Probing the role of point mutations in the *cyp*51A gene *from Aspergillus fumigatus* in the model yeast *Saccharomyces cerevisiae*

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Azole-resistant strains of *Aspergillus fumigatus* have been detected and the underlying molecular mechanisms of resistance characterized. Point mutations in the *cyp51*A gene have been proved to be related to azole resistance in *A. fumigatus* clinical strains and with different resistance profiles depending on the amino acid change (G54E, G54V, G54R, G54W, M220V, M220K, M220T, M220I). The aim of this work was to express *A. fumigatus cyp51*A genes in the yeast *Saccharomyces cerevisiae* in order to better assess the contribution of each independent amino acid substitution to resistance. A tetracycline regulatable system allowing repression of the endogenous essential *ERG11* gene was used. The expression of *Aspergillus cyp*51A alleles could efficiently restore the absence of *ERG11* in *S. cerevisiae.* In general, *S. cerevisiae* clones expressing. *A. fumigatus cyp*51A alleles from azole-resistant isolates showed higher MICs to all azoles tested than those expressing alleles from susceptible isolates. The azole susceptibility profiles obtained in *S. cerevisiae* upon expression of specific *cyp51*A alleles recapitulated susceptibility profiles observed from their *A. fumigatus* origins. In conclusion this work supports the concept that characteristics of specific *A. fumigatus cyp*51A alleles could be investigated in the heterologous host *S. cerevisiae.*

Keywords *Aspergillus fumigatus*, *Saccharomyces cerevisiae*, Cyp51A, azole resistance

Introduction

Invasive fungal diseases are an increasingly common complication in critically ill patients and often with fatal outcomes [1]. *Candida* species are the most common cause of fungal infections followed by *Aspergillus* spp. [2,3]. Among the latter, *A. fumigatus* is the main causative agent of invasive disease, normally affecting immunocompromised patients with persistent neutropenia suffering severe hematological malignancies or transplant recipients [4].

Despite the improvement and the development of novel antifungals, the mortality due to invasive aspergillosis remains very high [1,5]. The status of the host immune system is a critical parameter that will determine the outcome of fungal infections. However, other factors such as antifungal resistance might have a significant impact on the outcome of the infection. Triazole drug resistance in *A. fumigatus* is an emerging problem that has been documented in environmental strains and during azole therapy [6,7].

Most *A. fumigatus* strains are susceptible to the available antifungals used for the treatment of patients with invasive infections [8,9]. However, *A. fumigatus* strains with secondary resistance to azole drugs have been described and their resistance mechanisms have been thoroughly studied.

The cytochrome P450 14-α sterol demethylase, encoded by *cyp*51A gene (ERG11 in yeast), is the azole drugs target and responsible, at least in part, for the azole drugs' affinity

in *A. fumigatus* [10]. Azole-resistant *A. fumigatus* isolates of clinical origin have been found to have different mutations that are responsible for the increase in their azole MICs [11–14]. Different resistance profiles can be mainly attributed to single amino acid substitutions in Cyp51A. One mutation is at position glycine 54 including amino acid changes G54E, G54V, G54R, or G54W. Clinical strains with these mutations showed resistance to itraconazole and high MICs to posaconazole but not to voriconazole or ravuconazole [12]. The second important mutation is at methionine 220 including amino acid changes M220V, M220K, M220T and M220I [13], which yield resistance to itraconazole and reduced susceptibility to posaconazole, voriconazole and ravuconazole. However, the functional characterization of the individual contribution of each *cyp*51A mutation to azole resistance has never been tested for *A. fumigatus*.

In the present study, the wild type *cyp51*A and the different altered *cyp51*A alleles from susceptible and resistant *A. fumigatus* strains were expressed in the yeast *Saccharomyces cerevisiae* in order to address the role of each on azole resistance.

The heterologous expression of *A. fumigatus cyp*51A was performed by conditional expression of the yeast 14-α sterol demethylase gene (*ERG11*) and induced expression of several *cyp*51A cDNAs from *A. fumigatus*. A tetracycline regulatable system [15,16] which allows repression of gene expression was used. Since *ERG11* is essential in *S. cerevisiae*, this key system can test the Cyp51A functional complementation by growth restoration. The different yeast isolates containing each single *cyp*51A gene were used to asses the differences in azole drugs interaction between different mutated alleles.

Material and methods

Strains and growth conditions

A total of nine *A. fumigatus* strains were used in this work. Their identification names together with their *cyp51*A genetic background are indicated in Table 1. Their minimal inhibitory concentrations (MICs) to itraconazole, voriconazole and posaconazole obtained from previous studies [12,13] are summarized in Table 1. *S. cerevisiae* strains used in this work are listed in Table 2.

A. fumigatus strains were grown at 37°C in potato dextrose agar (Oxoid, Madrid, Spain) or malt extract agar (MEA). Conidia stocks were preserved in sterile distilled water at 4°C. *S. cerevisiae* strains were grown either in complete medium YEPD, containing 1% Bacto peptone (Difco), 0.5% yeast extract (Difco) and 2% glucose (Fluka) or in minimal media containing yeast nitrogen base (YNB) with 2% glucose (Fluka, Buchs, Switzerland) and without

Table 1 *Aspergillus fumigatus* strains used in this study with their respective Cyp51A amino acid substitutions and their azole susceptibility profiles.

Strains	Amino acid change	MICs mg/L			References
A. fumigatus	Cyp51A	ITZ	VRZ	POS	
CM237	–	0.25	0.5	0.06	–
CM2158	M220V	> 8	2	0.5	[13]
CM2159	M220K	> 8	1.2	2	[13]
CM2161	G54E	> 8	0.35	0.5	[12]
CM2162	G54V	> 8	0.25	0.35	[12]
CM2164	M220T	> 8	0.76	0.33	[13]
CM2266	G54W	> 8	0.71	16	[12]
ITZ8	G54R	> 8	0.5	0.5	[12]
PW6	M220I	> 8	0.25-1	0.5-1	[13]

amino acids (Difco), but complemented with all bases and amino acids except for selection without uracil (YNB-ura) or histidine (YNB-his). Selective media to induce expression media consisted in YNB-ura, 2% galactose (Fluka) and doxycycline (2 mg/ml) (Sigma). When isolates were grown on solid media, 2% agar (Difco) was added to each medium.

DNA preparations for transformation

All DNA oligonucleotides were purchased from Eurogentec S.A. (Belgium). The expression vector pYES2/CT was purchased from Invitrogen (Lausanne, Switzerland). Linear plasmids used for transformation were prepared in accord with the following procedure. Five μg of plasmid DNA were digested with HindIII and XhoI. After digestion the linear plasmid was precipitated with two volumes of ethanol 100% and 0.1 volume of 3M sodium acetate. After centrifugation, digested plasmids were washed once with 70% ethanol and dissolved in 10 ml of TE buffer. PCR products for transformation were prepared in the same way.

Table 2 *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Parent	References
DSY3886	*MAT**a** ura3-52 leu2Δ1 his3Δ200 GAL2* CMVp(*tetR'-SSN6*)::*LEU2trp1*::Tta	–	[16]
DSY3899	*MAT**a** ura3-52 leu2Δ1 his3Δ200 GAL2* CMVp(*tetR'-SSN6*)::*LEU2trp1*::Tta *ERG11*::kanMX-*tet*O$_7$	DSY3886	This work
DSY3961	*MAT**a** ura3-52 leu2Δ1 his3Δ200 GAL2* CMVp(*tetR'-SSN6*)::*LEU2trp1*::Tta *ERG11*::kanMX-*tet*O$_7$ *pdr5*Δ::HIS3kanMX	DSY3899	This work

Design of ERG11 conditional expression

The *Saccharomyces cerevisiae* strains used in this work were: (i) strain DSY3886 derived from Y40122 (*MAT*a *ura3-52 leu2Δ1 his3Δ200 GAL2* CMVp(*tetR'-SSN6*)::*LEU2 trp1*::Tta); and (ii) strain DSY3899, derived from DSY3886 but with *ERG11* under the control of doxycycline (*ERG11*::kanMX-*tet*O$_7$). To place the *ERG11* promoter under control of the *Tet* system in *S. cerevisiae* DSY3886, a linear DNA fragment was obtained from a PCR with pMC324 as a template and primers P1 (5′-GCAGCGC ACATACA ATGTGCGTGCAAGATTTGCCGGGTT GGACAACGTACGCTGCAGGTCGACGG-3′) and P2 (5′-TACGTATTCCAATGCCTCTCCAACGATTGAC TTGGTAGCAGACATAGGCCACTAGTGGATCTG-3′) which target integration at the *ERG11* promoter. The resulting isolate, DSY3899, was next used for deletion of the major efflux transporters *PDR5*. This was accomplished by transformation with a PCR-generated fragment obtained with the template pFA6a-His3MX6 [17] and primers PDR5F (5′-AAGTTTTCGTATCCGCTCGTTCGAAAGA CTTTAGACAAAACGGATCCCCGGGTTAATTAA-3′) and PDR5R: (5′-TCTTGGTAAGTTTCTTTTCTTAAC-CAAATTCAAAATTCTAGAATTCGAGCTCGTTTAAA C-3′). The resulting isolate was named DSY3961.

Induction of the cyp51A cDNAs expression from A. fumigatus in S. cerevisiae

A. fumigatus RNA was obtained from 16-h cultures as previously described [18]. *A. fumigatus cyp*51A cDNAs flanked by pYES2/CT regions for homologous recombination in *S. cerevisiae* were amplified by PCR using the set of primers Cy51F (5′-ACTACTAGCAGCTGTAATACGACT-CACTATAGGGAATATTAAGCTTAAAATGGTGTCGAT-GCTATTGCTCACGG-3′) and Cy51R (5′-AGGGTTAGG GATAGGCTTACCTTCGAAGGGCCCTCTAGACTCGAG CTTGGATGTGTCTTTAGAACGCTT-3′). pYES2/CT contains a polyhistidine (6xHis) tag for protein tagging at C-terminal end. Strain DSY3961 was transformed with 5 μl of digested plasmid and 5 μl of the *A. fumigatus cyp*51A previously amplified by PCR in order to perform homologous recombination in *S. cerevisiae*. Transformants were selected onto YNB-ura. To verify the system 4 μl of liquid YNB-ura overnight cultures (five 10-fold serial dilutions starting with OD = 0.4) of DSY3961 and DSY3961+ CM237cDNA were spotted in YNB-ura plates with galactose (2%) in the absence or presence of doxycycline (2 μg/ml).

After transformation with each *A. fumigatus* cDNA allele, 20 transformants were grown overnight in YNB-ura liquid media. Each transformant was screened for *erg11* function complementation by inoculating 4 μl of the yeast cultures in YNB-ura agar plates containing galactose (2%), and with and without doxycycline (2 μg/ml). *cyp51*A genes were amplified and sequenced for verification.

Inmunoblots

Protein extracts for immunoblotting were prepared by alkaline extraction from overnight cultures induced with galactose. Briefly, cells were resuspended in an Eppendorf tube with 1 ml water and 150 μl of a solution containing 1.85 M NaOH and 7.5% βmercaptoethanol. This mixture was incubated on ice for 10 min. Proteins were then precipitated with 150 μl of a 50% trichloroacetic acid solution and the suspension was left on ice for another 10 min. Precipitated proteins were centrifuged at maximal speed in a microcentrifuge for 5 min. The sediment was resuspended in 100 μl of loading buffer (40 mM Tris-HCl [pH 6.8], 8 M urea, 5% sodium dodecyl sulfate [SDS], 0.1 M EDTA, 1% βmercaptoethanol, and 0.1 mg/ml bromophenol blue) and incubated at 37°C for 30 min. Non-solubilized material was eliminated by a centrifugation step for 10 min. Ten microliters of solubilized yeast proteins was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred by Western blotting onto a nitrocellulose membrane. Immunodetection was performed with a polyclonal mouse anti His-tag antibody as previously described [19,20].

E-test Susceptibility testing

Susceptibility testing to azoles was performed with the E-test using the selective media YNB-ura, galactose and doxycycline. Standardization of growth conditions were performed prior to evaluating the *in vitro* azole drug susceptibility testing of individual *S. cerevisiae* clones expressing each of the different *cyp51*A cDNAs. Yeast cultures were grown overnight in YNB-ura with galactose (2%) and diluted to a density of 1 McFarlan. The clones were tested by E-test for fluconazole (Pfizer S.A., Madrid, Spain), itraconazole (Janssen Pharmaceutical S.A., Madrid, Spain), voriconazole (Pfizer S.A.) and posaconazole (Merck & Co, Madrid, Spain) in YNB-ura with galactose (2%) and doxycycline (2 μg/ml). Plates were incubated at 30°C. At least two clones for each *cyp51*A mutation, except for DSY3961+CM2162cDNA, were tested in order to average differences due to different copy number of plasmids between clones. The test was repeated at least two times.

Statistical analysis

MIC values were converted to log2 values to get a normalized distribution. The significance of the differences in MICs was determined by Student's *t* test (unpaired,

unequal variance). A *P* value of < 0.05 was considered significant.

Results

ERG11 *conditional expression in* S. cerevisiae

The functional complementation of *S. cerevisiae ERG11* with the different *cyp*51A alleles from the *A. fumigatus* azole-susceptible and azole-resistant strains was accomplished using the tetracycline regulatable system described in the methods section [15,16]. To facilitate azole susceptibility testing of individual *S. cerevisiae* clones expressing each of the different *cyp51*A cDNAs, a yeast mutant lacking the major efflux transporter (*PDR*5), and thus hypersusceptible to azole antifungals, was first constructed (DSY3961). The system was first verified comparing the growth of the strain DSY3961 and the strain DSY3961+CM237cDNA carrying *A. fumigatus* wild type

allele, in YNB-ura with galactose (2%) with and without doxycycline. Strain DSY3961 alone was unable to grow when doxycycline was added to the media, demonstrating the essential nature of *ERG11* in *S. cerevisiae* and its functional complementation by *A. fumigatus cyp*51A alleles (Fig. 1A).

Expressing A. fumigatus cyp51A *alleles in* S. cerevisiae

Functional complementation of *ERG11* by *cyp*51A alleles was performed by turning off the expression of *ERG11* with doxycycline and inducing the expression of *cyp*51A cDNAs from *A. fumigatus* controlled by the *GAL1* promoter with 2% galactose. After co-transformation of *cyp*51A with pYES2/CT, transformants were screened for each *Aspergillus* cDNA background. All *A. fumigatus cyp*51A alleles could functionally complement the absence of *ERG11* (Fig. 1B). Figure 1C illustrates Cyp51Ap immunodetection for clones expressing each *cyp*51A allele.
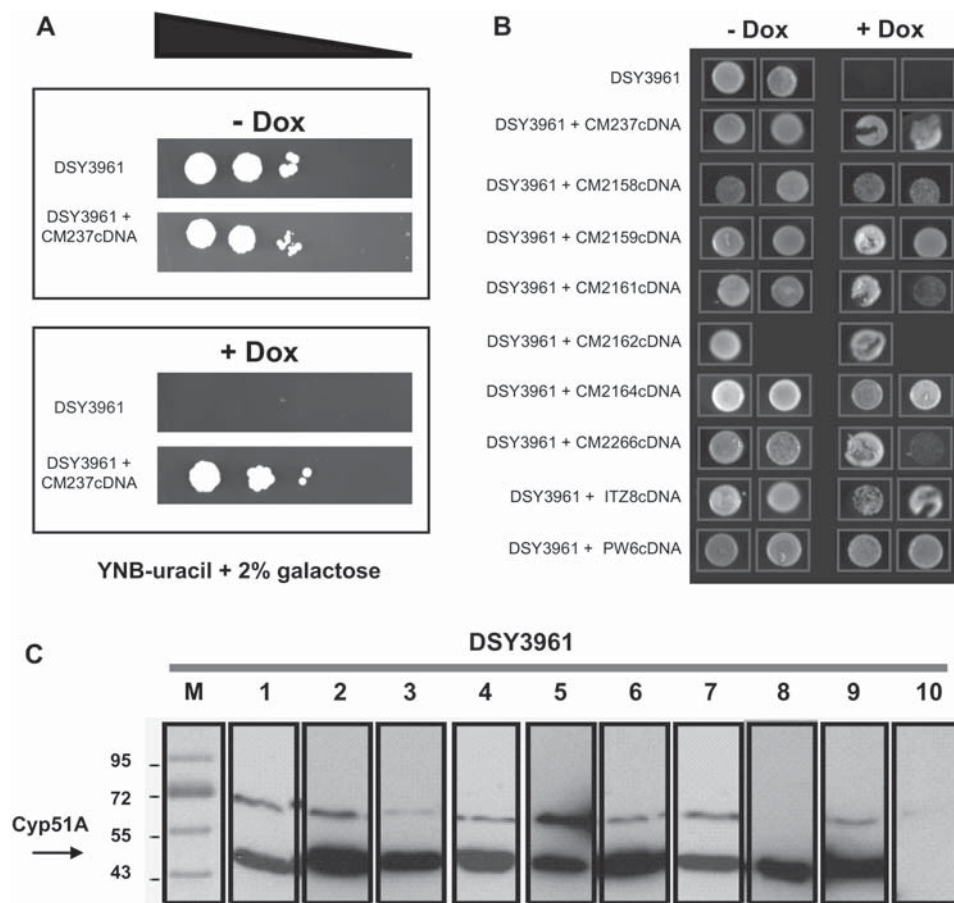
Fig. 1 (A) Serial dilutions of DSY3961 and DSY3961+CM237cDNA in YNB-ura with galactose (2%) in the absence or presence of doxycycline. (B) DSY3961 cDNAs mutated clones screening in YNB-ura with galactose (2%) in the absence or presence of doxycycline. (C) Inmunodetection of Cyp51A proteins for DSY3961+ (1) CM237cDNA, (2) CM2158cDNA, (3) CM2159cDNA, (4) CM2161cDNA, (5) CM2162cDNA, (6) CM2164cDNA, (7) CM2266cDNA, (8) ITZ8cDNA, and (9) Pw6cDNA. Number (10) is the negative control (DSY3961+vector pYES2/CT).

These clones were designated as DSY3961 plus each corresponding *A. fumigatus* strain cDNAs, i.e., CM237, CM2158, CM2159, CM2161, CM2162, CM2164, CM2266, ITZ8, and Pw6.

PCR amplification and sequencing of *cyp*51A from the selected clones confirmed that all of them conserved their original sequence and matched with wild type (azole-susceptible) *A. fumigatus cyp*51A sequences. One of the clones expressing CM2161cDNA and Pw6cDNA exhibited a nucleotide change compared with their original *cyp*51A sequence, thus resulting in non-synonymous but on a non-conserved region of the protein (H350Y and A435T, respectively).

Role of *A. fumigatus* cyp51A *alleles in azole resistance*

As expected, all clones showed high fluconazole MICs, which is in agreement with the intrinsic resistance of *A. fumigatus* to this azole. There were marked differences between the E-test MICs values for DSY3961+CM237cDNA clones (expressing *A. fumigatus* wild type *cyp*51A) and all of those expressing the *cyp*51A mutated alleles from *A. fumigatus* resistant strains. Some representative examples are shown in Fig. 2.

In general, all clones with *cyp*51A mutated alleles reproduced the susceptibility pattern of their corresponding *A. fumigatus* parental strain (Table 1). Azole susceptibility values obtained for the full set of *S. cerevisiae* clones are plotted in Fig. 3.

A. fumigatus resistant strains have an indistinct pattern of resistance to itraconazole with MICs $\geq$ 8 µg/ml. However, differences in itraconazole susceptibility were noticeable in *S. cerevisiae* cells, specially those expressing alleles with amino acid change at G54E, G54V, G54W and M220K (Figs. 2 and 3). Regarding voriconazole, while the highest MIC values were obtained with *A. fumigatus* isolates CM2158, CM2159 and PW6, only *S. cerevisiae* clones expressing alleles from CM2159 (M220K) and PW6 (M220I) displayed higher MICs to voriconazole as compared to the wild type. Given that the detection limit for the E-test voriconazole MIC assay is 0.002 µg/ml, our analysis could not detect any differences below this detection limit. Consistent with the *cyp*51A genetic background of CM2266 (G54W), the clones DSY3961+CM2266cDNA (G54W) reached the highest posaconazole MICs values, followed by clones carrying Cyp51Ap mutated alleles G54V, G54E and M220K (Table 1). In contrast, clones with changes at position M220T (DSY3961+CM2164cDNA) yielded the lowest MICs to posaconazole with no statistical significance with the wild type *cyp*51A (Figs. 2 and 3).

Slight differences in MICs values of clones carrying the same *cyp*51A alleles were observed (Fig. 3). These variations could be attributed to the different number of copies of plasmids expressing the *cyp*51A. Nevertheless, the statistical analysis showed that differences were significant (*P* value of < 0.05) in most cases.

Discussion

Strains of *A. fumigatus* are uniformly susceptible to the second-generation triazole drugs itraconazole, voriconazole, and posaconazole [8]. Resistance to antifungal drugs is a recognized problem occurring at a low frequency, although, resistance percentages between 6% and 12% were reported in some countries [6,21]. Secondary resistance to azoles involving acquisition of resistance in a susceptible strain accounts for all resistance in *A. fumigatus*. This resistance reflects genetic changes responsible for specific resistance mechanisms. The molecular mechanisms underlying azole resistance has been well documented in *A. fumigatus*. The initial finding was the existence of two different genes in *A. fumigatus*, encoding two different 14-α sterol demethylase proteins (*cyp*51A and *cyp*51B) [22]. However, it seems that Cyp51A is responsible for the basic susceptibility to azole drugs [10] and only *cyp51*A point mutations have been proven to be responsible for the azole resistance in *A. fumigatus*. Basically, these point mutations confer three different antifungal susceptibility profiles: (i) cross resistance to itraconazole and posaconazole linked to amino acid substitutions at glycine 54 (G54) [12,23,24]; (ii) a pattern of itraconazole resistance and high voriconazole, ravuconazole and posaconazole MICs linked to amino acid substitutions at methionine 220 (M220) [13]; and (iii) a pattern of multiple azole-cross-resistance associated with a higher *cyp*51A expression produced by insertion of a 34 bp tandem repeat sequence in the *cyp*51A gene promoter in combination with an amino acid substitution at Cyp51A leucine 98 (TRL98H) [14].

Moreover, different azole resistance profiles can be attributed not only to the amino acid position but also to the amino acid change at each position (Table 1). In an effort to understand the particular contribution of each amino acid change to azole resistance, we have applied a complementation system to study *A. fumigatus* genes that can functionally complement yeast essential genes. Particularly, the promoter *tet*O (doxycycline repressible) was used in this work to control the expression of *ERG11* in *S. cerevisiae* [15,16] and evaluate differences in azole susceptibility between *A. fumigatus cyp*51A alleles bearing the different point mutations. A similar approach has just been reported to characterize the impact of induced mutations in the *Mycosphaerella graminicola* 14-α eburicol demetilase on azole sensitivity [25]. Among *A. fumigatus* resistance mechanisms described above, we have focused on the amino acids changes at Cyp51A position G54 and M220. For obvious reasons, the third mechanism (TRL98H)
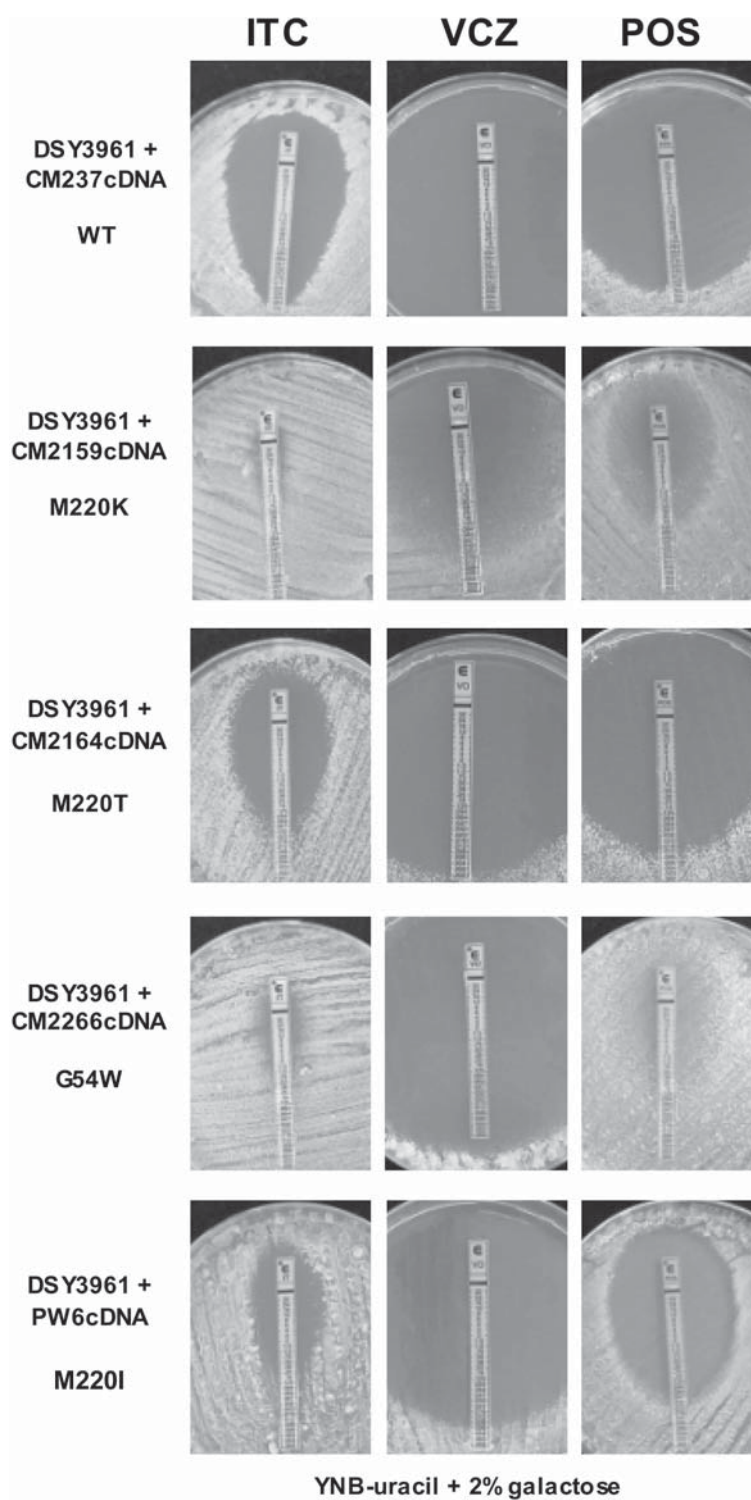
Fig. 2 E-test susceptibility testing to itraconazole (ITC), voriconazole (VCZ) and posaconazole (POS) for DSY3961+CM237cDNA carrying wild type allele (WT), and representative clones bearing different Cyp51Ap alleles: M220K, M220T, G54W and M220I.

Fig. 3 Plots of itraconazole (A) voriconazole (B) and posaconazole (C) MIC values for all *Saccharomyces cerevisiae* clones (DSY3961) expressing the indicated mutated *cyp51A* alleles. Means are indicated by horizontal bars.

responsible for multiple azole cross resistance could not be verified using this approach.

Each mutated *cyp51*A alleles contributed in a different way to azole resistance when itraconazole, posaconazole or voriconazole MICs were studied. In general, all clones bearing mutated alleles reproduced the susceptibility pattern of their original genetic background (Table 1 and Fig. 3). These findings verified that each amino acids change seems to have a different implication on azole drug resistance. It is noteworthy that using this system we could detect differences in ITC levels of susceptibility (Figs. 2 and 3), while that is not possible when testing ITC-resistant *A. fumigatus* strains (Table 1) whose MICs are consistently ≥ 8 mg/l [9].

The explanations for these results are possibly due to the way the drug interacts with its target and the conformational behaviour of the protein bearing the different amino acid changes. In that sense, Cyp51A homology models have already predicted different drug-protein interactions depending on the azole drug [26,27] and also the amino acid changes at Cyp51A [26,28]. These models predicted that G54 and M220 are located in loops in close proximity to the opening of channel 2. A change at G54, which is situated near to entry of the substrate access channel, has a dramatic effect on itraconazole and posaconazole. These two antifungals have long chains that may interact with the substrate access channel at different locations. Remarkably, when G54 was substituted by the large and hydrophobic tryptophan residue (W), it seems that this change is sufficient to interfere with the access to channel 2, which then prevents docking of posaconazole and/or itraconazole [26]. Modifications at G54 would not have any impact on the binding of voriconazole which has a more compact chemical structure than itraconazole or posaconazole. In contrast, mutations altering *A. fumigatus* Cyp51Ap at position M220 would cause an increase of MICs values to voriconazole [23,26]. It is encouraging that the results presented here compare well with the structural predictions. Clones expressing alleles with the amino acid change at G54W showed the highest MICs values to itraconazole and posaconazole, followed by the amino acids changes G54E and G54V. These results are also in good agreement with the MICs values for the *A. fumigatus* CM2266 (G54W) clinical strains which has been shown to be highly resistant to posaconazole *in vitro* and *in vivo* [9,29].

Among the substitutions at position M220 (M220K, M220I, M220T), the M220K change contributed to the highest MICs values to itraconazole and posaconazole. Because lysine (K) is the largest residue among the three amino acids, this result could be expected from a structural point of view. Lysine has a basic lateral chain and might affect the azole docking in a way that also results in decreased susceptibility to itraconazole and posaconazole.

However, modifications M220I and M220T had less impact on itraconazole resistance and particularly a changed M220T had no effect at all on posaconazole susceptibility. Finally, only clones bearing the Cyp51Ap substitution M220K and M220I showed increased MICs to voriconazole, which is similar to the azole resistance profile of *A. fumigatus* strains with these specific amino acid substitutions and correlates with the Cyp51Ap homology models. Therefore, we can conclude that the different substitutions at M220 would affect drug-target interactions depending either on the shape, size and nature of the substituted amino acid or on the azole drug.

In conclusion, we have designed a system to evaluate functional complementation of *ERG11* in *S. cerevisiae*. This system has been implemented for the first time to assess differences in azole susceptibility between *A. fumigatus cyp*51A alleles with different point mutations (resulting in eight different amino acid substitutions at G54 and M220 in *Aspergillus fumigatus* Cyp51A) that have been described as responsible for clinical azole resistance. This system is a valuable tool that can be used to address and predict resistance mechanism in *A. fumigatus*. It also provides opportunities to test other resistance mechanisms where others genes of interest could be expressed in a similar manner. Moreover, this system could probe amino acid changes/intrinsic resistance of Cyp51A from other filamentous fungi which are not easily genetically tractable i.e., *Scedosporium* spp., *Fusarium* spp. and many *Mucorales* spp., Finally, the system designed here can be used to test the efficacy of additional azole drugs with respect to existing *cyp*51A mutations, or de novo inhibitors synthesis to improve the treatment of infections produced by *A. fumigatus*.

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