Substitutions at Methionine 220 in the 14α-Sterol Demethylase (Cyp51A) of Aspergillus fumigatus Are Responsible for Resistance In Vitro to Azole Antifungal Drugs

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Substitutions at methionine 220 in the 14α–sterol demethylase (Cyp51A) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs.

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ABSTRACT

Five clinical isolates of *Aspergillus fumigatus* that exhibited a similar pattern of reduced susceptibility to itraconazole and other triazole drugs were analysed. Sequence analysis of genes encoding the 14α-sterol demethylases (*cyp51A* and *cyp51B*) revealed that all five strains harboured mutations in *cyp51A* resulting in the replacement of methionine at residue 220 by either valine, lysine or threonine. The mutated *cyp51*A genes were introduced into a wild type *A. fumigatus* wild type strain, the transformants exhibited reduced susceptibility to all triazole agents confirming that the mutations were responsible for the resistance phenotype.

Aspergillus fumigatus is one of the most prevalent airborne fungal pathogens causing infection worldwide with a high mortality and morbidity in the immunocompromised host (14). Although *A. fumigatus* is intrinsically resistant to fluconazole (FLZ), the newer triazoles such as itraconazole (ITC), posaconazole (POS), and voriconazole (VRC) are active both in vitro and in vivo against this species (2, 11, 24). However, a number of *A. fumigatus* isolates with in vitro ITC-resistance have been described (1, 4, 8, 9, 16, 19, 20), in some cases the resistance detected in vitro has been confirmed in animal models of infection (3, 7). Resistance to VRC has been recently detected in clinical and laboratory strains (E. Manavathu, I. Baskaran, S. Krishnan, G. Alangaden, P. Chandrasekar. Cytochrome P450 14-alpha sterol demethylase mutation dependent triazole cross-resistance in *Aspergillus fumigatus*. Abstract: M-471. page 444. and E. Manavathu, A. Espinel-Ingroff, G. Alangaden, P. Chandrasekar. Molecular Studies on VOR-resistance in a clinical isolate of *Aspergillus fumigatus*. Abstract: M-392. 43rd ICAAC Abstracts, American Society for Microbiology, September, 2003, page 440). In addition, it appears that some clinical *Aspergillus* strains are starting to show high MICs against the new triazole agents like posaconazole and ravuconazole (19, 24).

The triazoles inhibit the ergosterol biosynthesis pathway via the inhibition of 14α-sterol demethylase (Cyp51), an enzyme that removes the methyl group at position C-14 of precursor sterols. In *A. fumigatus*, there are two distinct but related Cyp51 proteins encoded by *cyp51A* and *cyp51B* (18). Two molecular mechanisms of resistance to azoles have been proposed in *A. fumigatus*: the first is reduced intracellular accumulation, due to either increased expression of efflux pumps (8, 30) or reduced penetration of the drug (15). The other

mechanism of resistance is through the modification of Cyp51 (8, 22). To date the most prevalent mechanism of resistance in *A. fumigatus* appears to be the modification of Cyp51, specifically mutations in *cyp51A*. These mutations, in both clinical strains and laboratory generated spontaneous mutants, have been associated with two different susceptibility profiles: (i) cross-resistance to ITC and POS has been associated with amino acid substitutions at glycine 54 (G54) (9, 16, 20) (ii) cross-resistance to VRC and ravuconazole (RAV) has been associated with amino acid substitutions at G448 (E. Manavathu, I. Baskaran, S. Krishnan, G. Alangaden, P. Chandrasekar. Cytochrome P450 14α-sterol demethylase mutation dependent triazole cross-resistance in *Aspergillus fumigatus*. Abstract: M-471. 43rd ICAAC Abstracts, American Society for Microbiology, September, 2003, page 444). A third pattern of azole resistance was recently reported (9, 19). This new pattern is characterized by high MICs for ITC, VRC, RAV and POS. The majority of the strains exhibiting this susceptibility profile harbour amino acid substitutions at methionine 220 (M220), an area of Cyp51A that was not previously associated with amino acid substitutions causing reduced susceptibility to azoles (9, 16, 20).

The objective of this work was to determine if the amino acid substitutions at M220 were sufficient to confer reduced susceptibility to azoles in *A. fumigatus*. To this end, *cyp51*A alleles encoding the different point mutations were introduced into a wild type (azole susceptible) *A. fumigatus* strain (CM-237), all three amino acid substitutions conferred reduced susceptibility to azoles.

Fungal strains and antifungal susceptibility testing. Eight clinical *A. fumigatus* strains from the Mycelial Collection of the Spanish National Centre for Microbiology (CNM-CM) were analyzed (Table 1). (i) strains with elevated MICs

to all azole drugs:CNM-CM-1252 (AF-90), CNM-CM-1245 (AF-91), CNM-CM-2158 (AF-1422), CNM-CM-2159 (F/6919), CNM-CM-2164 (SO/3829). (ii) susceptible strains (MIC ≤0.5 µg/ml) to all four triazoles: (CNM-CM-2739, CNM-CM-1369, CNM-CM-237). In addition, CM-237 was utilized for describing the sequence of the genes *cyp51A* and *cyp51*B (18). *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains for susceptibility testing.

Broth microdilution susceptibility testing was performed as described in the NCCLS document M38-P (21) with the modifications described previously (2, 23, 26). ITC (Janssen Pharmaceutical, Madrid, Spain), VRC (Pfizer S.A., Madrid, Spain), RAV (Bristol-Myers Squibb, Madrid, Spain), and POS (Schering-Plough Research Institute, Kenilworth, NJ) were obtained as powders from their respective manufacturers. The drugs were dissolved in dimethyl sulfoxide (DMSO) (Sigma, Madrid, Spain) at 1600 µg/ml, the final concentration range assayed was 8.0-0.015 µg/ml. Visual readings were performed using a microtiter reading mirror, the MIC was defined as the lowest concentration of drug that completely inhibited fungal growth after 48 hours of incubation at 35ºC. Susceptibility tests were performed at least twice with each strain on different days. Susceptibility results are shown in Table 1. The five strains exhibited reduced susceptibility to all four triazoles with variable MICs depending on antifungal drugs and strain, being POS the most active in vitro compound.

PCR amplification and sequence analysis of the *cyp51A* and *cyp51B* genes. Conidia from each strain were inoculated into 3 ml GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) and grown overnight at 37ºC.

Mycelial mats were recovered and subject to a DNA extraction protocol (18). The full coding sequences of *cyp51A* and *cyp51B* were PCR amplified as previously described (9). To rule out the possibility that any sequence changes identified were due to PCR induced errors, each mutant was independently analyzed twice.

Sequence analysis of *cyp51A* revealed a number of point mutations. The five ITC-resistant strains (CM-1252, CM-1245, CM-2158, CM-2159, CM-2164) each had a single nucleotide substitution in codon 220 (encodes methionine) resulting in the introduction of either valine, lysine or threonine (Table 1). The five ITC-resistant strains also harboured point mutations in *cyp51B*, however two of the three mutations detected were silent, the one missense mutation (resulting in the replacement of Asp by Glu at codon 387) was not conserved across the five isolates (Table 1). Sequence alignments of the region encompassing M220 revealed that this residue is not a strictly conserved amino acid between all yeasts and moulds, although this position is conserved in many fungal species and it is on the edge of a highly conserved region (Figure 1). Moreover, the fact that five ITC-resistant strains harbored the same mutation strongly suggests that the substitution is associated with azole resistance. In this regard, the region encompassing the mutation was sequenced in 22 *A. fumigatus* ITC-susceptible clinical strains; none of the 22 strains had mutations at codon 220.

Replacement of the wild type *cyp*51A gene with *cyp*51A alleles that encode substitutions at codon 220. The *cyp51*A alleles from strains CM-1252, CM-2159 and CM-2164 strains were PCR amplified and each was individually electroporated into the wild type *A. fumigatus* strain CM-237.

Electroporation was carried out using a protocol previously described for *A. nidulans* (28) and subsequently adapted for *A. fumigatus* (32), transformants were selected on media containing ITC as described previously (9). ITC-resistant transformants appeared after 2-7 days incubation. The transformants were labeled with a 'T' followed by a roman numeral (Table 1). The number of ITC-resistant transformants obtained using the *cyp51*A genes from strains CM-2159, CM-1252 and CM-2164 were four (T-III, T-VI, T-VII and T-XI), one (T-XII) and one (T-XXII), respectively. To confirm that each transformant contained only one copy of the *cyp51*A gene we performed a Southern blot analysis. Chromosomal DNA was extracted from each transformant, digested with either *Sal*I or *Bam*H1 (Amersham Biosciences, Madrid, Spain) and resolved by gel electrophoresis. Southern blots (27) were probed with a labeled fragment of *cyp51*A (18), in every case only a single band hybridized to the probe (data not shown). The *cyp51*A and *cyp51*B genes from the six transformants were sequenced; all six appeared to have incorporated the mutated *cyp51*A allele. With the exception of the original mutation at codon 220, none of the transformants had any other mutations in either *cyp51A* or *cyp51B*. The susceptibility of the transformants to triazoles was determined as described above, in general the transformants exhibited similar susceptibility profiles to the original clinical isolates (table 1).

In order to identify the gene replacement events we used a direct selection procedure. Consequently, there is a possibility that the mutations identified in the transformants arose spontaneously and were selected for through the inclusion of ITC in the transformation plates. However, three observations argue against this possibility. Firstly, the three alleles used in the

transformations each encoded a different substitution at residue 220. The mutations identified in the six transformants matched those present in the donor *cyp51*A allele, such congruence would be highly unlikely if the mutations had arisen spontaneously. Secondly, in control experiments, in which the donor DNA was replaced by water, no resistant isolates were detected. Finally, there have been no prior reports of spontaneous mutations arising in this area of the *A. fumigatus* Cyp51A protein in laboratory selected mutants (16, 20, Abstract: M-471. page 444, E. Manavathu, I. Baskaran, S. Krishnan, G. Alangaden, P. Chandrasekar. Cytochrome P450 14-alpha sterol demethylase mutation dependent triazole cross-resistance in *A. fumigatus***).**

To date there have been a number of reports that have identified polymorphisms in the *cyp51* gene from clinical *C. albicans* isolates that are responsible for and/or associated with azole antifungal resistance to fluconazole (10, 17, 29). In some filamentous fungi, one mutation (Y136F) has been correlated with resistance to different demethylase inhibitors (5, 6). In *A. fumigatus* specific mutations in *cyp51*A have been associated with decreases in susceptibility to ITC and POS (9, 16). In this report, substitutions at residue 220 were detected in five independent ITC-resistant isolates. The minor differences in the way the mutations impact the susceptibility to specific azoles presumably reflect differences in the way the azoles interact with the target protein. The clinical significance of these differences, if any, has yet to be determined.

The precise manner in which substitutions at M200 impact triazole binding is not immediately obvious. Recently, a three dimensional model of 14α–demethylase from *C. albicans* was built using the crystallographic coordinates of four prokaryotic P450 enzymes (12). The docking of both the

substrate and triazoles into the active site of the enzyme was explored. It was postulated that the long side chain of ITC interacted with the residues in the substrate access channel. This channel is lined by hydrophobic and aromatic residues such as F228, I231, F233, V234 and F235 (corresponding to F214, I217, F219, M220, and L221 in Cyp51A from *A. fumigatus*). Some of these residues are predicted to have direct interactions with either the substrate or the antifungal drug (12). From this model the substitutions at methionine 220 might be expected to disrupt drug binding. Recently, a homology model of Cyp51A from *A. fumigatus* was constructed based on the X-ray structure of the CYP51 orthologue from *Mycobacterium tuberculosis* (31). Some of the residues in the predicted *A. fumigatus* Cyp51 F-G loop (T215 and P230) are predicted to have contact with ITC and POS (31) but not directly with VCZ. Podust *et al*. crystallized the *Mycobacterium tuberculosis* Cyp51 in the presence of FLC (25). They suggested that substitutions causing azole-resistance in fungi are located in regions of the protein involved conformational changes associated with the catalytic cycle, rather than in residues that directly contact the drug (25). Also, it has been previously pointed out that it seems more important to look at the role of specific residue mutations in relation with their local environments (13). Further evidence will have to wait until the crystal structure for *A. fumigatus* Cyp51s is obtained in conjunction with the antifungal drugs. In the meantime, the identification of amino acid substitutions responsible for azole resistance continues to provide new insights into the way the drugs interact with Cyp51A. Such knowledge may aid in the development of more active molecules. Further investigations into the functional analysis of the Cyp51A and Cyp51B proteins of *A. fumigatus* are currently in process in our laboratory.

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Figure 1. Alignment of Cyp51 proteins. A segment of Cyp51A (amino acids 205-247) (GenBank AAK73659) and Cyp51B (amino acids 220-262) (GenBank AAK73660) from *A. fumigatus* with the corresponding CYP51 segments from: *Candida albicans* (CaP450; GenBank. AAF00598), *C. glabrata* (CaErg11; GenBank AAB02329), *C. tropicalis* (CtErg11; GenBank AAA53284), *Saccharomyces cerevisiae* (ScErg11; GenBank AAA34546), *Schizosaccharomyces pombe* (ScpCyp51; GenBank CAA90803), *C. krusei* (CtErg11; GenBank AAO83898), *Ustilago maydis* (UmErg11; GenBank CAA88176), *Cryptococcus neoformans (*CnErg11; GenBank AAF12370), *Penicillium digitatum* (PdCyp51; GenBank. CAD27793), *A. nidulans* (AnCyp51; GenBank AAF79204), *Erysiphe graminis* (EgCyp51; GenBank AAC97606), *Monilia fructuosa* (MfCyp51; GenBank AAL79180), *Venturia ineaqualis* (ViCyp51; GenBank AAF71293), *Venturia nashicola* (VnCyp51; GenBank CAC85409), *Leptosphaeria maculans* (LmCyp51; GenBank AAN28927), *Botryotinia fuckeliana* (BfCyp51; GenBank AAF85983), *Neurospora crassa* (NcCyp51; GenBank EAA34813), *Mycosphaerela graminicola* (MgCyp51; GenBank AAF74756), *Tapesia yallundae* (TyErg11; GenBank AAG44831), *Uncinula necator* (UnCyp51; GenBank AAC49812). The alignment was performed by MegAligne using Clustal V. (DNAstar, Inc., Lasergene, Madison, USA). Asterisks (*) indicate residues located in the F-G loop referred to in the text.