaller *et al.*, Sterol biosynthesis and azole tolerance is governed by the opposing actions of SrbA and the CCAAT binding complex. *PLoS Pathog.* **12**, e1005775 (2016).
11 P. Vergidis *et al.*, High-volume culture and quantitative real-time PCR for the detection of Aspergillus in sputum. *Clin. Microbiol. Infect.*, 10.1016/j.cmi.2019.11.019 (2019).
12 P. L. White, R. B. Posso, R. A. Barnes, Analytical and clinical evaluation of the PathoNostics AsperGenius assay for detection of invasive aspergillosis and resistance to azole antifungal drugs directly from plasma samples. *J. Clin. Microbiol.* **55**, 2356–2366 (2017).
13 A. M. Albarrag *et al.*, Interrogation of related clinical pan-azole-resistant *Aspergillus fumigatus* strains: G138C, Y431C, and G434C single nucleotide polymorphisms in cyp51A, upregulation of cyp51A, and integration and activation of transposon Atf1 in the cyp51A promoter. *Antimicrob. Agents Chemother.* **55**, 5113–5121 (2011).
14 A. Buied, C. B. Moore, D. W. Denning, P. Bowyer, High-level expression of cyp51B in azole-resistant clinical *Aspergillus fumigatus* isolates. *J. Antimicrob. Chemother.* **68**, 512–514 (2013).
15 J. M. Rybak *et al.*, Mutations in *hmg1*, challenging the paradigm of clinical triazole resistance in Aspergillus fumigatus. *MBio* **10**, e00437-19 (2019).
16 M. Misslinger *et al.*, The cytochrome b_5 CybE is regulated by iron availability and is crucial for azole resistance in A. fumigatus. *Metallomics* **9**, 1655–1665 (2017).
17 P. Zhai, J. Song, L. Gao, L. Lu, A sphingolipid synthesis-related protein OrmA in *Aspergillus fumigatus* is responsible for azole susceptibility and virulence. *Cell. Microbiol.* **21**, e13092 (2019).
18 S. D. Willger *et al.*, Dsc orthologs are required for hypoxia adaptation, triazole drug responses, and fungal virulence in *Aspergillus fumigatus*. *Eukaryot. Cell* **11**, 1557–1567 (2012).
19 D. Hagiwara *et al.*, A novel Zn2-Cys6 transcription factor AtrR plays a key role in an azole resistance mechanism of *Aspergillus fumigatus* by co-regulating cyp51A and cdr1B expressions. *PLoS Pathog.* **13**, e1006096 (2017).
20 J. Song, X. Liu, P. Zhai, J. Huang, L. Lu, A putative mitochondrial calcium uniporter in *A. fumigatus* contributes to mitochondrial Ca(2+) homeostasis and stress responses. *Fungal Genet. Biol.* **94**, 15–22 (2016).
21 R. S. Almeida *et al.*, Genetic bypass of *Aspergillus nidulans* crzA function in calcium homeostasis. *G3 (Bethesda)* **3**, 1129–1141 (2013).
22 F. Lamoth, P. R. Juvvadi, C. Gehrke, W. J. Steinbach, In vitro activity of calcineurin and heat shock protein 90 inhibitors against *Aspergillus fumigatus* azole- and echinocandin-resistant strains. *Antimicrob. Agents Chemother.* **57**, 1035–1039 (2013).
23 M. Bromley *et al.*, Mitochondrial complex I is a global regulator of secondary metabolism, virulence and azole sensitivity in fungi. *PLoS One* **11**, e0158724 (2016).
24 P. Bowyer *et al.*, Identification of novel genes conferring altered azole susceptibility in *Aspergillus fumigatus*. *FEMS Microbiol. Lett.* **332**, 10–19 (2012).
25 B. Geissel *et al.*, Azole-induced cell wall carbohydrate patches kill *Aspergillus fumigatus*. *Nat. Commun.* **9**, 3098 (2018).
26 M. Neubauer *et al.*, Mitochondrial dynamics in the pathogenic mold *Aspergillus fumigatus*: Therapeutic and evolutionary implications. *Mol. Microbiol.* **98**, 930–945 (2015).
27 K. Kroll *et al.*, The hypoxia-induced dehydrogenase HorA is required for coenzyme Q10 biosynthesis, azole sensitivity and virulence of *Aspergillus fumigatus*. *Mol. Microbiol.* **101**, 92–108 (2016).
28 X. Wei *et al.*, Screening and characterization of a non-cyp51A mutation in an *Aspergillus fumigatus* cox10 strain conferring azole resistance. *Antimicrob. Agents Chemother.* **61**, e02101–e02116 (2016).