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Resistance to itraconazole in *Aspergillus nidulans* and *Aspergillus fumigatus* is conferred by extra copies of the *A. nidulans* P-450 14α-demethylase gene, *pdmA*

Nir Osherov[a], Dimitrios P. Kontoyiannis[b], Angela Romans[a] and Gregory S. May[a]*

[a]Division of Pathology and Laboratory Medicine, and [b]Department of Internal Medicine Specialties, Section of Infectious Diseases, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

Triazoles selectively inhibit the cytochrome P-450-dependent C-14 lanosterol α-demethylase (P-450 14 α DM), a key enzyme in ergosterol biosynthesis in fungi. To investigate mechanisms of triazole resistance in a mould, we used *Aspergillus nidulans*, a genetically amenable model fungus closely related to more pathogenic members of the genus. We selected for genes that would give resistance to itraconazole following transformation with a high copy genomic library of *A. nidulans*. In all the resistant colonies that we isolated, resistance was conferred by extra copies of the *A. nidulans* P-450 14 α DM gene, *pdmA*. We determined that in *A. nidulans*, extra copies of *pdmA* increase the MIC for itraconazole 36 times over wild-type controls. Similarly, transformation of an *Aspergillus fumigatus* strain with pITZR1 resulted in increased resistance to itraconazole. Our results indicate that triazole resistance in clinical isolates of moulds may result from amplification or overexpression of the P-450 14 α DM and demonstrate the utility of *A. nidulans* as a promising model fungus for the analysis of drug resistance and susceptibility in the pathogenic fungus *A. fumigatus*.

Introduction

The dramatic increase in the incidence of fungal infections over the past decade and the limited arsenal of antifungal drugs available to treat them have led to the development of drug resistance. Triazoles are widely used antifungals that selectively inhibit cytochrome P-450-dependent C-14 lanosterol α-demethylase (P-450 14 α DM). P-450 14 α DM catalyses a key reaction in the biosynthesis of ergosterol in *Candida albicans*, *Saccharomyces cerevisiae* and other fungi.[1] Mutation or overexpression of P-450 14 α DM has been shown to result in fluconazole resistance in both *C. albicans* and *S. cerevisiae*.[2–5]

The susceptibility of fungi to different triazoles varies considerably from one species to another. Fluconazole, for instance, is less effective against *Aspergillus* spp. than it is against *C. albicans* or *S. cerevisiae*.[2,4,6] The molecular basis for this variation in susceptibility to the different triazoles is unknown. Itraconazole resistance in *Aspergillus fumigatus* has been described for both spontaneous laboratory mutants and clinical isolates and attributed to increased efflux of the drug and/or alterations of the target enzyme, P-450 14 α DM.[7,8] However, direct evidence for the effect of P-450 14 α DM gene dosage on itraconazole resistance is lacking.

Aspergillus nidulans is a well-studied, genetically amenable filamentous fungus that is closely related to more pathogenic species of *Aspergillus*, such as *A. fumigatus*. In this report, we exploited the facile molecular genetics of *A. nidulans* to address questions related to triazole resistance in *A. fumigatus*. We also describe the effect of itraconazole on the morphological development and viability of germinating *A. nidulans* conidia. We transform *A. nidulans* to itraconazole resistance with a plasmid library of *A. nidulans* genomic DNA. Our results show that increased expression of P-450 14 α DM in the model fungus *A. nidulans* produces resistance to itraconazole. Finally, we show that heterologous expression of the *A. nidulans* P-450 14 α DM gene, *pdmA*, in *A. fumigatus* leads to increased resistance to itraconazole. This is the first time that an unbiased genetic selection for drug resistance has been carried out in *A. nidulans* and the resulting findings then shown to be

*Corresponding author. Tel: +1-713-745-1945; Fax: +1-713-792-4840; E-mail: gsmay@mdanderson.org

relevant to the acquisition of resistance in *A. fumigatus*. These studies demonstrate the utility of *A. nidulans* as a model organism for the study of antifungal drug action and as a model fungus closely related to more pathogenic members of the genus *Aspergillus*.

Materials and methods

Strains, media and growth conditions

A. nidulans conidia (strain GR5, *pyrG89; wA3; pyroA4*)[9] and *A. fumigatus* conidia (strain AF293.1, *pyrG1*) were grown in minimal medium (70 mM $NaNO_3$, 7 mM KCl, 4 mM $MgSO_4$, 12 mM KPO_4 pH 6.8, trace elements, 1% glucose, 5 mM uridine and 10 mM uracil) at 37°C. Where indicated, itraconazole (Janssen Pharmaceutica, Titusville, NJ, USA) was added to the medium. To prepare itraconazole-containing agar plates, we first cooled the agar to 48°C and then added the appropriate concentration of the drug.

The *A. fumigatus* uracil auxotroph strain AF293.1 was derived by selection of *A. fumigatus* (strain AF293) on MAG solid medium (2% malt extract, 0.2% peptone, 1% dextrose, trace elements, 1 mg/L pyroxidine, 8.8 mg/L riboflavin and 2% agar), supplemented with 5 mM uracil, 10 mM uridine and 1 mg/mL 5-fluoroorotic acid (5-FOA) (MAGuu-FOA), following mutagenesis of conidia with 4-nitroquinoline 1-oxide as described previously for *A. nidulans*.[10]

Growth studies and microscopy

Viability assays were performed by incubating 1×10^6 conidia/mL in liquid minimal medium in the presence of 0.2 mg/L (*A. nidulans* conidia, MIC 0.055 mg/L) or 0.4 mg/L itraconazole (*A. fumigatus* conidia; MIC 0.625 mg/L) at 37°C for the indicated time periods. Conidia were subsequently collected using a cell culture scraper, washed in distilled water, suspended and spread on MAG solid medium plates to assess viability. Colonies were counted after 48 h at 37°C to determine viability. The experiment described (Figure 1) was repeated independently three times with both strains. Similar results were obtained each time.

Microscopy was performed after either 6 or 24 h of conidial germination in the presence or absence of itraconazole at the concentrations indicated using a Nikon Microphot-SA microscope and a Hamamatsu Model C2400 camera. Images were collected and transferred to Adobe Photoshop v.4.0. Several fields containing 500–1000 germlings were examined, and representative portions were recorded.

Genetic, molecular genetic and other methods

Methods for manipulating *A. nidulans* and molecular methods have been described elsewhere.[11,12] A detailed description of the AMA1-based genomic library and its transformation into *A. nidulans* has also been published previously.[9] *A. fumigatus* was transformed using the same procedure. The complementing gene that conferred itraconazole resistance was identified by random mutagenesis of the rescuing plasmid with a transposon, using an *in vitro* transposition kit (GPS-1 system, New England Biolabs, Beverly, MA, USA) as described previously.[9] The gene *pdmA* (P-450 demethylase A) was named in accordance with *A. nidulans* convention. It was fully sequenced using primers (provided in the GPS-1 system kit) unique for the transposon ends, and its possible function identified through a BLAST database search.

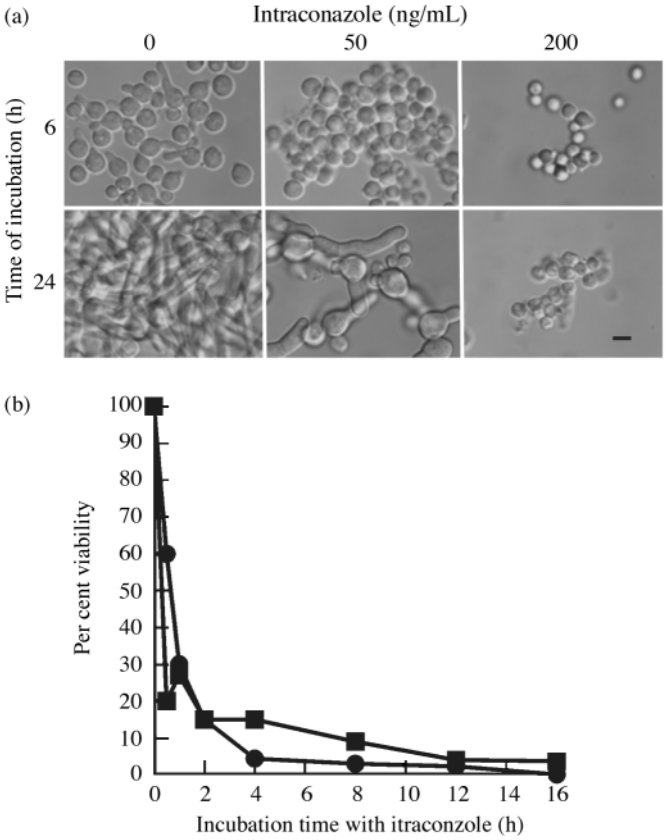

Figure 1. (a) Effect of itraconazole on conidial germination and hyphal growth in the *A. nidulans* strain GR5. Freshly harvested conidia were incubated in minimal liquid medium in the absence or presence of itraconazole for either 6 or 24 h and viewed by light microscopy. The scale bar in the lower right hand panel is 2 μm. (b) Effect of itraconazole on conidial viability as a function of incubation time. Freshly harvested *A. nidulans* GR5 (●) and *A. fumigatus* AF293 (■) conidia were incubated in liquid medium in the presence of 0.2 and 0.4 mg/L itraconazole, respectively.

Determination of MICs by Etest

The Etest MIC determinations were performed with itraconazole (range 0.002–32 mg/L), fluconazole (range

0.016–256 mg/L) and amphotericin (range 0.002–32 mg/L) strips used according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). Freshly harvested conidia were suspended in water at a concentration of 1×10^6 conidia/mL. Agar plates (150 × 15 mm; Falcon, Franklin Lakes, NJ, USA) were prepared with RPMI 1640 (pH 7.0). Plates were inoculated by dipping a sterile swab into the inoculum suspension and evenly swabbing the entire agar surface. Plates were dried for 15 min, and Etest strips were aseptically placed MIC scale side up on the agar surface. Plates were incubated at 37°C and MICs were recorded at 24, 48 and 72 h.

Results

Phenotypic effects of itraconazole on the growth and viability of A. nidulans

The effects of itraconazole on the growth and viability of *A. nidulans* have not been described previously. Such information could be useful both in determining the inhibitory concentration of itraconazole needed to develop the screen and in identifying morphogenetic differences between the response of *A. nidulans* and *A. fumigatus* to the drug.[13] *A. nidulans* conidia were germinated in liquid minimal medium at 37°C in the absence or presence of itraconazole (50 or 200 ng/mL). The effects of itraconazole on spore germination and hyphal growth were then assessed by light microscopy (Figure 1a). After 6 h of growth in the absence of drug, *A. nidulans* conidia swelled and 60% of the conidia formed germ tubes ($n = 50$). In contrast, at 50 ng/mL of itraconazole, conidia swelled but few formed germ tubes (5%, $n = 50$). At 200 ng/mL all the conidia failed to swell or germinate. After 24 h of growth in the absence of itraconazole a dense hyphal mat or mycelium formed. When grown in the presence of 50 ng/mL of itraconazole for 24 h, hyphal growth was significantly inhibited and the hyphae that did form were grossly abnormal and swollen (Figure 1a). At 200 ng/mL, conidia did not swell or germinate and appeared dark, as if lysis had occurred. These results agree well with those shown previously for *A. fumigatus*.[13]

We quantified the effect of high levels of itraconazole on conidial viability as a function of incubation time for both *A. nidulans* and *A. fumigatus* (Figure 1b). The conidia of both *Aspergillus* species rapidly lost viability over time in the presence of the drug. After 4 h in the presence of 400 ng/mL itraconazole, only 15% of the conidia of AF293 remained viable, and in the presence of 200 ng/mL of the drug, only about 5% of the conidia of GR5 remained viable. Our results, showing that itraconazole possesses fungicidal activity in *Aspergillus* spp., are in agreement with those described by Manavathu *et al.*[14] To determine whether the lethal effect of the drug was dependent on activation of conidial germination, we incubated *A. nidulans* GR5 conidia in phosphate buffer, which does not support germination in the presence or absence of itraconazole. Surprisingly, conidia lost viability at the same rate as those incubated in minimal media (data not shown), indicating that itraconazole kills both metabolically active and inactive conidia with equal efficiency.

Isolation of genes conferring itraconazole resistance

A. nidulans strain GR5 was transformed with an *A. nidulans* genomic DNA library made in an AMA1-based pRG3-containing autonomously replicating high copy vector.[9,15,16] This library was chosen because the AMA1 sequence allows autonomous non-integrating replication of the plasmid at high copy numbers and results in efficient rates of transformation (*c.* 20 000 *A. nidulans* transformants/μg plasmid DNA). Autonomous replication also facilitates recovery of the plasmid from transformants and subsequent cloning steps.[9] AMA1-based vectors are present at approximately 10 copies per nucleus, and the expression level of certain marker genes has been shown to be approximately proportional to copy number.[15] Moreover, high levels of expression of the plasmid-borne gene do not affect plasmid mitotic stability or copy number.[15] Self-replicating AMA1-based plasmids persist in the asexual life cycle of *A. nidulans* for several generations without undergoing chromosomal integration.[16]

Transformants were plated on minimal medium plates lacking uracil, containing 200 ng/mL itraconazole, four times the lethal dose (see Table). A total of approximately

Table. MICs (mg/L) of antifungal drugs for *Aspergillus* strains

Aspergillus strain	Itraconazole[a]	Fluconazole	Amphotericin B
GR5 pRG3-AMA1[b]	0.055	64	>32
GR5 pITZR1[b]	2.00	>256	>32
AF293.1 pRG3-AMA1[c]	0.625	>256	4
AF293.1 pITZR1[c]	2.50	>256	3

[a]Values presented are representative of one of three independent experiments.
[b]*A. nidulans* strain.
[c]*A. fumigatus* strain.

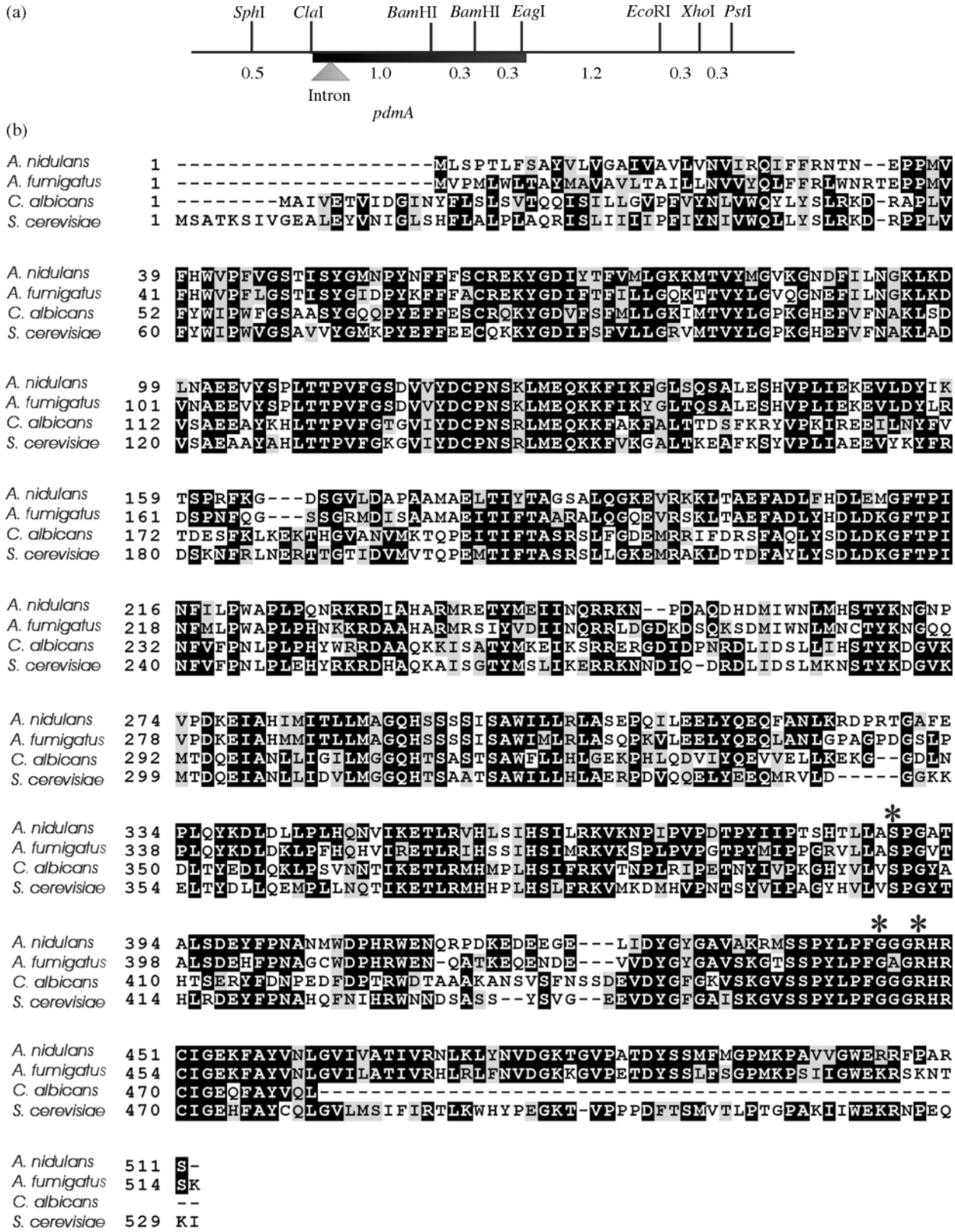

Figure 2. (a) Restriction enzyme map of the *pdmA* locus. The black solid region defines the *pdmA* coding region. (b) The amino acid sequence alignment of *A. nidulans pdmA* (GenBank accession number AF266481) to *A. fumigatus* CYP51 (AF222068), *C. albicans* CYP51A1 (AF153846) and *S. cerevisiae* Erg11p (M21484) was performed utilizing CLUSTAL_W 1.8. Sequence identities were 75, 48 and 51%, respectively. Mutations in the amino acid residues with an asterisk over them were shown to confer azole resistance in *C. albicans* isolates.[17]

150 000 *pyr*$^+$ transformants, representing a 25-fold coverage of the *A. nidulans* genome, were screened for itraconazole resistance. Plates typically contained 10^4 minute non-resistant *pyr*$^+$-transformed colonies that died after 48 h of growth, and a few *pyr*$^+$ itraconazole-resistant colonies that grew and sporulated normally. A total of 14 itraconazole resistant colonies were evident after 48 h of growth at 37°C. These colonies were retested for itraconazole resistance. The self-replicating AMA1-based plasmids found in these colonies were cloned into *Escherichia coli*. Restriction maps of the plasmid inserts recovered from the 14 independent, itraconazole-resistant transformant colonies showed considerable overlap and their inserts cross-hybridized with one another as determined by Southern blot hybridization.

When *A. nidulans* strain GR5 was transformed with one of these plasmids, pITZR1, containing a 5 kb genomic DNA insert (Figure 2a), all the transformant colonies were resistant to up to 2000 ng/mL itraconazole. Using insertional inactivation with a transposon, we mapped the functional gene and derived sequence from the ends of the transposon as described previously.[9] Sequencing identified the gene as the *A. nidulans* homologue of P-450 14 α DM, *pdmA* (GenBank accession number AF266481). The deduced protein sequence shows a high degree of identity to the P-450 14 α DM proteins from a number of other fungi including *A. fumigatus* (75%), *C. albicans* (48%) and *S. cerevisiae* (51%) (Figure 2b).

Bi-directional gene transfer between the closely related *A. nidulans* and *A. fumigatus* has been described previously.[18] In order to heterologously express *pdmA* in *A. fumigatus*, we transformed it as described previously for *A. nidulans*.[9] Transformation of pITZR1 into a *pyrG*$^-$ *A. fumigatus* strain (AF293.1) resulted in itraconazole resistance in transformant colonies.

To demonstrate that itraconazole resistance segregates with the pITZR1 plasmid, itraconazole-resistant *A. nidulans* (GR5 pITZR1) and *A. fumigatus* (AF293.1 pITZR1) transformant strains were plated on MAGuu-FOA agar plates to evict the pITZR1 plasmid. Loss of the plasmid resulted in the concomitant loss of uracil prototrophy and itraconazole resistance.

To demonstrate that GR5 pITZR1 and AF293.1 pITZR1 strains contain multiple copies of the pITZR1 plasmid we performed a Southern blot analysis of uncut genomic DNA prepared from these and control strains hybridized with a *Bam*HI fragment of *pdmA* (Figure 3). The results indicate that both strains contain multiple copies of the pITZR1 plasmid as compared with the single copy genomic *pdmA* gene in the GR5 pRG3-AMA1 control strain.

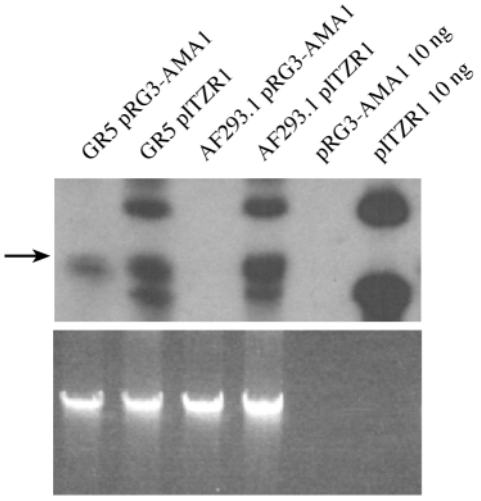

Figure 3. Southern blot analysis of the strains described in this study. Equal amounts of genomic DNA were loaded undigested and run on an agarose gel (lower panel). The DNA was transferred to a nitrocellulose membrane and probed with a *Bam*HI fragment of *pdmA* (top panel). Ten nanograms of pure empty vector (pRG3-AMA1) and pITZR1 plasmid were also run as controls. The arrow at the left of the figure indicates the position of migration of the genomic DNA.

Determination of MICs by Etest

We determined the level of itraconazole resistance in *A. nidulans* and *A. fumigatus* strains transformed with pITZR1 or the parent vector pRG3-AMA1 lacking an insert using Etest (Table). Recent studies have shown that Etest is a useful, rapid, highly reproducible alternative to the conventional broth-based susceptibility methods for assaying the *in vitro* susceptibility of both yeasts and *Aspergillus* species.[19,20] Strain GR5 transformed with pITZR1 was 36 times more resistant to itraconazole than when transformed with the empty parent plasmid. Strain AF293.1 transformed with pITZR1 was four times more resistant than the control strain. We also tested the susceptibility of these strains to fluconazole and amphotericin B by Etest. *A. fumigatus* transformed with the vector alone was resistant to fluconazole, and its susceptibility to amphotericin B was unchanged by the presence of pITZR1. In contrast, *A. nidulans* was naturally and surprisingly resistant to amphotericin B and developed greater resistance to fluconazole when transformed with pITZR1 (Table). Natural resistance to amphotericin B was seen in several unrelated isolates of *A. nidulans* (data not shown), indicating that it is not a strain-specific phenomenon.

Discussion

Advances in our understanding of the molecular events that take place during invasive growth in *C. albicans* and in the development of drug resistance in this organism have been expedited by the use of the related and genetically tractable model organism *S. cerevisiae*.[2] In contrast, research into the pathogenesis of filamentous fungi has

suffered from the lack of a suitable amenable model system. In this report, we provide 'proof of concept' for the use of the filamentous fungus *A. nidulans* as a model organism for the study of drug resistance in the closely related and considerably more pathogenic *A. fumigatus*. Using selection for itraconazole resistance in *A. nidulans*, we demonstrate that amplification of *pdmA* can produce resistance to triazole antifungals but not to the unrelated drug amphotericin B. We also demonstrate that transformation of *A. nidulans pdmA* in a strain of *A. fumigatus* is able to confer itraconazole resistance in this closely related species. Our results suggest that triazole resistance in clinical isolates of *Aspergillus* species may result from amplification or over-expression of the P-450 14 α DM.

Increased expression of *ERG16* (formerly *ERG11*), the gene encoding P-450 14 α DM in *C. albicans*, has been shown to correlate with increased azole resistance in clinical isolates of this organism.[4] However, because multiple genetic changes have taken place in these strains, including an increase in the expression of drug efflux pumps, the relative contribution of *ERG16* over-expression to overall resistance is unknown. In the budding yeast *S. cerevisiae*, over-expression of Erg11p has been shown to confer fluconazole resistance,[2] but this study is the first demonstration of a direct screen for dominant resistance-conferring genes in a relevant filamentous fungus closely related to pathogenic species.

In addition to the *pdmA* gene, we expected to isolate other genes that confer resistance to itraconazole, such as drug efflux pumps. There are several likely explanations for our failure to isolate such genes. One is that the AMA1-based transformation system we used does not express resistance-conferring genes other than *pdmA* at levels high enough to confer resistance. Another is that efflux pump genes alone cannot confer resistance to itraconazole in *A. nidulans*. Alternatively, these genes may only function effectively when over-expressed in combination with each other and/or with P-450 14 α DM. These hypotheses can be addressed directly in *A. nidulans*.

This work highlights the potential for studying the development of resistance to novel antifungal agents in *A. nidulans* and applying the results to *A. fumigatus*. Sophisticated, well-established genetic screens for resistant or hypersensitive mutants in *A. nidulans* can provide us with important molecular information that can be applied to related pathogenic *Aspergillus* spp.

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