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The *cdr1B* efflux transporter is associated with non-*cyp51a*-mediated itraconazole resistance in *Aspergillus fumigatus*

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Objectives: Recent increases in triazole resistance in *Aspergillus fumigatus* have been attributed primarily to target site (*cyp51A*) mutations. A recent survey of resistant isolates in Manchester showed that >50% of resistant isolates had no mutation in *cyp51A* or its promoter. We investigated the mechanisms of resistance in clinical azole-resistant isolates without *cyp51A* mutations.

Methods: Twelve azole-resistant isolates, 10 of which were itraconazole resistant, were studied. Bioinformatic comparisons between *Candida albicans* efflux genes and *A. fumigatus* genome data identified 20 putative azole transporter genes. Basal and azole-induced expression of these genes and *cyp51A* was quantified using RT–PCR with comparison with clinical azole-susceptible isolates. Function of high basal or itraconazole-induced expression transporters was tested by gene knockout in azole-susceptible and azole-resistant isolates.

Results: All susceptible strains showed minimal basal expression of *cdr1B* compared with 8 of 10 azole-resistant strains with high basal expression of this gene (>5-fold), 3 of which showed >30-fold increased expression. Knockout of this gene resulted in a 4-fold reduction in itraconazole, posaconazole and voriconazole MICs for a susceptible clinical isolate and a 4-fold reduction in itraconazole susceptibility in a clinical resistant isolate. One strain showed a >500-fold induction of *cyp51A*. No increase in basal expression or expression after induction was seen for the 18 remaining putative transporters.

Conclusions: The reasons behind the shift away from target site mutation in azole-resistant isolates from Manchester are unknown. The modest change in expression of *cdr1B* in azole-susceptible strains implies that only study of resistant isolates will lead to further understanding of resistance mechanisms in *A. fumigatus*.

Keywords: aspergillosis, drug resistance, efflux pumps, antifungal, azole resistance

Introduction

Fungal disease affects individuals with dysfunctional immune responses. The most commonly affected are those with hyperactive immune responses, such as atopic individuals, who may have complications of asthma caused by fungal allergy.[1,2] Immunocompromised individuals are subject to devastating, frequently fatal invasive mycoses.[3] Finally, ostensibly normal individuals can occasionally suffer from cavitating forms of fungal disease.[4] The two most important genera affecting human health are *Candida* and *Aspergillus*, with *Aspergillus fumigatus* being the most common cause of mould-related mycosis.[5]

Therapy for fungal disease is limited to a small number of drug classes, including azoles, echinocandins and polyenes. Azoles are the most commonly used antifungal therapy and resistance to azoles has been observed in *Candida* and more recently *Aspergillus*. The mortality of patients with multi-azole-resistant invasive aspergillosis was 88% compared with 30%–50% who were infected with azole-susceptible strains.[6] Time from diagnosis to resolution of the infection or death in invasive forms of fungal disease is typically a few weeks, whereas in allergic or chronic diseases patients may take antifungal medication for many years. In both cases drug resistance is highly problematic. Only azoles are orally active against *Aspergillus*, restricting

patients who harbour drug-resistant isolates to intravenous treatment options.

Azoles act by inhibiting the activity of the cytochrome p450 gene lanosterol 14-α-demethylase, a key enzyme in biosynthesis of ergosterol. It is thought that lack of ergosterol diminishes membrane rigidity and leads to fungal cell death.[7] Azole resistance in *Candida albicans* and *Candida glabrata* and other yeasts is well studied[8–19] and the majority of cases are thought to be attributable to overexpression of the drug efflux transporters *CDR1*, *CDR2* and *MDR1* or to mutations in the transcription factors *TAC1* and *MRR1* that lead to overexpression of the transporter genes.[14–16] Mutations at the azole target gene *cyp51A* are also frequently observed.[20,21] Alternatively, aneuploidy arising from duplications in chromosomal regions containing these genes or their orthologues has also been observed to lead to azole resistance in both *Candida* spp. and *Cryptococcus neoformans*.[22,23]

Itraconazole resistance was first reported in *A. fumigatus* in 1997 (in isolates obtained from the USA in the late 1980s).[24] Since then, many reports have described resistance in *A. fumigatus* clinical isolates in Belgium, Canada, China, Denmark, France, Norway, Spain, Sweden, the Netherlands, the UK, the USA[25–28] and recently India.[29] The global frequency is not clearly defined as many laboratories do not test the azole susceptibility of clinical *A. fumigatus* isolates or use a common standard method for susceptibility. A dominant target mutation (TR+L98H) appears to be common in the environment in the Netherlands and China.[28,30] In a recent survey of azole-resistant *A. fumigatus* in a UK referral clinic a striking rise in drug resistance was observed.[6,26,31] Until 2008 all observed resistance was attributable to mutations in the *cyp51A* gene; however, between 2008 and 2010 resistant isolates containing no mutations in this gene or promoter became more prevalent. In 2010, 51% of Manchester isolates that were resistant to azoles were non-*cyp51A*. This is a major concern as molecular assays developed to provide rapid diagnosis of *cyp51A*-based drug resistance are of no utility in this set of isolates.

Although several studies have demonstrated an increase in transcription of transporter genes in azole-susceptible isolates, previous attempts to define whether *A. fumigatus* can become clinically drug resistant in the same transporter expression-dependent manner as *C. albicans* have been inconclusive. Two major facilitator transporters, *AfuMDR1* and *AfuMDR2*, were identified by Tobin *et al.*[32] Slaven *et al.*[33] observed overexpression of *atrF* in a clinical azole-resistant isolate, AF72. Nascimento *et al.*[34] showed up-regulation of two transporters, *AfuMDR3* and *AfuMDR4*, in 2/23 laboratory mutants that were resistant to itraconazole, the latter of which has also been shown to be up-regulated in a biofilm phenotype.[35] da Silva Ferreira *et al.*[36] showed induction of five ABC transporters, *abcA–E*, and MFS transporters *mfsA–C* in response to voriconazole in a laboratory-derived clinical azole-susceptible isolate, CEA17. However, the relationship of these findings to clinical azole resistance has not been convincingly demonstrated.

Here we used the results from these previous studies, together with genes predicted by comparison with *C. albicans* drug resistance genes, to attempt to discover drug efflux transporters that are clinically important in the development or maintenance of azole resistance in *A. fumigatus*. Our rationale was to survey existing knowledge of azole transporters in *A. fumigatus*, then to test the function of candidate genes in clinical isolates of *A. fumigatus* that were resistant to azoles but that did not carry a *cyp51A* mutation, in order to avoid unnecessary complexity and to characterize the mechanism of resistance in the increasingly common non-*cyp51A* isolates.

Materials and methods

Strains and media

A. fumigatus clinical isolates, obtained from the Mycology Reference Centre Manchester (MRCM), UK were used throughout this work (Table 1). Itraconazole-resistant isolates were selected from a total of 230 *A. fumigatus* clinical isolates received between 1 January 2008 and 31 December 2009.[6,26] The MICs for the isolates were determined using the broth microdilution method as previously described.[37] An A1160 Δ*Ku80 pyrG*$^{+}$ strain (here referred to as A1160 *pyrG*$^{+}$) was constructed in this study and served as a parental strain for transporter gene deletion mutants. *A. fumigatus* isolates and strains were typically stored as stock spore suspensions in sterile glycerol nutrient broth at −80°C and subsequently subcultured on Sabouraud dextrose agar (SDA) (Oxoid) for transformation or Vogel's minimal agar[38] containing 1% glucose for MIC determination and incubated at 37°C for 24 h before spores were used. Spores were harvested in 1× PBS with 0.05% Tween 20, counted using an Improved Neubauer haemocytometer (Weber Scientific International, Cambridge, UK) and adjusted to 1×10^{5} cfu/mL for MIC determination or to 1×10^{6} cfu/mL for transformation, DNA or RNA cultivation. For transformation a YPS medium was used, composed of 2% yeast extract (Oxoid), 0.5% peptone (Oxoid), 0.9 M sucrose (Sigma), 5 mM Tris base (Sigma) and 1.5% technical agar (Oxoid) (pH 6 for transformation of hygromycin cassettes and pH 8 for transformation of phleomycin cassettes).

Quantification of gene expression by RT–PCR

RT–PCR was performed on an Mx3005p real-time PCR machine (Stratagene) essentially as previously described.[39] Negative controls consisted of no-RNA or no-template wells with or without reverse transcriptase. Primers were optimized and tested individually to minimize primer–dimer artefacts. After the RT–PCR, melting curve determination was performed and 5 μL of each sample was analysed by gel electrophoresis on a 1% agarose gel to ensure that there was no non-target amplification. The RT–PCR was typically performed in 25 μL reactions containing 50 ng of *A. fumigatus* RNA, 2 pmol of each forward and reverse primer and 12.5 μL of Brilliant SYBR Green qRT–PCR Mix (Stratagene). PCR was carried out under the following conditions: 50°C for 60 min (for reverse transcription) then 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 1 min and extension at 72°C for 1 min. Results were analysed using the $2^{-\Delta\Delta Ct}$ method. Primers used are shown in Table S1 (available as Supplementary data at *JAC* Online). Statistical analysis followed the method suggested by Yuan *et al.*,[40] essentially using two group *t*-tests and non-parametric analogous Wilcoxon tests on paired samples, usually delta Ct values from the same gene in different isolates ranked against the same gene in Af293, using SPSS.

Construction of the A1160 ΔKu80 pyrG^{+} strain

An A1160 Δ*Ku80 pyrG*$^{+}$ strain was constructed as a parental strain for transporter gene deletion mutants. The CEA17Δ*akuB*^{ku80} *pyrG*$^{-}$ strain (FGSC A1160),[33] containing a single base-pair mutation in *pyrG*,[40] was transformed with a functional *A. fumigatus pyrG* gene derived from A1163 by PCR using primers Afpyrg-F and Afpyrg-R (Table S1) as described below.

Table 1. Description of azole-resistant and -susceptible strains used in this study and treatment history and underlying diseases of the individuals from whom the fungi were isolated

Patient	Sex	Age (years)	Fungal diagnosis	Strain	*cyp51A* mutation	MIC (mg/L)			Previous antifungals	Time on azoles	Underlying diagnoses
						ITC	VRC	POS			
1	M	55	CPA	F17727	—	>8	>8	>8	ITC, VRC	>5 years	TB, AS
2	F	58	CPA	F18149	—	>8	>8	2	ITC, VRC	>2 years	NA
3	F	55	CPA	F19980	—	>8	>8	0.5	POS	NA	NA
4	M	45	subacute IA	F17999	H147Y, G448S	>8	>8	0.5	VRC	NA	lung SSC, chemotherapy
5	F	42	ABPA	F18085	A248T	>8	4	0.25	ITC	>1 year	NA
6	F	17	ABPA > CPA	F20140	—	>8	2	0.5	VRC	>5 years	CF
7	F	66	ABPA	F18304	—	>8	2	0.125	ITC, VRC	>2 years	RA, bronchiectasis
8	F	35	ABPA	F20063	—	>8	1	0.5	ITC	>1 year	asthma
9	M	62	IA	F20451	—	>8	1	0.25	NR	NA	AML, neutropenic
10	F	53	IA	F18454	—	0.5	>8	0.06	VRC	1 week	reno-pancreatic failure
11	M	7	ABPA	F18329	—	1	4	0.25	NA	NA	CF
12	M	42	ABPA	F15483	—	0.5	1	0.06	NA	NA	NA
13	M	NA	IA	Af293 (CBS 101355)	—	0.5	0.25	0.06	NA	NPE	aplastic anaemia
14	M	NA	NA	Af210	—	0.5	0.25	0.06	NA	NPE	Crohn's disease, peritonitis
15	M	NA	IA	CEA10 (CBS 144.89)	—	0.5	0.25	0.06	NA	NPE	NA

M, male; F, female; CPA, chronic pulmonary aspergillosis; IA, invasive aspergillosis; ABPA, allergic bronchopulmonary aspergillosis; ITC, itraconazole; POS, posaconazole; VRC, voriconazole; NA, data not available; NPE, no previous exposure; TB, *Mycobacterium tuberculosis* infection; AS, ankylosing spondylitis; SSC, squamous cell carcinoma; CF, cystic fibrosis; RA, rheumatoid arthritis; AML, acute myeloid leukaemia.

Construction of transporter gene deletion cassettes

Gene knockout cassettes were constructed by a modified PCR fusion method.[41,42] Four sets of primers were used in order to create each deletion cassette (Table S1) for AFUA_1G14330 (*cdr1B*) and AFUA_1G05010 (*MFS56*). Primer sets P1–P2 and P5–P6 were used to amplify the upstream and downstream flanking regions of the genes, respectively. Primers P3–P4 amplified a 2.8 kb hygromycin B phosphotransferase cassette from pAN7-1 including the *gpdA* promoter and *TtrpC* terminator (*hph* cassette).[43] The above primers were used in three separate PCRs (first-round PCR) consisting of ~100 ng of Af293 genomic DNA or 10 ng of plasmid DNA, 0.2 μM of forward and reverse primers, 200 μM dNTPs, 2 U of the LongAmp *Taq* DNA polymerase (NEB) and 1× reaction buffer in a total volume of 25 μL. Cycling conditions were: 1 cycle at 94°C for 30 s, then 35 cycles at 94°C for 15 s, 55°C for 15 s and 68°C for 3 min. A single elongation step at 68°C for 5 min was performed at the end of the reaction. Upstream and downstream flanking regions of the genes were column purified using a Qiagen PCR purification kit. The hygromycin cassette was gel purified using a Qiagen gel purification kit.

Primers P7 and P8 (nested) were used in the second-round PCR to fuse the three first-round PCR fragments. The reaction mixture was composed of 1 μL of gel-purified first-round PCR products (~100 ng each), 0.4 μM of forward and reverse primers, 400 μM of dNTPs, 4 U of the LongAmp *Taq* DNA polymerase and 1× reaction buffer in a total volume of 50 μL. The reaction was run at 55°C annealing and 5 min elongation as in Weidner *et al.*[44] Direct PCR products of both Δ*cdr1B*::*hph* and Δ*MFS56*::*hph* were used to delete transporter genes in A1160 Δ*Ku80 pyrG*$^{+}$ and F20063 clinical isolates.

For double *cdr1B* and *MFS56* knockout, a ~2.15 kb phleomycin cassette from pAN8-1 under the *gpdA* promoter and *TtrpC* terminator[45] was constructed with the same primers, P1–P8, as for the *hph* cassette because they share the same amplification regions in the *gpdA* promoter and *TtrpC* terminator. This cassette was used for transformation of A1160 Δ*Ku80 pyrG*$^{+}$ Δ*MFS56*::*hph*, generating a A1160 Δ*Ku80 pyrG*$^{+}$ Δ*cdr1B*::*phleo*Δ*MFS56*::*hph* mutant (A1160 *pyrG*$^{+}$ Δ*cdr1B*Δ*MFS56*).

Fungal transformation

Fungal transformations were performed using an optimized Ballance and Turner method.[46] Briefly, spores (2×10^{6}/mL) were grown in 50 mL of liquid SDA broth containing 100 μg/mL ampicillin distributed into three 9 cm Petri dishes for 14–16 h at 37°C (static culture). The hyphae were harvested by filtration through a single layer of lens tissue, washed once in 1× PBS, then digested in 20 mL of sterile 50 mM $CaCl_2$/0.6 M KCl solution (pH 7.6) containing 1 g of Glucanex 200G (Novozyme) at 30°C for 3 h with gentle mixing (100 rpm) to form protoplasts. The digestion mixture was filtered through two layers of lens tissue and the protoplasts were centrifuged at 800 **g** for 5 min at 4°C. The pellet was resuspended in 1 mL of cold 50 mM $CaCl_2$/0.6 M KCl solution and protoplasts were counted using an Improved Neubauer haemocytometer, then adjusted to give 1×10^{6} per 100 μL aliquot. Fifteen microlitres of the second-round PCR product was added to each aliquot and mixed with 20 μL of 40% polyethylene glycol (PEG) 6000 (prepared in 50 mM $CaCl_2$/0.6 M KCl) followed by incubation on ice for 25 min. Subsequently, 200 μL of 40% PEG6000 was added and the protoplasts were incubated for 5 min at room temperature. The mixtures were transferred onto solid YPS medium supplemented with 200 μg/mL hygromycin B and/or 150 μg/mL zeocin (Melford) and incubated at 37°C until the appearance of resistant colonies. These were streaked on SDA supplemented with 200 μg/mL hygromycin B and/or 150 μg/mL zeocin (Melford) and a single colony from each strain was grown on the same medium for DNA extraction and other subsequent analyses (below).

DNA extraction

DNA extractions were performed on spores using an optimized cetyl trimethyl ammonium bromide (CTAB) DNA extraction method. Briefly, spores were harvested in PBS/Tween 20, centrifuged at 6000 **g** for 2 min and resuspended in 1 mL of extraction buffer (2% CTAB, 100 mM Tris, 1.4 M NaCl and 10 mM EDTA, pH 8.0) and transferred to a 2 mL screw-top tube containing 425–600 μm washed glass beads (filled to the 300 μL mark; ~50 mg) (Sigma). Subsequently they were vortexed for 10 min at maximum speed (Genie-2) and incubated for 10 min at 65°C. Vortexing and heating steps were repeated. The spores were centrifuged at maximum speed for 2 min and the supernatant was transferred to a 2 mL tube containing 4 μL of 100 mg/mL RNase (Qiagen) and incubated at 37°C for 15 min. Then 700 μL of chloroform:isoamyl alcohol (24:1) was added, the tube was mixed and centrifuged at maximum speed for 2 min. The aqueous phase was transferred to a 1.5 mL Eppendorf tube and 0.6 volumes of isopropanol was added. The tube was mixed and centrifuged as above. Isopropanol was decanted and the pellet was washed in 500 μL of 70% ethanol and centrifuged for 1 min at 8000 **g**. The alcohol was decanted and the tube was pulse-centrifuged. The residual alcohol was removed using a gel-loading tip and the pellet was resuspended in 100 μL of H_2O. This protocol provided large amounts of good-quality genomic DNA.

Analysis of mutants

The *pyrG* locus of the A1160 Δ*Ku80 pyrG*$^{+}$ strain was sequenced with primers PyrGMutsq-F and PyrGMutsq-R to confirm the *pyrG*$^{-}$ gene replacement. Primers PyrGck-F and PyrGck-R were used to confirm correct integration. All transporter gene deletion mutants were screened by PCR with two sets of primers: P1–P4 and P5–P6, which only generated PCR products if correct integration events occurred. Additionally, primers P9 and P10 were used to confirm gene deletion. These primers match the sequence of the target gene. The A1160 Δ*Ku80 pyrG*$^{+}$ and F20063 DNAs were used as the template for positive PCR control reactions.

RNA extraction

Six baffled conical flasks containing 40 mL of Vogel's medium with 1% glucose were inoculated with spores to a final concentration of 1×10^{6} spores/mL then incubated at 37°C with shaking at 250 rpm for 14–16 h. Itraconazole was added to one set of three flasks to a final concentration of 4 mg/L for >8 mg/L resistant isolates. An equivalent volume of DMSO was added to the other set of three control flasks. Subsequently, fungal mycelia were collected by centrifugation at 3000 **g**, then 100 mg of fungal mycelium was transferred to a red-cap tube containing 1 mL of RNA*pro*™ Solution (Lysing Matrix). RNA was extracted using a FastRNA® Pro Red kit (Qbiogene) according to the manufacturer's instructions. RNA was measured spectrophotometrically then diluted to 25 ng/μL. Aliquots of RNA were stored immediately at −80°C until use.

Results

Selection and characterization of non-cyp51A-resistant isolates

Susceptibilities to the antifungals itraconazole, posaconazole and voriconazole were determined for 64 azole-resistant clinical isolates provided by the MRCM during 2008–09 as previously described.[6,26] *Candida krusei* ATCC 6258 was used as a control strain for each MIC plate. All control results (data not shown) were within the acceptable target range. MIC values for resistant *A. fumigatus* isolates were consistent with clinical laboratory

results obtained at the time of isolation. The entire *cyp51A* gene and 1 kb promoter was sequenced in each isolate. Briefly, isolates were subjected to PCR to amplify overlapping regions of the *cyp51A* gene using primer pairs cyp51_6-F and cyp51_8-R, cyp51_4-F and cyp51_2-R, cyp51_1-F and cyp51_7-R and cyp51_3-F and cyp51_5-R (Table S1), amplicons were sequenced in both directions and the resulting assembled sequence was examined to determine the presence of non-synonymous single-nucleotide polymorphisms (SNPs) or other mutations. Strains that did not carry mutations or that carried mutations not thought to be associated with azole resistance were selected for further analysis (Table 1). One strain, F17999, carrying *cyp51A* mutation G448S, known to be associated with resistance to voriconazole and posaconazole,[6] was also included for comparison.

There is no correlation between azole treatment and resistance in either duration or azole type. For example isolate F19980 was isolated from an individual with a history of posaconazole treatment yet the isolate is resistant to voriconazole and itraconazole, but not posaconazole (Table 1). Strain F17727 was isolated from an individual with a history of itraconazole and voriconazole treatment yet is resistant to itraconazole, voriconazole and posaconazole. Alternatively, F18454 is derived from an individual with a short period of voriconazole treatment and shows only voriconazole resistance. The underlying disease, type of fungal disease, age or sex also appear not to correlate with resistance. These observations must be treated with some caution. It is possible that individuals had been treated with widely available fungicides such as itraconazole without the treatment being recorded; however, the restricted use of voriconazole and posaconazole would indicate that records of usage are more accurate.

Bioinformatic analysis of potential A. fumigatus azole transporter genes

In order to determine whether the *A. fumigatus* genome carried the same complement of drug-resistance genes previously identified in *C. albicans* and to clarify the diverse historical descriptions of *A. fumigatus* transporters associated with drug resistance for further study, an analysis of orthology was carried out by the Best-Reciprocal-Hits from BLAST method. Essentially, *C. albicans* gene sequences (*CDR1*, *CDR2*, *MDR1*, *TAC1* and *MRR1*) were compared with the *A. fumigatus* genome (Af293) using BLASTX, TBLASTN and BLASTN using an $1E^{-20}$ cut-off. *AfuMDR1–2*, *AfuMDR3–4*, *abcA–E*, *mfsA–C*, *ABC11*, *MFS56* [50] and *atrF* were compared with the *C. albicans* genome (SC5314 build 19) in a similar manner. Resulting BLAST hits in the *A. fumigatus* genome were then compared back with *C. albicans* and hits in the *A. fumigatus* genome were compared back with the *C. albicans* genome using a cut-off score of $1E^{-20}$ to assess orthology (Table S2, available as Supplementary data at *JAC* Online).

CDR1 and *CDR2* show homology (45%–50% identity) to the same set of *A. fumigatus* genes annotated as ABC transporters. However, when these *A. fumigatus* genes were compared with the *C. albicans* genome they revealed homology to *CDR1* and *CDR4*, suggesting that there is no true orthologue of *CDR2* in *A. fumigatus*. In this case AFUA_2G15130 and AFUA_1G14330 would be paralogous to each other and orthologous to *CDR1*. For clarity we term these genes *cdr1A* (AFUA_2G15130, previously annotated as *CDR4*) and *cdr1B* (AFUA_1G14330), previously referred to as *abcC* and *ABC11*,[36,47] throughout. *CaMDR1* had weak homology (30%–34% identity) to a group of *A. fumigatus* genes annotated as MFS transporters that in turn showed homology to the *C. albicans* gene *MDR1*. The *C. albicans* genes *TAC1* and *MRR1* and the *A. fumigatus* genes *abcB*, *AfuMDR3*, *mfsA* and *mfsC* appear to possess no true orthologues in the *C. albicans* genome. The *A. fumigatus abcA* gene[34] is the same as *AfuMDR1*[32] and, with *abcA*, *abcD*, *abcE* and *AfuMDR2*, *AfuMDR4* appears to be orthologous to a group of *C. albicans* mitochondrial ABC transporters containing *MDL1*, *MDL2* and *HST6*. *A. fumigatus mfsB* shows orthology to *C. albicans DHA1*. The lack of orthology in these groups reflects the fact that the different organisms may possess different complements of transporters. Orthology of suspected drug transporters to proteins annotated as glycerol or peptide transporters may reflect 'rewiring' of transporter function or simply incorrect annotation. From 11 *A. fumigatus* transporter genes previously implicated in azole resistance, only *abcC* (*ABC11*), *abcB* and *mfsB* are orthologous to known *Candida* efflux genes.

Analysis of basal and azole-induced transporter gene transcription in resistant and susceptible A. fumigatus isolates

To determine whether the non-target resistance observed in the Manchester strains could be explained by an increase in transporter expression in a manner analogous to known *C. albicans* drug resistance mechanisms, RT–PCR was used to analyse a set of 20 transporter genes as well as *cyp51A* in minimal medium with and without sub-MIC levels (1 mg/L itraconazole in Vogel's glucose) of itraconazole. The set of transporters was chosen on the basis of homology to known *C. albicans* azole resistance genes, *CDR1* and *MDR1* (AFUA_2G15130, AFUA_1G14330, AFUA_1G17440, AFUA_5G00790, AFUA_3G07300, AFUA_2G11580, AFUA_2G16860, AFUA_1G06440 and AFUA_1G10370; Table S2), known for induction in response to azole in wild-type (azole-susceptible) Af293, consisting of *abcA–E* and *mfsA–C*,[33] *MDR1*, *MDR2*, *AfuMDR3*, *AfuMDR4*[34] and *atrF*,[33] and for probable involvement in azole resistance from a previous insertional mutagenesis screen in our laboratory (*CDR1-B=abcC*: AFUA_1G14330, *MFS56*: AFUA_1G05010).[47] Expression levels for the 20 transporter genes and *cyp51A* were determined by RT–PCR using *β-tubulin* expression as a comparator. Both basal levels (Figure 1) and fold induction compared with basal level after addition of azole (Figure 2) were determined in the azole-susceptible strain Af293. Two other azole-susceptible controls, Af210 and CEA10, were also analysed (not shown), but showed <10% variation in expression for the genes tested when compared with Af293. Most genes were poorly expressed during normal growth, with expression levels typically <20% of the *β-tubulin* comparator (Figure 1). Expression was not detectable for *AfuMDR3*. Fourteen of the genes were induced >2-fold upon exposure to azole, 10 of which were induced 4-5-fold by azole (Figure 2). The *C. albicans MDR1* orthologue AFUA_2G11580 was induced 14.2-fold on azole exposure, although this is compared with a very low basal expression. The *cdr1A* orthologue was induced 6.4-fold in response to azole treatment and the *CDR4* orthologue AFUA_1G17440 was induced 7.1-fold. Basal *cyp51A* expression in Af293 was 0.13-fold that of *β-tubulin* and was induced 5.3-fold in the presence of azoles.

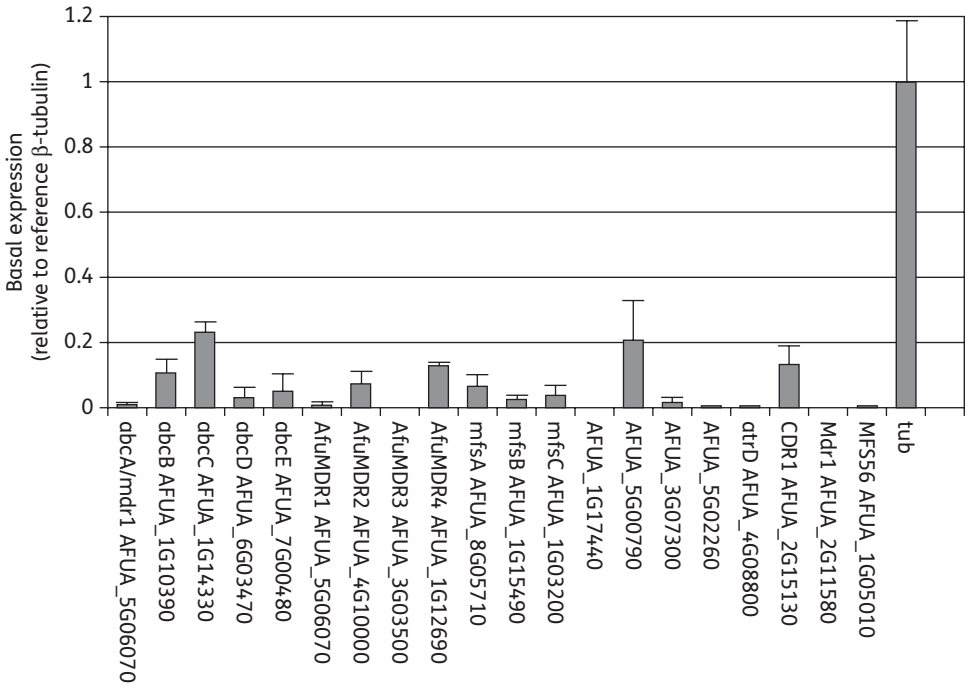

Figure 1. Basal levels of transporter expression in Af293. RNA was extracted from mycelia growing without addition of azole. RT–PCR was used to quantify expression for each gene shown as previously described. Three biological replicates were performed with three technical replicates from each sample. Mean values shown are relative to *β-tubulin* (tub). *AbcC*=*cdr1B*. Error bars represent standard deviations. Gene names and orthology can be found in Table S2.

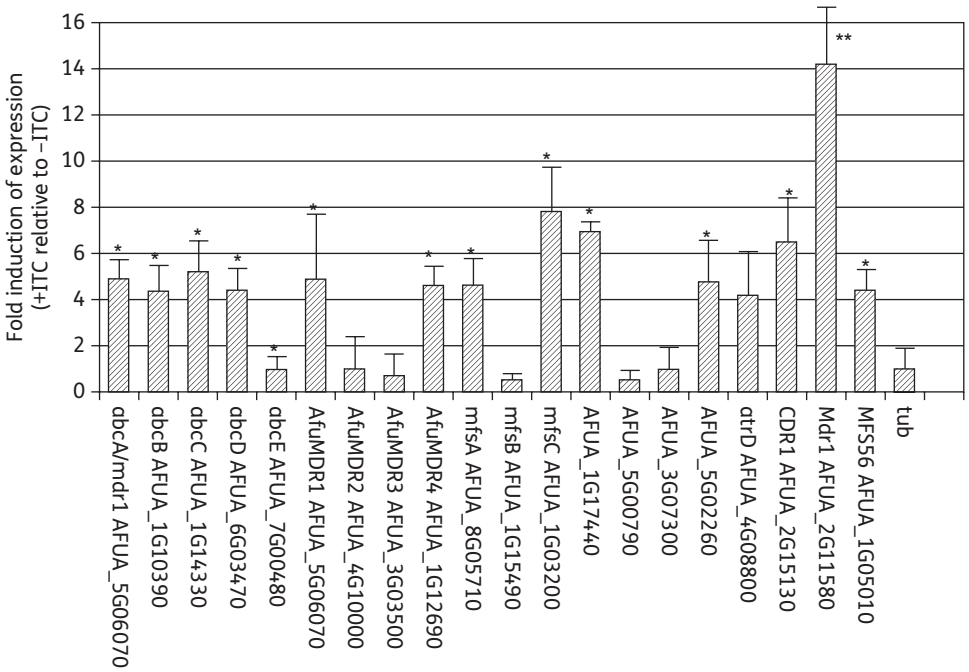

Figure 2. Fold induction of transporter genes in response to itraconazole in Af293. RNA was extracted from mycelia exposed to 1 mg/L itraconazole (ITC) for 4 h. RT–PCR was used to quantify expression for each gene shown as previously described. Three biological replicates were performed with three technical replicates from each sample. Values shown represent fold changes in expression relative to the fold change in *β-tubulin* (tub) expression upon exposure of the culture to 1 mg/L itraconazole. Error bars represent standard deviations. *$P<0.05$ for Wilcoxon paired comparison ranked against *β-tubulin* fold change in expression. **$P<0.01$ for Wilcoxon test ranked against *β-tubulin* fold change in expression. Gene names and orthology can be found in Table S2.

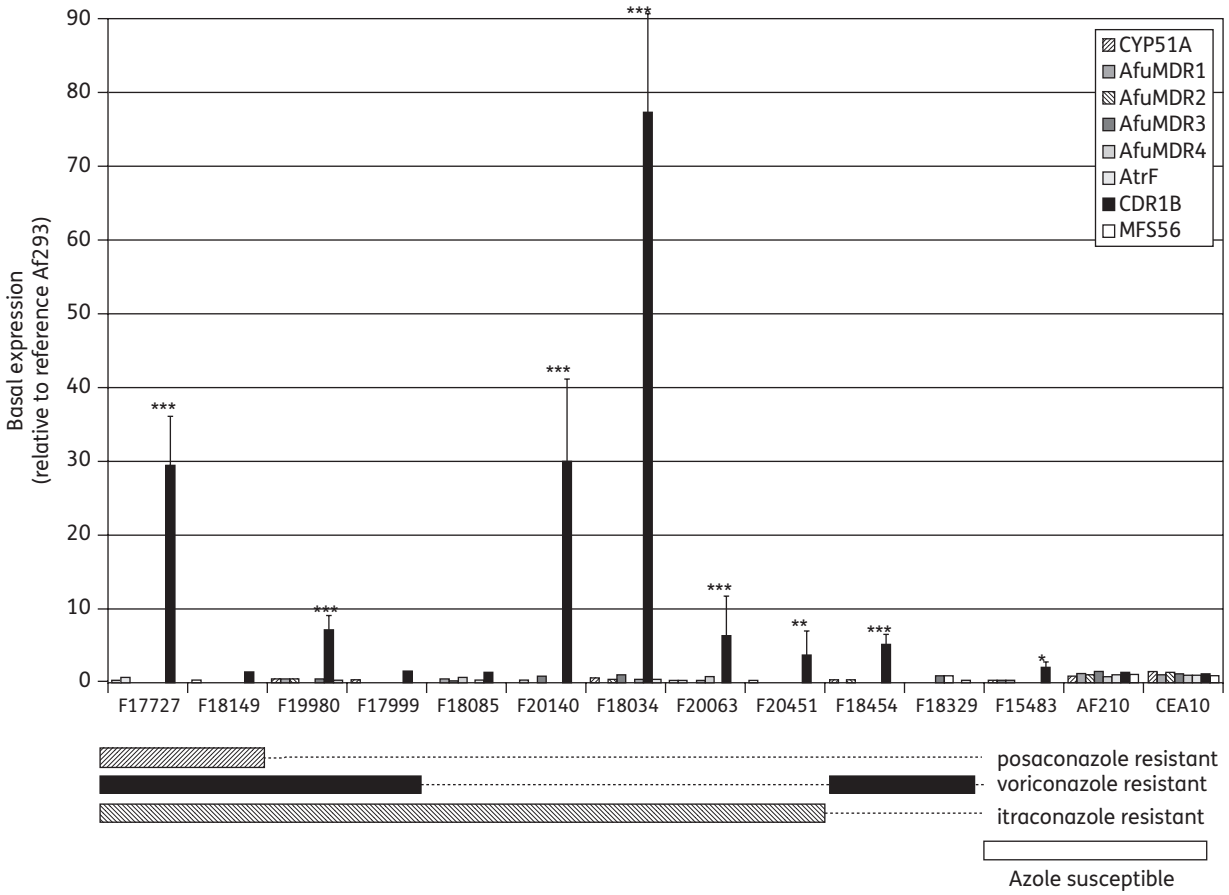

Figure 3. Fold difference in basal expression compared with Af293. Mycelia were not exposed to azoles and values shown represent basal expression of a given gene in an isolate/basal expression of the same gene in Af293. Three biological replicates were performed with three technical replicates from each sample. Values were calculated from expression for each gene using RT–PCR with efficiency >0.95 comparable to *β-tubulin* with equivalent total RNA input for each isolate. ***$P<0.001$ for Wilcoxon test ranked against Af293 basal expression for each gene as shown in Figure 1. **$P<0.01$ for Wilcoxon test ranked against Af293 basal expression for each gene. *$P<0.05$ for Wilcoxon test ranked against Af293 basal expression for each gene. Bars underneath the histogram show which isolates are resistant to the relevant azole (see Table 1 for details). Error bars represent standard deviations. CYP51A, *cyp51A* (AFUA_4G06890); Afu*MDR1*, AFUA_5G06070; AfuMDR2, AFUA_4G10000; AfuMDR3, AFUA_3G03500; AfuMDR4, AFUA_1G12690; AtrF; CDR1B, AFUA_1G14330; MFS56, AFUA_1G05010.

Over-induction of efflux transporters in clinical azole-resistant isolates

We reasoned that efflux transporters important in azole resistance would be more strongly up-regulated in clinical azole-resistant isolates compared with susceptible isolates and performed expression analysis on the clinical azole-resistant isolates in our collection using azole-susceptible isolates as controls. Several transporters (*abcA*, *abcB*, *abcD*, *abcE*, *mfsA–C* and *cdr1A*) showed non-significant differences in induction between any azole-resistant mutant and azole-susceptible comparator strain Af293 and are not shown in Figures 3 and 4 for clarity. *AfuMDR3* and *AfuMDR4* showed a small reduction in both basal expression and induction. Eight of 10 non-*cyp51A* azole-resistant strains showed significantly higher basal levels of expression of *cdr1B* compared with Af293; in particular F20140, F18304 and F18454 showed >25-fold greater basal expression levels than Af293. F19980 (7.2-fold), F20063 (6.5-fold), F20451 (3.6-fold), F18454 (5.1-fold) and F15483 (2.1-fold) also showed significantly raised levels of basal expression in comparison with Af293 (Figure 3).

Ten of 12 azole-resistant isolates showed significantly higher levels of induction of *MFS56* than Af293 (Figure 4); notably F20063, F18085 and F17999 showed >300-fold overexpression of *MFS56* compared with Af293. Several other isolates also exhibited high overexpression of *MFS56* (F19980, 27.8-fold; F20140, 35.6-fold; and F20451, 17.7-fold). F17999 also showed increased induction of *atrF* (31.7-fold). One strain, F18085, showed overexpression of *AfuMDR1* (36.5-fold) and two others, F18304 and F19980, showed high-level induction of *cyp51A* (550.9- and 21-fold, respectively).

Test of gene function in azole-susceptible strain by gene knockout

In order to confirm whether the overexpressed transporters *AfuMDR1–4*, *cdr1B*, *MFS56* and *atrF* played a functional role in

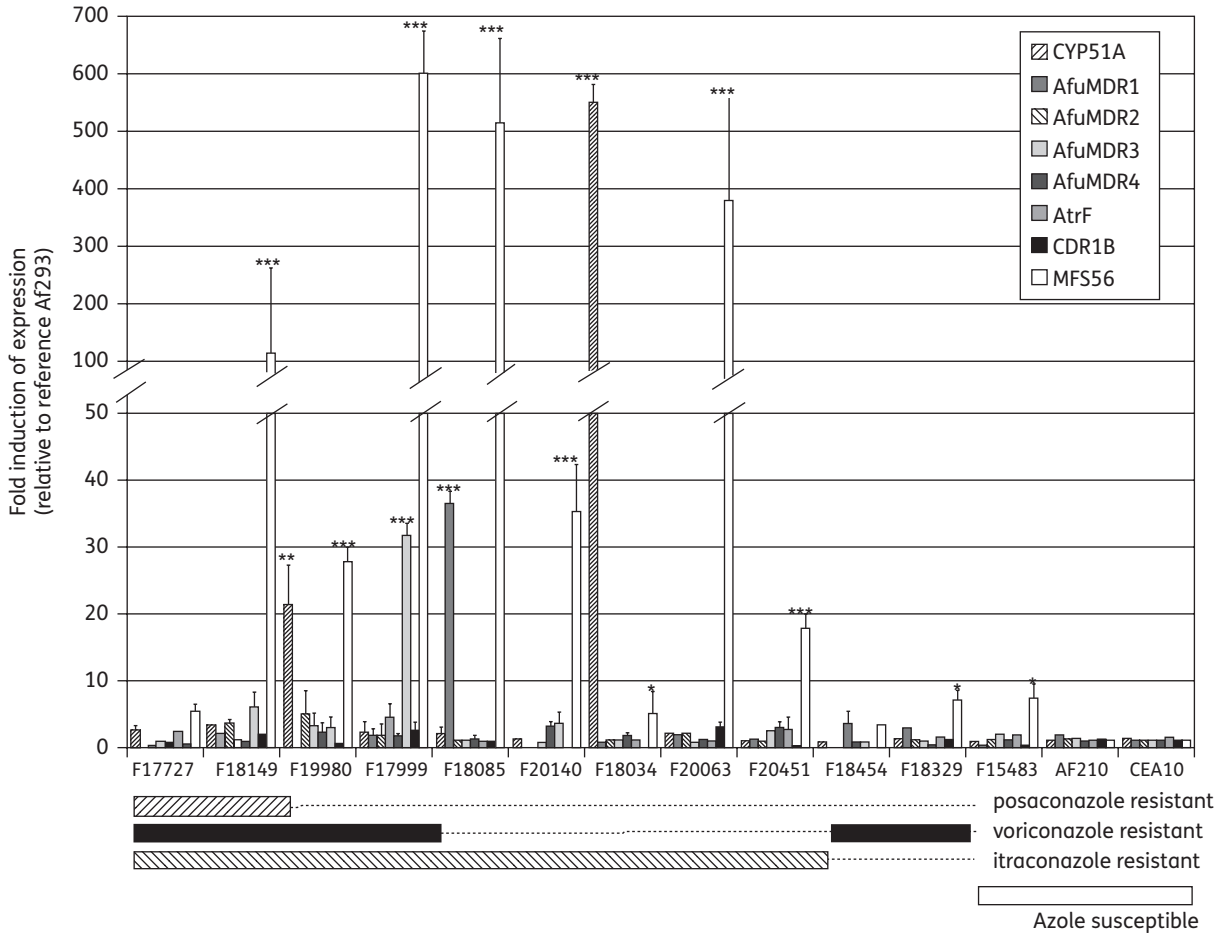

Figure 4. Fold difference in induction of gene expression relative to Af293. Mycelia were exposed to 1 mg/L itraconazole and values shown represent induced expression of a given gene in an isolate/induced expression of the same gene in Af293. Three biological replicates were performed with three technical replicates from each sample. Values were calculated from fold change in expression for each gene relative to fold change induction observed in Af293 (Figure 2). ***$P<0.001$ for Wilcoxon test ranked against Af293 induction of expression for each gene as shown in Figure 2. **$P<0.01$ for Wilcoxon test ranked against Af293 induction of expression for each gene. *$P<0.05$ for Wilcoxon test ranked against Af293 induction of expression for each gene. Bars underneath the histogram show which isolates are resistant to the relevant azole (see Table 1 for details). CYP51A, *cyp51A* (AFUA_4G06890); Afu*MDR1*, AFUA_5G06070; AfuMDR2, AFUA_4G10000; AfuMDR3, AFUA_3G03500; AfuMDR4, AFUA_1G12690; AtrF; *CDR1*B, AFUA_1G14330; MFS56, AFUA_1G05010.

azole resistance, these genes were knocked out in A1160 Δ*Ku80 pyrG*$^+$. These genes were selected on the basis of their induction by azole in either azole-susceptible or azole-resistant isolates or as controls suspected to play no role in resistance (*AfuMDR2*, *AfuMDR3* and *AfuMDR4*).

Genes were knocked out and verified using PCR. In order to exclude random mutational effects from transformation, two individual knockout transformants were selected for each gene knockout. After analysis and two rounds of single-spore plating, knockouts were tested for MICs of itraconazole in triplicate (Table 2). Most knockouts had MICs that were identical to those for the wild-type. However, the *cdr1B* knockouts reproducibly showed an MIC of itraconazole, voriconazole and posaconazole 4-fold lower than that for the parental strain. The *AfuMDR1* knockout reproducibly showed an MIC of itraconazole, voriconazole and posaconazole 2-fold lower. The double knockout A1160 *pyrG*$^+$Δ*cdr1B*Δ*MFS56* strain showed the same MIC change as the single A1160 *pyrG*$^+$Δ*cdr1B* mutant.

In order to directly test transporter function in the azole-resistant clinical isolates, the *MFS56* and *cdr1B* genes were knocked out in F20063, which showed a 6.3-fold induction of *cdr1B* and >300-fold induction of *MFS56*. Attempts to transform the other high-level-expression clinical isolates were unsuccessful. For F20063, the MICs of itraconazole, voriconazole and posaconazole were >8, 1 and 0.5 mg/L, respectively (Tables 1 and 2). MICs of voriconazole and itraconazole were determined for parental and three independent knockout strains for the *cdr1B* knockout (F20063Δ*cdr1B*) and four independent knockout strains for the *MFS56* knockout (F20063Δ*MFS56*) (Table 2). For all knockouts the MICs of voriconazole and posaconazole were unchanged. F20063Δ*cdr1B*-5, F20063Δ*cdr1B*-6 and F20063Δ*cdr1B*-7 showed MICs of itraconazole of 2 mg/L (4-fold lower than F20063), whereas F20063Δ*MFS56*-2, F20063Δ*MFS56*-4, F20063Δ*MFS56*-6 and F20063Δ*MFS56*-7 had unchanged MICs of itraconazole of >8 mg/L.

Table 2. Azole MICs (mg/L) for knockout strains and parental isolates

Strain	Itraconazole	Voriconazole	Posaconazole
CEA10	0.5	1	0.25
A1160 *pyrG*$^+$	0.5	1	0.25
Af210	0.5	1	0.25
Af293	0.5	1	0.25
F20063	>8	1	0.5
A1160 *pyrG*$^+$ Δ*cdr1B*-1	0.125	0.25	0.06
A1160 *pyrG*$^+$ Δ*cdr1B*-2	0.125	0.25	0.06
A1160 *pyrG*$^+$ Δ*MFS56*-1	0.5	1	0.25
A1160 *pyrG*$^+$ Δ*MFS56*-2	0.5	1	0.25
A1160 *pyrG*$^+$ Δ*MDR1*-1	0.25	1	0.125
A1160 *pyrG*$^+$ Δ*MDR1*-2	0.25	1	0.125
A1160 *pyrG*$^+$ Δ*MDR2*-1	0.5	1	0.25
A1160 *pyrG*$^+$ Δ*MDR2*-2	0.5	1	0.25
A1160 *pyrG*$^+$ Δ*MDR3*-1	0.5	1	0.25
A1160 *pyrG*$^+$ Δ*MDR3*-2	0.5	1	0.25
A1160 *pyrG*$^+$ Δ*MDR4*-1	0.5	1	0.25
A1160 *pyrG*$^+$ Δ*MDR4*-2	0.5	1	0.25
A1160 *pyrG*$^+$ Δ*atrF*-1	0.5	1	0.25
A1160 *pyrG*$^+$ Δ*atrF*-2	0.5	1	0.25
F20063Δ*cdr1B*-5	2	1	0.5
F20063Δ*cdr1B*-6	2	1	0.5
F20063Δ*cdr1B*-7	2	1	0.5
F20063Δ*MFS56*-2	>8	1	0.5
F20063Δ*MFS56*-4	>8	1	0.5
F20063Δ*MFS56*-6	>8	1	0.5
F20063Δ*MFS56*-7	>8	1	0.5
A1160 *pyrG*$^+$ Δ*cdr1B*Δ*MFS56*	0.125	0.25	0.06

Discussion

Here we attempted to identify efflux transporters associated with clinical azole resistance in *A. fumigatus*. A small bioinformatic meta-analysis was performed to identify potential transporter genes of interest and to clarify existing nomenclature. Nineteen *A. fumigatus* transporter genes were defined through bioinformatic analysis and a further two genes were added from a previous insertional mutagenesis screen.[45] One of these genes, *ABC11* (*cdr1B*), was identical to *abcC*, giving a total of 20 genes of interest. Fourteen of these genes, including the *C. albicans CDR1* orthologue *cdr1A*, were >2-fold up-regulated in the azole-susceptible clinical isolate Af293 (Figure 2), but were not induced in a different manner in clinical azole-resistant strains. Since Af293 remains azole susceptible, this strongly suggests that these genes are not involved in azole resistance.

It was surprising that none of the transporters that are up-regulated in response to azole in azole-susceptible isolates differ significantly (>2-fold) in their induction by azole in the non-*cyp51A*-resistant isolates. This result appears counter-intuitive; however, the observed up-regulation does not confer resistance on the drug-susceptible strains and may possibly reflect secondary effects of azole on metabolism. Large increases in basal level of *cdr1B* and induction of *MFS56* were associated with resistance. *MFS56* is a major facilitator family transporter not previously reported to be involved in azole efflux in any organism. *A. fumigatus cdr1B* (*abcC*) is an orthologue of *CDR1*, although it has not previously been defined as such as *A. fumigatus* carries a paralogue of this gene (*cdr1A*, AFUA_1G17440) that is slightly more closely related to *CDR1*. This gene is not over-induced in resistant isolates. Eight of 11 azole-resistant isolates in this study displayed basal levels of *cdr1B* >5-fold greater than that observed in susceptible isolates, together with high induction of *MFS56* expression (F20063, F18085 and F17999). F18304 also showed high-level induction of *cyp51A*. This strain is resistant to itraconazole, but susceptible to other azoles. This suggests that models of multi-azole resistance based solely on *cyp51A* mutation or expression may be incomplete. The findings do not explain the variable levels of resistance to other azole drugs and therefore other factors are likely to be involved in these cases. One isolate, F18329, that displayed intermediate voriconazole resistance, but normal itraconazole and posaconazole susceptibility, did not display any major change in expression of *MFS56* or *cdr1B* when exposed to azole. However, *AfuMDR1* basal levels were 3.3 times that observed in azole-susceptible strains and this may account for the slightly elevated MIC of voriconazole. F18085 showed little change in either basal or induced levels of *cdr1A*, but showed high induction of *MDR1*, although knockout of this gene appeared to have no effect on azole susceptibility in susceptible isolates (Table 2). Our data show that *cdr1B* inactivation primarily affects the itraconazole MIC. As the other transporters studied in this paper appear not to be involved, it is likely that either non-transporter-mediated mechanisms of resistance or other transporters mediate voriconazole and posaconazole resistance in these strains. One isolate, F18454, showed increased expression of *cdr1B*, but an MIC of itraconazole of 0.5 mg/L. This suggests that other mechanisms may be required for full itraconazole resistance. Additionally, it can be seen from Figure 4 that expression patterns of *cdr1B* do not completely correlate with patterns of azole resistance. This highlights the probable existence of non-*cyp51A* multifactorial resistance mechanisms in these isolates.

Disruption of candidate transporter genes in an azole-susceptible strain resulted in few changes in azole susceptibility. Only the *cdr1B* knockout led to a 4-fold reduction in the MICs of all azoles tested in a clinical azole-susceptible isolate (Table 2). *cdr1B* is slightly up-regulated in response to azole in our experiments and was also observed to be up-regulated (as *abcC*) by da Silva Ferreira *et al.*[36] When *cdr1B* and the highly induced *MFS56* gene were disrupted in a non-*cyp51A*-mediated azole-resistant isolate, F20063, which displayed high over-induction of *MFS56* and 6.2-fold over-induction of *cdr1B*, only the *cdr1B* knockout affected azole susceptibility. Notably, this knockout only affected susceptibility to itraconazole; the MIC decreased 4-fold from >8 to 2 mg/L. A number of possible explanations for this observation can be made. Firstly, it may be that *cdr1B* is largely responsible for itraconazole resistance in this strain, although knockouts do not return to wild-type levels of drug susceptibility. Additionally, the MIC of voriconazole is unchanged, whereas in wild-type *cdr1B* knockouts the MIC of voriconazole shows 4-fold greater susceptibility. Surprisingly, the *MFS56* knockout in F20063 does not increase susceptibility to any tested azole in this strain, given the very high level of induction by azoles. In this case the residual MIC of itraconazole of 2 mg/L for the *cdr1B* knockouts strongly suggests the presence of other mechanisms that may also be responsible for resistance

to voriconazole and posaconazole. One limitation of these experiments is that induction profiles were only determined using itraconazole. Hence it is possible that different profiles could occur when isolates were treated with posaconazole or voriconazole and that such profiles could explain the observed differences in azole susceptibility.

One possible scenario that could underpin the development of resistance in *A. fumigatus* is intermediate resistance mediated by transient up-regulation of a transporter such as *cdr1B* or *MFS56*, allowing fungal survival until stable resistance can arise, e.g. through mutation in the *cyp51A* gene. This has not occurred in the non-*cyp51A* resistant strains in this study. Resistance in these isolates is at the highest MIC measurable and is stable through many generations. The patients from whom these strains were isolated did not receive any unusual type, dose or duration of azole treatment and there appears to be no relationship between treatment duration and multi-azole resistance.

The qualitative similarity between the non-*cyp51A* strains and *Candida* azole resistance is clear: similar classes of transporter gene are up-regulated in both cases. *cdr1B* is an orthologue of *CDR1*. Orthologues of other *C. albicans* resistance-related transporters, such as *CDR2*, *CDR4* and *MDR1*, do not play a discernible role in azole resistance in these isolates. Most azole resistance in *C. albicans* is attributed to transporter-mediated mechanisms, whereas in *Aspergillus* target site mutations have been more frequently documented. The reasons behind the shift away from target site mutation in azole-resistant isolates from Manchester is unknown, but the change is alarming in that it has the potential to confound recent molecular diagnostic tests using detection of mutations in the *cyp51A* gene to detect azole resistance.

The lack of correlation of transcriptional response between the azole-resistant strains and azole-susceptible 'wild-type' strains in this study implies that the use of azole-susceptible strains to gain insight into resistance mechanisms may not be a productive route to understanding of azole resistance in *A. fumigatus*, although *in vivo* investigations or experiments using different inductive media might increase the utility of such systems. As overexpression appears to be a route to resistance in *A. fumigatus*, a transcriptome-based approach might be a useful means of clarifying resistance mechanisms, although our results demonstrate that overexpression of a gene does not necessarily correlate with functional significance.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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