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Antimicrob Agents Chemother. 2005 Jun;49(6):2536-8.

Targeted gene disruption of the 14-α sterol demethylase *(cyp51A)* in *Aspergillus fumigatus* and its role in azole drug susceptibilities.

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Key words: *Aspergillus fumigatus,* azole drugs, gene disruption, CYP51.

Running title: 14-α sterol demethylase gene inactivation in *A. fumigatus*

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ABSTRACT

Aspergillus fumigatus 14α-sterol demethylase (Cyp51A) role in azole drug susceptibilities was assessed. Targeted disruption of *cyp51A* in azole susceptible and resistant strains resulted in decreased azole MICs from 2-40 fold. The *cyp51A*$^{-}$ mutants were morphologically indistinguishable from the wild type strain retaining the ability to cause pulmonary disease in neutropenic mice.

Aspergillus fumigatus is one of the most prevalent airborne fungal pathogens causing invasive aspergillosis in the immunocompromised host (18). Attributable mortality rates remain excessively high and about 80% of the patients with invasive aspergillosis die despite treatment with available antifungal agents (9, 22). The biosynthesis of ergosterol is a complex process involving many enzymes in which the 14-alpha sterol demethylase is the target for azole antifungals by selectively inhibiting the yeast and fungal forms over plants and mammals forms (17,39). The basic knowledge of the fungal cytochrome P450, encoding 14-α sterol demethylases (EDM, CYP51, ERG11 or $P450_{14DM}$) gene family may facilitate additional studies, resulting in fungal inhibitors with increased activity or broader spectrum. In *A. fumigatus*, there are two distinct but related Cyp51 proteins encoded by *cyp51A* and *cyp51B* (23) but their contribution to the ergosterol biosynthesis pathway have not been assessed yet. The existence of two *cyp*51 genes in *Aspergillus* spp. suggest that the product of these genes might perform different function in the cell, perform similar function having different localization, or have different substrate affinities (23). Functional analysis of *A. fumigatus* genes encoding Cyp51 will contribute to a better understanding of the enzyme inhibition by azole drugs at the molecular level and eventually it will lead to improvements in antifungal drugs design. Erg11 activity has been shown not to be essential in yeast such as *Candida albicans* or *C. glabrata* (13, 35) but to date, there are not reports on Cyp51 functional studies in any filamentous fungi. *A. fumigatus* is intrinsically resistant to fluconazole (FLC), unlike itraconazole (ITC) and voriconazole (VRC), which have been shown to have good *in vitro* and *in vivo* activity against these species (6, 12, 14). However, a number of isolates with in vitro itraconazole resistance have been already described (3, 8, 11, 14, 25). Although different mechanisms of resistance to azoles have been proposed in *A. fumigatus* (10, 20, 27, 29, 36) to date, the most prevalent mechanism of resistance in *A. fumigatus* appears to

be mutations in the *cyp51A*. These mutations are responsible for two different azole drugs susceptibility profiles: (i) cross-resistance to ITC and posaconazole (POS) that has been linked to amino acid substitutions at glycine 54 (G54) (11, 21, 27) and (ii) cross-resistance to all azole drugs which has been associated with amino acid substitutions at methionine M220 (24)**.** Therefore, we have started doing the functional analysis of the *A. fumigatus* Cyp51A by targeted disruption of the *cyp*51A gene in an *A. fumigatus* wild type strain and in two ITC-resistant *A. fumigatus* strains of clinical origin and the analysis of their phenotypes.

Strains and plasmids: (A) The *A. fumigatus* strain CM-237 (23). (B) Two clinical *A. fumigatus* strains (CNM-CM-1252 (AF-90) and CNM-CM-796) from the filamentous fungi collection of the Spanish National Centre for Microbiology (CNM-CM) with elevated MICs to azole drugs (Table 1). The isolate CNM-CM-796 showed a glycine 54 amino acid substitution in the Cyp51A whereas CNM-CM-1252 showed a methionine 220 amino acid substitution. Those Cyp51A amino acid substitutions produced a different pattern of azole resistance as described in previous works (11, 24). The fungi were grown at 37ºC in potato dextrose agar (Oxoid, Madrid, Spain), minimal media (MM)(4), Sabouraud dextrose agar (Oxoid), malt extract agar (Oxoid) and RPMI agar (Sigma-Aldrich, Madrid, Spain). Spore stocks were made in sterile distilled water. *Escherichia coli* JM109 was grown in Luria Bertani (LB) medium (33), supplemented with ampicillin (100 µg/ml), for propagation of plasmids for DNA purification. For standard cloning and sub-cloning procedures the vector pGEM-3Z and pGEM-T (Promega, Madrid, Spain) were used.

Molecular cloning and DNA Sequencing: The full coding sequences of *cyp*51A of *A. fumigatus* was PCR amplified as previously described (23) and cloned into pGEM-T vector system (Promega, Madrid,

Spain) to obtain plasmid pUM100. Restriction digestion of plasmid pID621 (kindly provided by Dr. D.W. Holden) was used to obtain the SalI 1.4 kb fragment of hygromycin B (*hph*) resistance cassette for the construction of the disruption vector. The 1.4 kb *hph* cassette was inserted in the XhoI restriction site of pUM100 to create pUM102. A linear 3.0 kb DNA fragment obtained by SacI/SacII double digestion of pUM102 was used for *A. fumigatus* CM-237 strain transformation (Figure 1A).

***Aspergillus* transformations:** *A. fumigatus* transformation experiments were achieved by electroporation using a protocol previously described for *A. nidulans* (34) and later adapted for *A. fumigatus* (40) with subsequent modifications (11). Hygromicin B (Sigma) (130 μg/ml) was used for transformants selection. The plates were incubated at 37ºC for a week. Hygromycin resistant transformants were appearing at variable times ranging from 2 to 5 days. Mutants were named by a letter (A) followed by a number. For genomic DNA isolation, *A. fumigatus* mycelia mats were recovered and subjected to a DNA extraction protocol described previously (11, 38). Genomic DNAs from hygromycin resistant transformants and the parental strain were digested with two different restriction enzymes (SalI and EcoRV) (Amersham Biosciences, Madrid, Spain). Southern analysis was performed as previously described (33) using a *cyp*51*A* gene probe as described elsewhere (23).

Antifungal susceptibility testing: Broth microdilution susceptibility testing was performed as described in the NCCLS document M38-A (28) with the modifications described previously (5, 30, 32). Itraconazole, ketoconazole (KTC) (both from Janssen Pharmaceutical S.A., Madrid, Spain), voriconazole, fluconazole (both from Pfizer S.A., Madrid, Spain), ravuconazole (RVC) (Bristol-Myers Squibb, Madrid, Spain), and amphotericin B (Sigma Aldrich Quimica, S.A., Madrid, Spain) were obtained as standard powder from their respective manufacturers. Fluconazole was assayed using

concentration ranges from 3200 to 0.03 μg/ml, with a initial stock solution of 128,000 μg/ml in DMSO. Susceptibility tests were performed at least three times with each strain on different days.

RNA extraction and LightCycler PCR: Total RNA extraction from the *A. fumigatus* CM-237 parental strain and the derived CM-A8 mutant strain and reverse transcription (RT) reactions were performed following the conditions previously described (23). Amplification of cDNAs was carried out using the LightCycler PCR and detection system (Roche Diagnostics, Barcelona, Spain). Primers sets used for cDNAs amplification were: primer A1 and A2 for *cyp*51A and primer B1 and B2 for *cyp*51B (23). Also, a primer set Tub-1 (5' AACCAAATTGGTGCCGC 3') and Tub-2 (5' CACGGATCTTGGAGATC 3'), that amplify a 697 bp product (nucleotides 253 to 950 including three intron sequences), was used for the amplification of the β-tubulin *A. fumigatus* house keeping gene (*Tub1*) (GenBank accession number: AY048754). LightCycler PCRs were set up in a final volume of 20 μl with the FastStart DNA Master SYBR Green (Roche Diagnostic, Madrid, Spain) using 2 mM $MgCl_2$, each primer at 0.5 μM and 2 μl of cDNA. After one step at 95°C for 10 minutes the samples were cycled 45 times, each cycle consisting of 10 s at 95 °C, 5 s at 54°C and 20 s at 72 °C. A positive control (using DNA) and negative controls (no RNA and No Reverse-Transcriptase) were included in each experiment. Each assay included duplicate reactions and was repeated three times on different days. A standard curve with serial dilutions of cDNA was performed to each PCR reaction. The results were normalized using data obtained for the *A. fumigatus* house-keeping *Tub1* gene. The concentration of β-tubulin was determined once for each cDNA sample and used to normalize the genes tested from the same cDNA sample. The method described by Pfaffl (31) was employed to relative quantification. PCR efficiencies were calculated from the curves slopes given by LightCycler software (Roche Diagnostic).

Total ergosterol content determinations: For sterol analysis 10^6 spores/ml from strains CM-237 and CM-A8 were cultured in minimal liquid medium for 18 hours at 37°C and at 150 rpm in an orbital shaker incubator (C25KC, New Brunswick Scientific). Fungal mycelia were harvested by vacuum filtration and divided in two parts. Humidity was calculated by drying one of the parts until constant weight was obtained (72 h). Ergosterol was extracted using the protocol described by Arthington-Skaggs (1). Ergosterol content was analysed by HPLC using a μ-Bondapack C18 column (Waters LC Module I plus; Waters Corporation, Madrid, Spain), 100 % methanol as mobile phase and a flow rate of 1 ml/min. The UV detector was set at 282 nm. Under these conditions ergosterol had a retention time of 7.6 min. The quantities of ergosterol were estimated by calculating the area under each peak. The data process was done with Millenium^{32} and Millenium^{32} photodiode array detector (PDA) software (Waters Corporation, Madrid, Spain). A calibration curve was produced using standards of 5, 10, 25, 50 and 100 μg/ml of pure ergosterol (Sigma). The experiments were repeated four times. The humidity was eliminated to express the results in μg of ergosterol per mg of dried mycelia mass.

Pathogenicity test: The pathogenicity of the *cyp*51A (CM-A8) mutant strain was assessed in neutropenic mice (ICR, SPF, six weeks old, CRIFFA, Barcelona, Spain) as previously described by Smith *et* al. (37). The experiments were carried out with eight mice per group, inoculated with 10^4 spores/mouse (in 30 μl via intranasal) of CM-237 strain or the *cyp*51A deficient strain (CM-A8). A control group with the mice immunosupressed but without inoculation of fungal spores was also included. Mice were closely monitored twice a day for clinical symptoms (starry fur, hunched appearance, lethargy) and moribund mice were humanely killed and that moment was considered the day of death. Survival was recorded for 15 days and animals that survive at day 15 were humanely

killed and dissected for lung homogenisation and platting onto Sabouraud dextrose agar. The stability of the *cyp*51A gene disrupted CM-A8 strain was checked by Southern analysis as described above.

Data analysis: The significance of the differences in MICs and ergosterol content was determined by Student's *t* test (unpaired, unequal variance). A *P* value < 0.01 was considered significant. Both, on-scale and off-scale MICs results were included in the analysis. The off-scale MICs were converted to the next concentration up or down. In order to approximate a normal distribution, the MICs were transformed to log2 values to established susceptibility differences between isolates. Kaplan-Meier survival analysis was used to determine the cumulative proportion of severe ill mice that were sacrificed in the groups inoculated with the different *A. fumigatus* strains. Statistical analysis was done with the Statistical Package for the Social Sciences (SPSS, version 12.0) (SPSS S.L., Madrid, Spain).

RESULTS AND DISCUSION

Construction of *A. fumigatus cyp*51A deficient strains

For the construction of the *A. fumigatus cyp*51A$^-$ mutant strains, the *cyp*51A gene was inactivated in the CM-237 wild type strain and in two ITC-resistant strains. Plasmid pUM102, used for the disruption experiments, contained the *hph* gene (hygromycin resistance gene) (7) and *cyp*51A flanking DNA sequences of 630 bp and 984 bp (Figure 1A). The resistance marker interrupted *cyp*51A immediately upstream from a highly conserved region. A linear fragment of approximately 3.0 kb released by double digestion SacI/SacII of pUM102 was used to electroporate spores of the *A. fumigatus* CM-237 parental strain. Genomic DNAs from twelve hygromycin-resistant transformants were extracted and analyzed by Southern hybridization using a 925 bp fragment from the *cyp*51A gene as a probe. The

digestion of genomic DNA from hygromycin resistant transformants with EcoRV should produce a 4.6 kb fragment instead the 3.2 kb EcoRV fragment obtained when digesting genomic DNA from the parental strain (as the result of gene replacement with the linear fragment that includes the 1.4 kb *hph* gene)(Figure 1B). To further confirm the gene disruption event another restriction enzyme (SalI) was used. A gene replacement event would also result in the loss of 4.0 kb SalI fragment and the generation of a 5.4 kb SalI fragment (Figure 1C). One transformant had undergone gene replacement (named CM-A8) and the rest of transformants were the result of an ectopic integration (Figure 1B) and discarded for further analysis. In order to assess the *cyp*51*A* contribution to azole drug resistance, two ITC resistant strains were also chosen for *cyp*51A^{-} gene inactivation: (CNM-CM-796 (from now onward CM-796) and CNM-CM-1252 (from now onward CM-1252), described in material and methods. Southern analysis of several transformants obtained from different transformation experiments showed that the appropriate *cyp*51A^{-} gene knock out strains (named CM-A41 and CM-A83) were obtained from each of the ITC-resistant parental strains (CM-796 and CM-1252 respectively) (Figure 1C).

Azole drug susceptibility patterns

Geometric mean and range of minimum inhibitory concentration (MIC) determinations for all the antifungal drugs tested (amphotericin B, itraconazole, voriconazole, ravuconazole, fluconazole and ketoconazole) with the CM-237 parental strain, the ITC-resistant strains (CM-796 and CM-1252) and their derived *cyp*51A^{-} mutant strains (CM-A8, CM-A41 and CM-A83) are shown in table 1. Notably, a decrease in the MICs of the *cyp*51A^{-} mutant strains were noted for all azole drugs tested but specially

for FLC and KTC and also, for ITC in ITC-resistant strains. MICs for AMB were unchanged for all the strains.

Morphology of *cyp*51A deficient strain CM-A8

No differences were found between *cyp*51A$^-$ CM-A8 strain and CM-237 wild type strain of *A. fumigatus* when they were examined macroscopically with respect to colony morphology and colony radial growth rate on different solid media. Microscopically, no differences were found on terms of hyphae morphology or conidial production between *cyp*51A$^-$ CM-A8 and CM-237 wild type strains (data not shown). The total ergosterol content of both the wild type and *cyp*51A$^-$ mutant strains was also similar. The arithmetic mean and standard deviation of the four times repetitions of the ergosterol content was 5.56 $\pm$ 0.34 μg of ergosterol by mg of dry mass for CM-237 strain and 5.98 $\pm$ 0.82 μg/mg for CM-A8 mutant strain ($P > 0.01$).

Gene expression determination

Three different RNA extractions from cultures growth in MM media were performed to compare the expression of *cyp*51B gene in the wild type strain (CM-237) and the mutant strain (CM-A8). No significant differences ($P > 0.01$) were found in the *cyp*51B expression levels between the *cyp*51A$^-$ CM-A8 strain and the CM-237 wild type strain. The mean of the three repetitions yield a ratio of 1.01 with a standard deviation of 0.36. No PCR products were obtained when the same cDNAs were amplified with the primers set A1 and A2 confirming the absence of a functional *cyp*51A gene in the CM-A8 mutant strain.

Virulence of the *cyp51A* deficient mutant strain CM-A8

The virulence capacity of strain CM-237 and the *cyp*51A$^-$ deficient mutant CM-A8 were compared in a neutropenic murine model of pulmonary aspergillosis. There were no significant differences in the onset of the illness or any reduction of mortality between the CM-237 strain and the *cyp*51A$^-$ mutant CM-A8 strain confirming its pathogenicity (figure 3)($P > 0.01$). The stability of the *cyp*51A$^-$ disrupted CM-A8 strain was checked by Southern analysis as described above.

Although there are not reports of *cyp51* functional analysis in any filamentous fungi, Erg11/Cyp51 inactivation has been shown to be essential in *S. cerevisiae* but not in other yeast (2, 13, 35). Viable strains with an *Erg11/cyp*51 gene disruption have been obtained for *C. albicans* and *C. glabrata* and both have similar phenotypes with respect to morphology and azole drug resistance and minor differences in ergosterol precursors accumulation (13, 26, 35). Since, azole drugs resistance mechanisms in *A. fumigatus* seem mostly related to amino acid substitutions in Cyp51A (11, 21, 24, 27), we have started by constructing *A. fumigatus cyp*51A deficient strains from an ITC-susceptible strain (CM-237) and also, from two ITC-resistant strains. The resulting *cyp*51A deficient mutants (CM-A8, CM-A41 and CM-A83) were morphologically similar to the wild type (CM-237) strain indicating that *cyp*51A gene in *A. fumigatus* is not essential for viability. The susceptibility testing of *cyp*51A deficient mutant to antifungal compounds indicate an increase of susceptibility to all azole drugs tested, but especially to FLC and KTC. (comparing geometric means MIC values, the *cyp*51A$^-$ mutant is 17 times more sensitive to FLC, and 40 times more sensitive to KTC, than the wild-type strain). The possibility of matching the lower MICs to antifungal compounds with increased membrane

permeability resulting from altered membrane sterol is discarded because the *cyp*51A mutant strain CM-A8 have equal amounts of total ergosterol than wild type strain (CM-237) and also susceptibility to other drugs such as AmB was unchanged. The marked increased susceptibility to FLC and KTC is noteworthy since *A. fumigatus* has shown intrinsic resistance to both antifungals. This susceptibility pattern suggests that the still active Cyp51B could be more susceptible to these two antifungals and that the *A. fumigatus* c*yp51B* gene is, in fact, the true homologue of yeast Erg11. Therefore, Cyp51A will be responsible for the strong FLC and KTC resistance showed by *Aspergillus* spp. Still, the FLC and KTC MICs of *A. fumigatus* are higher than the MICs observed with both antifungals drugs for yeast. This fact could be explained by evolutionary specie specific enzyme differences that could have occurred in order to adapt to different sterol precursors. Also, not all yeast has similar susceptibility patterns to FLC and KTC as it happens with *C. krusei* and *C. glabrata* (5).

The lack of apparent morphologic defect and the unchanged total ergosterol content of the *cyp*51A^{-} mutant strain (CM-A8) were quite expected findings since the *A. fumigatus* mutant strain still carries a functional *cyp*51B gene copy.

The results obtained here suggest that we have block only one of the two possible routes and that the *cyp*51A deficient strain (CM-A8) came overcome ergosterol biosynthesis with only one active route supported by the Cyp51B activity. The normal amounts of total ergosterol content showed by in the *cyp*51A deficient mutant (CM-A8) will support this theory. Also, the absence of *cyp*51B increased expression showed by the *cyp*51A deficient strain compared to the wild type strain was quite an expected result because in different yeast have been shown that global *Erg* genes up regulation, and especially *Erg11* expression, is dependant of ergosterol depletion caused by inhibition of 14-alpha sterol demethylase (15). Alternatively, since Cyp51 activities seems interchangeable

between mammals, yeast and filamentous fungi but with higher activity for their respective endogenous substrates in vivo (17, 19), the *A. fumigatus* Cyp51A and Cyp51B might have a polyvalent role for different substrates (lanosterol and eburicol). In that case, we will have to wait until the analysis of *A. fumigatus cyp51B* gene knock out strains to know if the polyvalent role of each enzyme is totally reciprocal. These experiments are underway in our laboratory.

Virulence studies in filamentous fungi cyp51 deficient mutants are lacking but some work has been done with *C. glabrata* strains Erg11 depleted by using a regulatable promoter. Under conditions of promoter repression the *C. glabrata* Erg11 depleted strain showed a growth defect, become resistant to FLC but this Erg11 diminishing effect would not affect cell growth in mice (26). Another study with *C. glabrata* Erg11 gene deleted strains showed an increase susceptibility to oxidative killing by H_20_2 and neutrophils, although membrane perturbations associated with the absence of 14-alpha sterol demethylase activity and sterol alterations could be responsible for the enhanced killing and phagocitosis of *Erg11* deficient *C. glabrata* strains (16). The virulence levels of strain CM-237 and the *cyp*51A$^-$ deficient mutant CM-A8 were compared in a neutropenic murine model of pulmonary aspergillosis. There were no significant differences in the onset of the illness or any reduction of mortality between the CM-237 strain and the *cyp*51A$^-$ mutant CM-A8 strain confirming its pathogenicity and thus, providing a model for the in vivo testing of new and existing antifungal compounds in *A. fumigatus* strains deficient in one Cyp51 activity.

The findings in this work lead to several conclusions: (i) in *A. fumigatus* two 14-alpha sterol demethylases have been shown and each of them might has different drugs affinities. If each enzyme interacts differently with azole drugs the possibility of a synergistic effect using different azole drug combination should be tested. (ii) Cyp51A seems to be responsible for the intrinsic FLC and KTC

resistance of *A. fumigatus.* (iii) the availability of the *A. fumigatus cyp*51A^{-} mutant could be used as an screen in vitro for identification of inhibitory compounds that specifically target the Cyp51B activity. (iv) Finally, findings in vitro could be confirmed by testing antifungal compounds in the murine model of invasive aspergillosis.

Acknowledgments

This work was supported in part by grants: MPY1120/03 from Instituto de Salud Carlos III and SAF2002-02089 from the Ministry of Science and Technology. E. Mellado held a contract Ramón y Cajal from Ministry of Science and Technology. L. Alcazar-Fuoli has a predoctoral fellowship from Instituto de Salud Carlos III. We are grateful to J.P. Latge for helpful suggestions. We thank Gema del Rio for invaluable technical assistance. We also thank Pfizer, Janssen Pharmaceutical, and Bristol-Myers Squibb and for supplying antifungal powders.

LITERATURE CITED

1. **Arthington-Skaggs B.A., D.W. Warnock, and C.J. Morrison.** 2000. Quantitation of *Candida albicans* ergosterol content improves the correlation between in vitro antifungal susceptibility test results and in vivo outcome after fluconazole treatment in a murine model of invasive candidiasis. Antimicrob. Agents Chemother. **44**:2081-5.
2. **Bard, M., N.D. Lees, T. Turi, D. Craft, L. Cofrin, R. Barbuch, C. Koegel, and J.C. Loper.** 1993. Sterol synthesis and viability of erg11 (cytochrome P450 lanosterol demethylase) mutations in *Saccharomyces cerevisiae* and *Candida albicans*. Lipids. **28:**963-967.
3. **Chryssanthou, E.** 1997. In Vitro Susceptibility of Respiratory Isolates of *Aspergillus* species to Itraconazole and Amphotericin B. Acquired Resistance to Itraconazole. Scand. J. Infect. Dis. **29:** 509-512.
4. **Cove, D.J.** 1966. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochem. Biophys. Acta. **113**: 51-56.
5. **Cuenca-Estrella M., T.M. Diaz-Guerra, E. Mellado, and J.L. Rodriguez-Tudela.** 2001. Influence of glucose supplementation and inoculum size on growth kinetics and antifungal susceptibility testing of *Candida* spp. J. Clin. Microbiol. **39:**525-32.
6. **Cuenca-Estrella M., J.L. Rodriguez-Tudela, E. Mellado, J.V. Martinez-Suarez, and A. Monzon.** 1998. Comparison of the in vitro activity of voriconazole (UK-109,496), itraconazole and amphotericin B against clinical isolates of *Aspergillus fumigatus.* J. Antimicrob. Chemother. **42:** 531-533.

7. **Cullen D., S.A. Leong, L.J. Wilson, and D.J. Henner.** 1987. Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, hph. Gene. **57:**21-6.
8. **Dannaoui, E., F. Persat, M.F. Monier, E. Borel, M.A. Piens and S. Picot.** 1999. In-vitro susceptibilities of *Aspergillus* spp. isolates to amphotericin B and itraconazole. J. Antimicrob. Chemother. **44:** 553-555.
9. **Denning D.W.** 1998. Invasive aspergillosis. Clin. Infec. Dis. 26: 781-805.
10. **Denning DW, K. Venkateswarlu, K.L. Oakley, M.J. Anderson, N.J. Manning, D.A. Stevens, D.W. Warnock, and S.L. Kelly.** 1997. Itraconazole resistance in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **41:** 1364-1368.
11. **Diaz-Guerra T.M., E. Mellado, M. Cuenca-Estrella, and J.L. Rodriguez-Tudela.** 2003. A point mutation in the 14-alpha sterol demethylase gene *cyp51*A contributes to itraconazole resistance in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **47:**1120-1124.
12. **Fortun J., P. Martin-Davila, M.A. Sanchez, V. Pintado, M.E. Alvarez, A. Sanchez-Sousa, and S. Moreno.** 2003. Voriconazole in the treatment of invasive mold infections in transplant recipients. Eur. J. Clin. Microbiol. Infect. Dis. **22:**408-413.
13. **Geber, A., C.A. Hitchcock, J.E. Swartz, F.S. Pullen, K.E. Marsden, K.J. Kwon-Chung, and J.E. Bennett.** 1995. Deletion of the *Candida glabrata* ERG3 and ERG11 genes: effect on cell viability, cell growth, sterol composition, and antifungal susceptibility. Antimicrob. Agents Chemother. **39:** 2708-2717.
14. **Gomez-Lopez A., G. Garcia-Effron, E. Mellado, A. Monzón, J.L. Rodriguez-Tudela, and M. Cuenca-Estrella.** 2003. In vitro activities of three licensed antifungal agents against spanish clinical isolates of *Aspergillus* spp. Antimicrob. Agents Chemother. **47:**3085-3088.

15. **Henry K.W., J.T. Nickels, and T.D. Edlind.** 2000. Upregulation of ERG genes in *Candida* species by azoles and other sterol biosynthesis inhibitors. Antimicrob. Agents Chemother. **44:**2693-700.

16. **Kan V.L., A. Geber, and J.E**. **Bennett.** 1996. Enhanced oxidative killing of azole-resistant *Candida glabrata* strains with ERG11 deletion. Antimicrob. Agents Chemother. **40:**1717-1719.

17. **Lamb D.C., D.E. Kelly, and S.L. Kelly.** 1998. Molecular diversity of sterol 14alpha-demethylase substrates in plants, fungi and humans. FEBS Lett. **425:**263-265

18. **Latgé J.-P**. 1999. *Aspergillus fumigatus* and Aspergillosis. Clinical Microbiol. Rev. **12:** 310-350.

19. **Lepesheva GI, Waterman MR.** 2004. CYP51-the omnipotent P450. Mol. Cell. Endocrinol. **215:**165-170

20. **Manavathu, E.K., J.A. Vazquez, and P.H. Chandrasekar**. 1999. Reduced susceptibility in laboratory-selected mutants of *Aspergillus fumigatus* to itraconazole due to decreased intracellular accumulation of the antifungal agent. Inter. J. Antimicrob. Agents. **12:** 213-219.

21. **Mann P.A., R.M. Parmegiani, S.Q. Wei, C.A. Mendrick, X. Li, D. Loebenberg, B. DiDomenico, R.S. Hare, S.S. Walker, and P.M. McNicholas**. 2003. Mutations in Aspergillus fumigatus resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14-alpha demethylase Antimicrob. Agents Chemother. **47:**577-581.

22. **Marr KA, R.A. Carter, F. Crippa, A. Wald, and L. Corey.** 2002. Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. Clin. Infect. Dis. **34:**909-917.

23. **Mellado, E., T.M. Diaz-Guerra, M. Cuenca-Estrella, and J.L. Rodriguez-Tudela.** 2001. Identification of two different 14-α sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. J. Clin. Microbiol. **39:** 2431-2438.

24. **Mellado E., G. Garcia-Effron, L. Alcazar-Fuoli, M. Cuenca-Estrella, and J.L. Rodriguez-Tudela.** 2004. Substitutions at Methionine 220 in the 14alpha-Sterol Demethylase (Cyp51A) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs. Antimicrob. Agents Chemother. **48:**2747-2750.

25. **Mosquera, J., and D.W Denning.** 2002. Azole cross-resistance in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **46:** 556-557.

26. **Nakayama H., N. Nakayama, M. Arisawa, and Y. Aoki.** 2001. In vitro and in vivo effects of 14alpha-demethylase (ERG11) depletion in *Candida glabrata*. Antimicrob. Agents Chemother. **45:**3037-3045

27. **Nascimento A.M., G.H. Goldman, S. Park, S.A. Marras, G. Delmas, U. Oza, K. Lolans, M.N. Dudley, P.A. Mann, and D.S. Perlin.** 2003. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. Antimicrob. Agents Chemother. **47:**1719-1726.

28. **National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution antifungal susceptibility. Testing of filamentous fungi: Approved Standard Document M38-A. 22(16) NCCLS.

29. **Osherov, N., D.P. Kontoyannis, A. Romans, and G.S. May.** 2001. Resistant to itraconazole in *Aspergillus nidulans* and *Aspergillus fumigatus* is conferred by extra copies of the *A. nidulans* P-450 14-alpha-demethylase gene, *pdm*A. J. Antimicrob.

Chemother. **48:**75-81.

30. **Petrikkou E., J.L. Rodriguez-Tudela, M. Cuenca-Estrella, A. Gomez, A. Molleja, and E. Mellado.** 2001. Inoculum standardization for antifungal susceptibility testing of filamentous fungi pathogenic for humans. J. Clin. Microbiol. **39:** 1345-1347.
31. **Pfaffl M.W.** 2001. A new mathematical model for relative quantification on real-time RT-PCR. Nucleic Acids Res. **29**:e45
32. **Rodriguez-Tudela J.L., E. Chryssanthou, E. Petrikkou, J. Mosquera, D.W. Denning, and M. Cuenca-Estrella.** 2003. Interlaboratory evaluation of hematocytometer method of inoculum preparation for testing antifungal susceptibilities of filamentous fungi. J. Clin. Microbiol. **41:** 5236-5237.
33. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

34. **Sanchez, O., and J. Aguirre.** 1996**.** Efficient transformation of *Aspergillus nidulans* by electroporation of germinated conidia. Fungal Genetics Newsletter **43**:48-51.
35. **Sanglard D., F. Ischer, T. Parkinson, D. Falconer, and J. Bille.** 2003. *Candida albicans* mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. Antimicrob. Agents Chemother. **47:**2404-2412.
36. **Slaven J.W., M.J. Anderson, D. Sanglard, G.K. Dixon, J. Bille, I.S. Roberts, and D.W. Denning.** 2002. Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, *atr*F, in the presence of itraconazole in an itraconazole resistant clinical isolate. Fungal Genet. Biol. **36:**199-206.
37. **Smith J.M., C.M. Tang, S. Van Noorden, and D.W. Holden.** 1994. Virulence of *Aspergillus fumigatus* double mutants lacking restriction and an alkaline protease in a

low-dose model of invasive pulmonary aspergillosis. Infect. Immun. **62:**5247-54.

38. **Tang, C.M., J. Cohen, and D.W. Holden.** 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol. Microbiol. **6:** 1663-1671.
39. **Van den Bossche H., G. Willemsens, W. Cools, F. Cornelissen, W.F. Lauwers, and J.M. van Cutsem.** 1980. In vitro and in vivo effects of the antimycotic drug ketoconazole on sterol synthesis. Antimicrob. Agents Chemother. **17:**922-928.
40. **Weidner G., C. d'Enfert, A. Koch, P.C. Mol, and A. A. Brakhage.** 1998. Development of a homologous transformation system for the human pathogenic fungus *Aspergillus fumigatus* based on the *pyr*G gene encoding orotidine 5'-monophosphate decarboxylase. Curr. Genet. **33:**378-385.

Figure Legends

Figure 1. (A) Fragments of the *A. fumigatus cyp51*A gene and construct (plasmid pUM102) used for creating the *cyp*51A deficient mutant strain. Unfilled boxes indicate the hygromycin-resistant cassette (*hph*), hatched bars represent *cyp*51A genomic sequence and the striped arrow represent full *cyp*51A coding sequence. Sites for restriction enzymes are: S, SalI; X, XhoI and E, EcoRV. **(B)** Southern hybridization analysis of *cyp51*A$^-$ single mutant strain CM-A8, two transformants with ectopic integration (CM-A4 and CM-A5) and the wild type strain (CM-237) Genomic DNAs were digested with EcoRV and hybridized using a 925 bp PCR fragment from the *cyp51A* gene as a probe (black box). **(C)** Southern hybridization analysis of the wild type CM-237 strain the *cyp*51A$^-$ mutant strain CM-A8 the ITC-resistant parental strains (CM-1252 and CM-796) and the corresponding *cyp*51A$^-$ mutant strains CM-A83 and CM-A41. Genomic DNAs were digested with SalI and probed as before. Sizes of expected bands are indicated on the side in kb.

Figure 2.- Survival of immunocompromised mice infected with the wild type CM-237 strain (○) and the *cyp*51A deficient strain CM-A8 (▲) of *A. fumigatus*. An inoculum of 10^4 spores/ mouse were used for each strain. A control group with mice immunocompromised but without inoculum was also included (♦).