Title: Species-specific differences in C-5 sterol desaturase function influence the outcome of azole antifungal exposure.

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Running Title: Fungal sterol C5-desaturase activity

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Abstract.

The azole antifungals inhibit sterol 14α-demethylase (S14DM), leading to depletion of cellular ergosterol and the synthesis of an aberrant sterol-diol that disrupts membrane function. In *Candida albicans*, sterol diol production is catalyzed by the C-5 sterol desaturase enzyme encoded by *ERG3*. Accordingly, mutations that inactivate *ERG3* enable the fungus to grow in the presence of the azoles. The purpose of this study was to compare the propensity of C-5 sterol desaturases from different fungal pathogens to produce the toxic diol upon S14DM inhibition and thus contribute to antifungal efficacy. The coding sequences of *ERG3* homologs from *C. albicans* (*CaERG3*), *Candida glabrata* (*CgERG3*), *Candida auris* (*CaurERG3*), *Cryptococcus neoformans* (*CnERG3*), *Aspergillus fumigatus* (*AfERG3A-C*) and *Rhizopus delemar* (*RdERG3A/B*) were expressed in a *C. albicans erg3Δ/Δ* mutant to facilitate comparative analysis. All but one of the Erg3p-like proteins (AfErg3C) at least partially restored sterol C-5 desaturase activity, and to corresponding degrees rescued the stress and hyphal growth defects of the *C. albicans erg3Δ/Δ* mutant - confirming functional equivalence. Each C-5 desaturase enzyme conferred markedly different responses to fluconazole exposure in terms of the minimal inhibitory concentration (MIC) and residual growth observed at supra-MIC concentrations. Upon fluconazole-mediated inhibition of S14DM, the strains expressing each homolog also produced varying levels of 14α-methylergosta-8,24(28)-dien-3β,6α-diol. The RdErg3A and AfErg3A proteins are notable for low levels of sterol diol production and failing to confer appreciable azole sensitivity upon the *C. albicans erg3Δ/Δ* mutant. These findings suggest that species-specific properties of C5-sterol desaturase may be an important determinant of intrinsic azole sensitivity.

Introduction.

Mortality rates associated with invasive fungal infections (IFIs) remain alarmingly high, despite the availability and appropriate use of three major classes of antifungal drugs (1). The azoles antifungals block synthesis of the membrane lipid ergosterol through inhibition of sterol 14α-demethylase (S14DM – Erg11p). This leads to depletion of cellular ergosterol, and the conversion of the accumulated lanosterol into 14α-methylergosta-8,24(28)-dien-3β,6α-diol, an abnormal sterol species that disrupts membrane function, leading to growth arrest (2). Diol production involves the addition of a polar hydroxyl group at the C6 position by the sterol C-5 desaturase enzyme (Erg3p), which is believed to perturb lipid bilayer packing, creating membrane disorder and dysfunction. Several well-characterized mechanisms are known to contribute to azole resistance in *Candida albicans*, one of the most important human fungal pathogens. This includes elevated expression of the target protein (3, 4), mutations that reduce the target enzymes affinity for the azoles (5-7), as well as enhanced expression of drug efflux pumps (8). Mutations that inactivate sterol C-5 desaturase (Erg3p), the enzyme responsible for converting the lanosterol/14α-methylfecosterol that accumulates upon inhibition of S14DM, into the 'toxic' diol species, also confer azole resistance (2). In contrast to the aforementioned resistance mechanisms, inactivation of the *ERG3* gene results in complete azole insensitivity, rather than merely an increase in MIC (minimal inhibitory concentration). Loss of Erg3p activity leads to the accumulation of 14α-methylfecosterol following azole treatment instead of 14α-methylergosta-8,24(28)-dien-3β,6α-diol, which is apparently compatible with *C. albicans* growth (2). While azole-resistant *erg3* null mutants can be readily selected *in vitro* (9), and a number have been described among azole-resistant clinical isolates (2, 10-12), their occurrence is less commonly reported than strains with elevated drug efflux or an altered target enzyme. This could reflect the fact that loss of Erg3p

function itself blocks a late step in ergosterol biosynthesis (12), and therefore alters membrane composition and function. As a result, *C. albicans erg3* null mutants are sensitive to some physiological stresses (13-16), and have reduced hyphal growth under some conditions (12, 14, 17) – a phenotype that has been associated with diminished virulence (18, 19). Accordingly, when tested in the standard mouse model of disseminated infection, most *C. albicans erg3* null strains tested have reduced virulence (14, 17, 20, 21). Data presented by Sanglard and colleagues indicates that compensatory mutations can restore the virulence of *erg3* deficient strains (12), and it is possible that certain strain specific genetic determinants are permissive (11, 22). Nonetheless, while inactivation of Erg3p enhances *C. albicans* growth in the presence of the azole antifungals, the associated fitness defects could potentially disfavor the selection of *erg3* null mutants in the clinical setting. However, we recently reported that reduced *ERG3* transcription was sufficient to confer *in vitro* fluconazole resistance upon *C. albicans* without diminishing stress tolerance, hyphal growth or virulence in a mouse model of disseminated infection (21). These data suggest that complete loss of Erg3p activity is not necessary to reduce fungal sensitivity, and qualitative or quantitative differences in Erg3p function could be a key determinant of azole susceptibility. Conceivably, differences in the intrinsic as well as relative C5-desaturase and hydroxylase activities of Erg3p enzymes from individual fungal species may affect the consequences of azole exposure. The objective of this study was to determine if differences in the function of Erg3p homologs may influence sensitivity to the azole antifungals.

Materials and Methods.

Growth conditions. *C. albicans* was routinely grown on YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C, supplemented with uridine (50 μg/mL) when necessary. Transformant selection was carried out on minimal YNB medium (6.75 g/L yeast nitrogen base

without amino acids, 2% dextrose, 2% Bacto agar), supplemented with the appropriate auxotrophic requirements as described for *S. cerevisiae* (23, 24) or 50 μg/mL uridine.

Plasmid construction. All oligonucleotides used in this study are listed in Supplemental Table 1. The putative sterol C5-desaturase coding sequences for each of the described species were identified through BLAST searches of their respective genome sequence databases using the predicted *C. albicans* protein sequence. Alignments and phylogenetic analysis were conducting using the Phylogeny.fr program (http://www.phylogeny.fr/index.cgi). The coding sequence of each *ERG3* homolog was optimized according to the codon bias of a subset of highly expressed, ribosomal *C. albicans* proteins using the OPTIMIZER program (25). Synthetic sequences incorporating *Sal*I and *Mlu*I sites either side of each optimized coding sequence were produced by IDTDNA (Supplemental Table 2), amplified from the supplied DNA template using primers AMPF1 and AMPR1, and cloned between the *Sal*I and *Mlu*I sites of the pKE4 expression vector (26). Each construct was then sequenced using primers TEF1PRSEQF and ADH13'UTRSEQR, to confirm the correct insertion and coding integrity.

***C. albicans* strain construction.** The *erg3Δ/Δura3Δ/Δ* mutant was described in a previous study (21), and was transformed with each of the pKE4 based expression constructs, or vector alone following digestion with *Nhe*I (to linearize the plasmids), using the lithium acetate method (27). Individual prototrophic transformant clones were isolated following selection on medium lacking uracil or uridine. Correct integration of the respective vectors (and thus full restoration of the *URA3-IRO1* locus) in each clone was confirmed by the amplification of a 2.1 Kb product following PCR analysis of purified genomic DNA with the LUXINTDETF + LUXINTDETR primer pair.

Sterol extraction and quantitation. Strains were grown overnight at 37°C, 200 rpm for 16 hours and then sub-cultured to an OD_{600} of 0.25 into 10 ml YPD broth supplemented with 5 mg/L

fluconazole or 0.5% DMSO vehicle alone, and grown for 6 hours at 37°C. Non-saponifiable lipids were extracted using alcoholic KOH as reported previously (28). Samples were dried in a vacuum centrifuge (Heto) and were derivatized by the addition of 100 µl 90% BSTFA/10% TMS (Sigma), 200 µl anhydrous pyridine (Sigma) and heating for 2 h at 80°C. TMS-derivatized sterols were analyzed and identified using GC/MS (Thermo 1300 GC coupled to a Thermo ISQ mass spectrometer, Thermo Scientific) with reference to retention times and fragmentation spectra for known standards. GC/MS data files were analyzed using Xcalibur software (Thermo Scientific) to determine sterol profiles for all isolates and for integrated peak areas. Percentages of total sterols are given as the mean of 3 replicates.

RNA isolation and RT-PCR. Each *C. albicans* strain was grown overnight in YPD at 30°C then sub-cultured to an OD_{600nm} of 0.2 and then incubated at 30°C with shaking for 6 hrs. Cells were pelleted by centrifugation before total cellular RNA was extracted using the hot phenol method (29). cDNA was synthesized from total RNA using the Verso cDNA Synthesis Kit (Thermo Scientific), in accordance with the manufacturer's instructions. Synthesized cDNA (20 ng) was used for the amplification of the recombinantly expressed *ERG3* coding sequences by PCR, using ADH1R as a reverse primer, and either CaERG3RTF, CgERG3RTF, CaurERG3RTF, CnERG3RTF, AfERG3ARTF, AfERG3BRTF, AfERG3CRTF, RdERG3ARTF or RdERG3BRTF as a reverse primer, with 25 cycles of amplification (Supplemental Table 1). Transcript expression was then confirmed by running 5 µl of each product on a gel.

Antifungal susceptibility testing. Antifungal susceptibility testing of all the strains included in this study was performed using the broth micro-dilution method described in the CLSI document M27-A3 (30) in a 96-well plate format. All drugs for susceptibility testing used in this study were diluted in DMSO in 2-fold dilutions at 200 times the final concentration. RPMI 1640 medium

(Sigma-Aldrich) was prepared according to the CLSI document; the medium was buffered with morpholinepropanesulfonic acid (MOPS) and pH adjusted using NaOH and HCl. Plates were incubated without shaking for 24 or 48 hours at 35°C. The content of each well was carefully resuspended by pipetting up and down before OD_{600nm} was measured using a Biotek Cytation 5 plate reader.

Growth kinetic analysis. Each strain was sub-cultured into YPD broth supplemented with 1 or 5 µg/ml fluconazole, or 0.5% DMSO vehicle at approximately 1 X 10^4 cells/ml, and 200 µl of each cell suspension transferred to the wells of a round bottomed 96-well plate. The plate was then incubated at 30°C within a BioTek Cytation 5 plate reader, and OD_{600nm} read at 30-minute intervals. Background was measured from well with medium alone (no cells) and subtracted from each reading, before OD_{600nm} was plotted as a function of time. The post 8-hour V_{max} (i.e. V_{max} following the initiation of azole-mediated growth inhibition) and T_{INT} (period of time elapsed between reaching an OD of 0.25 and 0.75) was calculated using the Gen 5 reader software, and expressed as a percentage of the same parameters for the SC5314 wild-type control strain grown in the absence of fluconazole. Each experiment was conducted on three separate occasions, and the means and standard deviations of V_{max} and T_{INT} are presented.

Stress tolerance and hyphal growth assays. *C. albicans* strains were grown overnight in YPD broth at 30°C, the cell density was adjusted to 10^7 ml^{-1} in sterile water and serial 1:5 dilutions performed in a 96-well plate. Cells were then applied to YPD agar, or YPD agar supplemented with 500 mM $CaCl_2$, or 0.05% SDS using a sterile multipronged applicator. For hyphal growth analysis, 2.5 µl from a cell suspension with 10^7 cells/ml was spotted on M199 agar or 10% FBS (fetal bovine serum) agar plates, followed by incubation for 96 h at 37°C.

Results.

Sterol C5-desaturase homologs from different fungal pathogens are functionally distinct. BLAST searches of genome sequence databases were used to identify coding sequences from human fungal pathogens that share significant homology with *C. albicans* C5-desaturase (Erg3p). This yielded a single coding sequence from each of *Candida glabrata* (*CgERG3*), *Candida auris* (*CaurERG3*) and *Cryptococcus neoformans* (*CnERG3*), three of the most important pathogenic yeast, with the predicted protein products sharing 59.4, 70.3 and 42.4% identity at the protein level respectively (Supplemental Table 3). Three Erg3p orthologs were previously reported in *Aspergillus fumigatus* (*AfERG3A-C*)(31) and two were identified in *Rhizopus delemar* (*RdERG3A* and *RdERG3B*), two of the most significant infectious molds. Phylogenetic analysis indicated that the three *Candida* desaturases are closely related, while CnErg3p is much more divergent (figure 1). The two *R. delemar* paralogs are very similar to each other (85% identical), while AfErg3A and AfErg3B are also relatively closely related (56.1% identical), but more closely related to the *Candida* orthologs than to the *Rhizopus* proteins. However, the predicted AfErg3C protein is dissimilar to the other two *A. fumigatus* paralogs (42.8 and 41.4% identical to AfErg3A and AfErg3B respectively), sharing greater similarity to CnErg3p (60% identity). To compare the function of sterol C5-desaturase from these pathogens, the coding sequence of each homolog was adapted for expression in *C. albicans* and cloned into the pKE4 expression vector, to drive high levels of transcription from the constitutive *TEF1* promoter (P_{TEF1}). Each construct (or vector alone), was then introduced into a *C. albicans erg3Δ/Δ* strain (21), generating an isogenic panel of strains, each expressing a distinct C5-desaturase enzyme. Comparable levels of transcription of each coding sequence was confirmed by RT-PCR (Supplemental figure 1). Analysis of the sterol content of each strain confirmed ergosterol as the major sterol species identified within the strain expressing *CaERG3* (~88% – table 1). The strains expressing *CaurERG3*, *CnERG3*, *RdERG3B*,

AfERG3A and *AfERG3B* orthologs had a similar sterol composition including levels of ergosterol, indicating comparable levels of C5-sterol desaturase activity. While the *CgERG3*, and to a greater extent the *RdERG3A* expressing strain, had lower levels of C5-desaturase activity, as evidenced by reduced ergosterol content and elevated levels of ergosta-7,22-dienol and episterol. In contrast, the composition of the *AfERG3C* expressing strain was essentially the same as the *erg3Δ/Δ* mutant - completely lacking ergosterol, and accumulating significant levels of ergosta-7,22-dienol and episterol (ergosta-7,24(28)-dienol) - indicating that *AfERG3C* does not encode a functional enzyme.

To further confirm and compare the function of each homolog, we conducted several simple phenotypic assays. All except the *AfERG3C* expression construct restored the capacity of the *erg3Δ/Δ* mutant to grow in the presence of high concentrations of calcium (figure 2A). However, the *CgERG3*, *RdERG3A* and *AfERG3C* expressing strains remained sensitive to the detergent SDS, and the *AfERG3A* strain partially sensitive (figure 2A), indicating abnormal membrane function - presumably as a result of C5-desaturase insufficiency. Finally, hyphal growth was compared on M199 and 10% FBS agar plates, conditions under which neither the *erg3Δ/Δ* mutant nor *AfERG3C* expressor formed filaments (figure 2B). All other strains produced filamentous borders at the colony margin, although these were slightly but reproducibly reduced in the *CgERG3* and *AfERG3A* expressing strains, and more noticeably in the *RdERG3A* strain. Collectively, these data indicate that the *C. auris* and *C. neoformans* sterol C5-desaturases as well as the *R. delemar* and *A. fumigatus* Erg3B enzymes are functionally equivalent to the *C. albicans* enzyme. The *C. glabrata*, RdErg3A and AfErg3A enzymes have intermediate levels of activity and therefore incompletely complement the phenotypic defects of the *C. albicans erg3Δ/Δ* mutant, while the *AfERG3C* gene is unlikely to encode a functional C5-desaturase.

Sterol C5-desaturase homologs confer different degrees of azole toxicity upon *Candida albicans*. We next compared the relative sensitivity of each strain to fluconazole using the standard CLSI broth microdilution susceptibility testing method. The minimum inhibitory concentration (MIC) for the wild-type control was 0.25 μg/ml, while the *erg3*Δ/Δ strain was >64 μg/ml at the 24-hour timepoint. Expression of *C. albicans*, *C. glabrata* and *C. auris ERG3* genes fully restored azole sensitivity to the *C. albicans erg3Δ/Δ* mutant, yielding similar MICs (0.25, 0.5 and 0.5 μg/ml respectively) to the wild-type (*ERG3*/*ERG3*) control strain (figure 3A). However, the residual 'trailing' growth observed at supra-MIC concentrations was slightly higher for the *C. glabrata* and *C. auris* expressing strains by the 48-hour time point (figure 3B). Expression of *C. neoformans ERG3* also restored fluconazole sensitivity, but with an MIC (1 μg/ml) 2-4-fold higher than the other strains. In addition, trailing growth observed at the 48-hour time point was substantially higher than the strains expressing the other yeast desaturase enzymes. Expression of *RdERG3B* conferred azole sensitivity upon the *erg3Δ/Δ* mutant to a similar extent as *CaERG3* (figure 3C). However, the *RdERG3A*, *AfERG3A* and *AfERG3C* (all MIC >64 μg/ml) expression constructs failed to restore azole sensitivity to the *erg3Δ/Δ* mutant. This is consistent with *AfERG3C* encoding a non-functional protein, however, the azole resistance of the *RdERG3A* and *AfERG3A* expressors to some extent uncoupled this phenotype from the stress and morphogenesis defects of the *erg3Δ/Δ* mutant. The *AfERG3B* expressing strain had an intermediate fluconazole sensitivity with an MIC of 8 μg/ml, ~16-fold greater than the wild-type and *CaERG3* expressing control at the 24-hour time point. In addition, it yielded much greater levels of trailing growth at the 24-hour time point, such that it appeared insensitive by 48 hours (figure 3D). Time course experiments were conducted in YPD broth at 30°C to provide a more detailed evaluation of each strain's growth kinetics following fluconazole exposure. Our comparison focused upon two main parameters: 1) the

maximum growth rate ($\mathbf{V_{max}}$) attained *after* 8 hours of fluconazole exposure, a time point after which growth inhibition occurred; and 2) the time interval between reaching an OD_{600} of 0.25 and 0.75 ($\mathbf{T_{INT}}$). In the absence of fluconazole, the V_{max} of the *erg3Δ/Δ* was slightly less (~80%) than the wild-type controls, as was the maximum OD_{600} attained at stationary phase, consistent with a fitness consequence of ablating Erg3p function. Expression of all variants except *AfERG3C* at least partially restored V_{max}. The addition of either 1 or 5 μg/ml fluconazole dramatically reduced the V_{max} for all strains (figure 4A), but the reduction was less severe for the *erg3Δ/Δ* mutant (~40-45% of wild-type in absence of fluconazole) than for the wild-type controls (20-25%). Some variation was observed in the V_{max} of the recombinant strains in the presence of fluconazole, with strains expressing the *RdERG3A* and *RdERG3C* isoforms having a similar Vmax to the *erg3Δ/Δ* mutant, suggesting it did not confer any sensitivity to fluconazole. T_{INT} was a more revealing parameter, with 1 and 5 μg/ml fluconazole extending the interval by ~3.5-fold for the two wild-type control strains, but just 1.5-fold for the *erg3Δ/Δ* mutant (figure 4B). Significant variation in the T_{INT} was observed for the recombinant strains in the presence of fluconazole, with the differences especially pronounced at the higher (5 μg/ml) concentration. The interval was longest for the CaErg3p expressing strain indicating it conferred the greatest sensitivity. Of the yeast enzymes, CnErg3p expression conferred the shortest T_{INT}. The RdErg3A and AfErg3C expressing strains were again indistinguishable from the deletion mutant, further indicating they do not contribute to azole sensitivity in *C. albicans*, while RdErg3B expression significantly extended T_{INT}. Interestingly, the increase in T_{INT} upon fluconazole exposure was relatively modest for all three of the *A. fumigatus* desaturase expressing strains.

Finally, we compared the sterol content of each strain in the presence of fluconazole. Ergosterol content was dramatically reduced for all strains expressing a functional desaturase, with levels of

lanosterol, eburicol and 4,14 dimethyl zymosterol increasing. In order to compare the propensity of each C5-desaturase to catalyze the formation of the 'toxic' 14 methyl ergosta-8,24(28)-dien-3-6-diol, the relative diol content was normalized to total C5-desaturase activity observed in the absence of fluconazole (table 2). Based on the levels of diol accumulation we classified the C5-desaturase enzymes into three categories: 1. Those with a high propensity to catalyze the formation of toxic diols in the presence of fluconazole (>5% normalized diol content) – CaErg3p, CaurErg3p, CnErg3p and AfErg3B; 2. Desaturases that catalyze the formation of intermediate levels of diol formation in the presence of fluconazole (>1 but <5% normalized diol content) – CgErg3p, AfErg3B and RdErg3B; and 3. Those which produce minimal amount of diols in the presence of fluconazole (<1% normalized diol content) – notably RdErg3A and the non-functional AfErg3C. A general trend was seen towards higher levels of accumulation in the strains most sensitive to fluconazole, but the correlation was imperfect (figure 5A-D). Interestingly, the correlation between C5-desaturase enzymes activity levels, levels of diol production upon azole exposure and degree of growth inhibition were imperfect. Of the yeast orthologs examined, CnErg3p was notable as its expression produced C5-desaturase sufficiency as determined through analysis of sterol content and full rescue of the stress tolerance and hyphal growth defects of the *C. albicans* null mutant. In addition, it facilitated the production of high levels of diols upon fluconazole exposure. Yet it also elevated fluconazole MIC by 4-fold versus the other yeast enzymes and conferred elevated levels of trailing growth. Paradoxically, while expression of CgErg3p was not sufficient to restore normal stress tolerance, full desaturase activity (as determined through sterol profiles), and the production of reduced diol levels in the presence of fluconazole versus CaErg3p, it did not confer any obvious advantage in the presence of fluconazole. Expression of AfErg3B was also notable for fully restoring C5-desaturase activity, stress tolerance and hyphal growth to the *C. albicans erg3Δ/Δ*

mutant, but without fully reversing the azole tolerance phenotype. The fluconazole MIC was elevated by approximately 8-fold, with substantial trailing growth observed despite production of significant levels of diol upon fluconazole exposure. Despite the imperfect correlation between levels of diol production and the antifungal efficacy of the azoles of C5-desaturase, our data support the notion that differences in Erg3p function are likely to affect the propensity of individual fungal species to produce the 'toxic' diol species upon S14DM inhibition. This in turn may result in different physiological consequences and sensitivity to azole exposure.

Discussion.

The relative sensitivity of infectious fungal species to antifungal drugs is a multifactorial trait that is determined by target-specific characteristics, as well as a variety of other physiological variables. In the case of the azoles, inhibition of fungal growth is thought to be primarily a consequence of two effects: 1). depletion of cellular ergosterol that regulates membrane fluidity; and 2). the accumulation of aberrant biosynthetic intermediates - most notably the Erg3p dependent production of the 'toxic' diol species from the accumulated lanosterol. While it is often stated that *erg3* null mutants of *C. albicans* are azole resistant, it is not clear that the continued growth observed in the presence of the azoles reflects true resistance. Indeed, the *erg3* phenotype shares many of the characteristics of the trailing growth phenotype (32, 33), in that it appears to be condition dependent. For *C. albicans* isolates, trailing growth is most prominent when using the CLSI broth microdilution susceptibility testing protocol (RMPI pH 7, 35°C). These isolates typically appear azole susceptible when observed at the 24-hour time point, but continued growth makes them appear resistant when observed at 48-hours. We recently reported that while an *erg3Δ/Δ* mutant appears insensitive to the azoles under the standard conditions of the CLSI protocol – even at the 24-hour time point - growth inhibition is observed when temperature or

medium pH is adjusted (conditions under which trailing growth is eliminated), revealing MICs in a similar range to wild-type (32). Patients and experimental animals infected with trailing isolates generally respond well to treatment with the azoles (26, 34), suggesting this phenotype does not affect clinical outcomes. However, two recent studies have challenged this assertion by suggesting that the trailing phenotype may be associated with reduced antifungal efficacy in experimental animals and higher rates of recurrence in patients following azole therapy (35, 36). The analysis of growth kinetics described herein confirms that azole exposure actually causes pronounced reductions in the *erg3Δ/Δ* mutants growth rate. As such, *C. albicans erg3* null mutants are not insensitive to the azoles, but are more tolerant than wild-type. Either way, we previously found that the *erg3Δ/Δ* mutant did have an increased capacity to survive azole exposure in mouse models of both vaginal as well as disseminated infection, although this was to a large extent obscured by the virulence defects of the *erg3Δ/Δ* mutant in the disseminated model (21).

Herein we attempted to determine if differences in the activity or substrate specificity of sterol C5-desaturase enzymes from different fungal pathogens may be an important determinant of intrinsic azole susceptibility. Specifically, we compared the propensity of these enzymes to catalyze the formation of the toxic sterol diols upon S14DM inhibition. To facilitate a direct comparative analysis of Erg3p function in the absence of other species-specific variables, each homolog was expressed in a *C. albicans erg3Δ/Δ* mutant. The use of synthetic coding sequences enabled us to adapt for codon usage in *C. albicans* and correct for the effects of different codon usage in the native coding sequences. In the absence of an antibody, we were not able to directly compare expression levels of the recombinant C5-desaturase enzymes. Insertion of an epitope tag at the C-terminus inactivates Erg3p (unpublished results), and there were concerns that epitope insertions at the N-terminus may also alter the normal catalytic function of these enzymes. For these reasons,

319 total sterol C5-desaturase activity in strains expressing each homolog was compared through
320 analysis of cellular sterol content and used to normalize levels of diol measured upon azole
321 exposure. In this way, our data can be viewed as comparing the relative capacity of each homolog
322 to act as a hydroxylase upon the 14α-methyl fecosterol substrate that is formed upon S14DM
323 inhibition. Our results indicate significant variation in the propensity of Erg3p enzymes from each
324 species to produce the toxic diol upon azole exposure. In addition, that C5-desaturase enzymes
325 from different fungal pathogens confer different levels of azole sensitivity when expressed in *C.*
326 *albicans*. In the case of some variants this certainly correlated with a lower intrinsic catalytic
327 efficiency, for example, expression of the RdErg3A and AfErg3A proteins did not restore azole
328 sensitivity of the *C. albicans erg3Δ/Δ* strain but also produced lower levels of C5-desaturase
329 activity than the CaErg3p expressing and wild-type control strains. This was further indicated by
330 only partial restoration of the stress and hyphal growth defects of the deletion mutant. While the
331 reduced catalytic efficiency of these enzymes could translate to reduced levels of diol production
332 in the presence of fluconazole and therefore azole insensitivity, these enzymes also appear to have
333 a low propensity to catalyze the formation of the toxic diol, possibly indicating a difference in
334 substrate specificity. Surprisingly, despite being very closely related (83.9% identical and 94.2%
335 similar), the two *R. delemar* paralogs conferred very different phenotypes upon the *C. albicans*
336 *erg3Δ/Δ* mutant with RdErg3B fully restoring fluconazole-mediated growth inhibition but
337 RdErg3A completely failing to do so. This implies that relatively small differences in protein
338 sequence and structure may have a profound impact on the efficiency with which C5-desaturase
339 enzymes catalyze the formation of the toxic sterol diols.
340 Aside from S14DM binding affinity and expression levels, membrane permeability and drug efflux
341 mechanisms, the inherent capacity of a fungus to tolerate the two aforementioned consequences of

S14DM inhibition and the associated membrane dysfunction determines sensitivity to the azole antifungals. Thus, in addition to a variable predilection to form the toxic diol species, the capacity to endure ergosterol depletion and/or the presence of the sterol diol is likely to vary depending upon several species (and even strain) specific characteristics, including: 1). the dependency of essential membrane proteins upon ergosterol, and their ability to function in the presence of the alternative sterols formed upon S14DM inhibition; 2). thresholds of tolerance of the physiochemical properties of membranes affected by azole exposure, such as fluidity, permeability, topology and organization of subdomains; 3). the precise composition of sterols that accumulate upon S14DM inhibition and their functionality; and 4). the capacity of stress responses to mitigate membrane damage/dysfunction. The variable consequences of S14DM inhibition in different fungal species provides ample evidence that these physiological considerations are species-specific. For example, while azole mediated inhibition of S14DM leads to the death of *A. fumigatus* cells, it only results in growth arrest for *Candida* species (37). Furthermore, despite conferring azole sensitivity when expressed in a *C. albicans erg3Δ/Δ* mutant, loss of Erg3p activity in *A. fumigatus* or *C. glabrata* does not appreciably affect the azole sensitivity of these species (22, 31). This could indicate that while the 14α-methylfecosterol that accumulates in *erg3* null mutants following S14DM inhibition is sufficient to support *C. albicans* growth, it may not in these other species. The *in vivo* consequences of azole mediated S14DM inhibition (and by inference response to therapy) are potentially further complicated by the capacity of some fungal pathogens to acquire exogenous sterols from their mammalian host (38-41).

In conclusion, our data further support the notion that loss of Erg3p activity enhances azole tolerance rather than confers true azole resistance. In addition, variation in the relative sterol

desaturase and hydroxylase activities of this enzyme affects the formation of toxic sterols upon S14DM inhibition and is therefore potentially an important determinant of azole tolerance.

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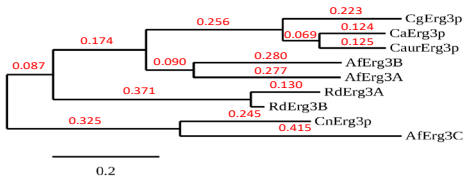

Figure 1. Phylogenetic relationship of sterol C5-desaturase like enzymes from human fungal pathogens. Homologs of *C. albicans* Erg3p were identified through BLAST searches of genome sequence databases of *C. glabrata* (CgErg3p), *C. auris* (CaurErg3p), *C. neoformans* (CnErg3p), *A. fumigatus* (AfErg3A/B/C) and *R. delemar* (RdErg3A/B). The predicted protein products were then aligned and their phylogenetic relationships evaluated using the phylogeny.fr server (http://www.phylogeny.fr/index.cgi).

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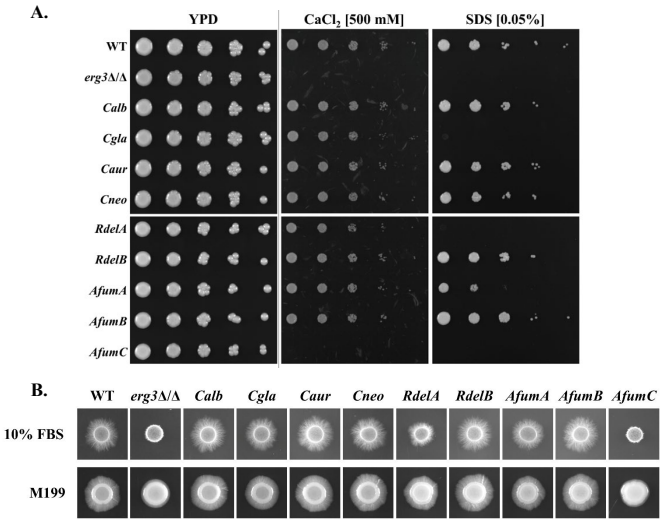

Figure 2. Heterologous expression of C-5 desaturases from different fungal pathogens rescues the stress tolerance and hyphal growth defects of a *C. albicans erg3Δ/Δ* mutant to varying extents. *C. albicans erg3*Δ/Δ strains expressing Erg3p homologs from *Candida albicans* (*Calb*), *C. glabrata* (*Cgla*), *C. auris* (*Caur*), *Cryptococcus neoformans* (*Cneo*), *Rhizopus delemar* (*RdelA* and *RdelB*), or *Aspergillus fumigatus* (*AfumA, AfumB*, and *AfumC*) were suspended at 1 x 10^7 cells/ml in sterile deionized water and serial 1:5 dilutions prepared. The wild-type (WT) *C. albicans* strain GP1, and the *erg3*Δ/Δ mutant harboring the pKE4 expression vector alone were used as controls. (A) Each cell suspension was then applied to YPD agar plates, or YPD agar supplemented with the indicated concentrations of $CaCl_2$ or SDS using a sterile multipronged applicator. Plates were incubated at 30°C for 48 hours and then imaged. (B) Each strain was suspended at 1 x 10^7 cells/ml in sterile deionized water and 2.5 µl spotted onto either M199 or 10% FBS agar plates. The resulting colonies were imaged after 96 hours incubation at 37°C.

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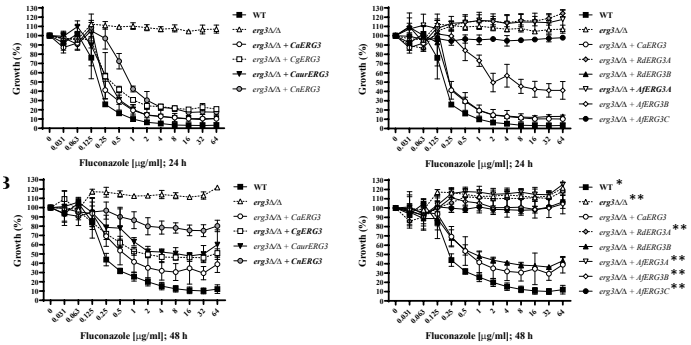

Figure 3. C-5 sterol desaturase homologs from different fungal pathogens confer varying degrees of fluconazole sensitivity upon a *Candida albicans erg3Δ/Δ* mutant. The fluconazole susceptibility of *C. albicans erg3Δ/Δ* strains expressing Erg3p homologs from infectious yeast species (A and B) or infectious molds (C and D) was evaluated using the CLSI broth microdilution protocol. The wild-type (WT) *C. albicans* strain GP1, and the *erg3*Δ/Δ mutant harboring the pKE4 expression vector alone were used as controls. The *CaERG3* expressing strain is also included in all panels as an additional reference. Growth was measured as OD_{600nm} after 24 (A and C) and 48 (B and D) hours incubation at 35°C and expressed as a percentage of the growth in the minus drug (DMSO alone) control wells for each strain. Data in all panels are the mean and standard deviation of four biological replicates. To compare levels of trailing growth of each strain, % growth at 64 µg/ml fluconazole was compared to the *erg3*Δ/Δ + *CaERG3* strain using the two-tailed t-test. * $p < 0.01$; ** $p < 0.001$.

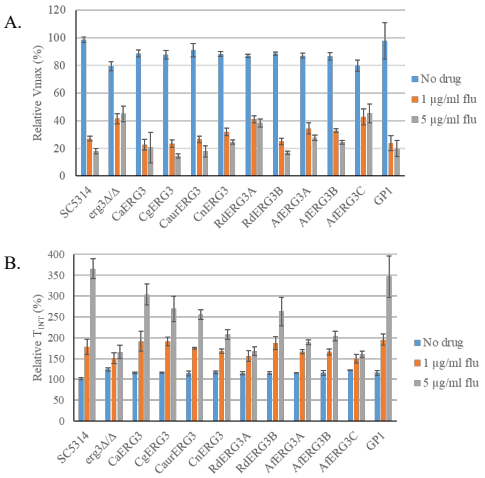

Figure 4. C-5 sterol desaturase homologs from different fungal pathogens alter the capacity of *Candida albicans* to grow in the presence of fluconazole. *C. albicans erg3Δ/Δ* strains expressing the indicated Erg3p homologs were grown in YPD broth supplemented with 1 or 5 µg/ml fluconazole, or with DMSO vehicle alone (no drug control), and growth monitored as OD_{600nm} at 30-minute intervals. The wild-type *C. albicans* strains SC5314 and GP1, and the *erg3*Δ/Δ mutant harboring the pKE4 expression vector alone were used as controls. The maximum growth rate achieved after the 8-hour time point (V_{max} – panel A) and the time interval between reaching an OD of 0.25 and 0.75 (T_{INT} – panel B) were calculated and expressed as a percentage of the same parameters for the SC5314 control strain grown in the absence of fluconazole. Data in all panels are the mean and standard deviation of three biological replicates.

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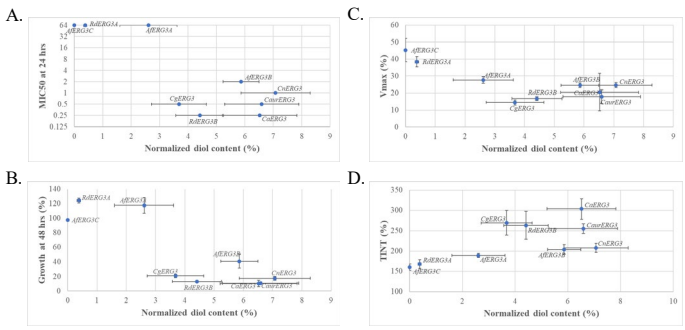

Figure 5. Erg3p homologs that catalyze high levels of diol production upon LDM inhibition are associated with more severe growth inhibition. The normalized diol content for *C. albicans* strains expressing the indicated Erg3p isoforms grown in the presence of fluconazole (as reported in table 2) was plotted against (A) the MIC_{50} (minimum inhibitory concentration causing 50% growth inhibition) of fluconazole at the 24 hour time point (see figure 3A and C); (B) the relative growth of each strain measured at the 48 hour time point in the presence of 64 µg/ml fluconazole (see figure 3B and D); (C) the post 8-hour V_{max} when grown in the presence of 5 µg/ml fluconazole – expressed relative to wild-type in the absence of fluconazole (see figure 4A); and (D) the T_{INT} parameter expressed as a percentage of wild-type in the absence of fluconazole (see figure 4B). The mean of each parameter is plotted with the error bars indicating standard deviations.

Table 1. Sterol profile of untreated *Candida albicans* strains. *Sterols that have not been identified (the mass ion and/or fragmentation pattern were unclear). The content of sterols indicative of Erg3p activity is shown in bold text. ND: not-detected.

	erg3Δ/Δ		*Δ/Δ + CaERG3*		*Δ/Δ + CgERG3*		*Δ/Δ + CaurERG3*		*Δ/Δ + CnERG3*		*Δ/Δ + AfERG3A*		*Δ/Δ + AfERG3B*		*Δ/Δ + AfERG3C*		*Δ/Δ + RdERG3A*		*Δ/Δ + RdERG3B*	
	mean	±SD	**mean**	±SD	**mean**	±SD	**mean**	±SD	**mean**	±SD	**mean**	±SD	**mean**	±SD	**mean**	±SD	**mean**	±SD	**mean**	±SD
Ergosta-5,8,22,24(28)-tetraenol			**1.1**	0.4	**0.6**	0.1	**0.8**	0.1	**1.3**	0.2	**0.8**	0.1	**0.5**	0.1			**0.5**	0.0	**1.0**	0.1
Ergosta-5,8,22-trienol			**0.7**	0.2	**0.2**	0.0	**0.4**	0.0	**2.1**	0.1	**0.2**	0.1	**0.7**	0.1			**0.1**	0.0	**0.6**	0.1
Zymosterol	**0.8**	0.2	**2.1**	0.6	**1.8**	0.5	**1.6**	0.3	**1.6**	0.6	**1.8**	0.3	**1.2**	0.4	**1.4**	0.3	**2.1**	0.2	**1.5**	0.8
Ergosta-8,22-dienol	**5.2**	0.8													**0.1**	0.0				
Ergosterol			**87.8**	2.2	**82.5**	0.7	**87.1**	1.1	**87.1**	1.3	**87.5**	1.3	**89.5**	2.2			**60.3**	1.4	**88.4**	1.6
Ergosta-8,22,24(28)-trienol	**1.5**	0.2													**4.6**	0.5				
Ergosta-7,22-dienol	**62.9**	3.8	**0.5**	0.1	**4.3**	0.5	**0.5**	0.2	**0.8**	0.3	**1.5**	0.2	**0.6**	0.2	**62.8**	1.7	**17.4**	0.5		
Ergosta-5,7,22,24(28)-tetraenol											**0.3**	0.1	**0.6**	0.2						
Fecosterol (Ergosta-8,24(28)-dienol)	**5.4**	0.5	**0.3**	0.2	**0.6**	0.1	**0.4**	0.2	**0.3**	0.2	**0.4**	0.0	**0.4**	0.4	**6.0**	0.1	**2.0**	0.6	**0.5**	0.2
Ergosta-8-enol	**2.1**	0.1			**0.2**	0.0			**0.5**	0.1					**2.4**	0.3	**0.8**	0.3	**0.4**	0.1
Ergosta 5,7,24(28) trienol			**1.0**	0.6	**0.4**	0.2	**0.7**	0.1			**0.8**	0.3	**0.5**	0.3			**0.4**	0.1	**1.0**	0.5
Ergosta-5,7-dienol			**1.9**	0.6	**1.5**	0.1	**2.7**	0.3	**1.4**	0.2	**1.2**	0.1	**1.5**	0.2			**1.6**	0.3	**2.2**	0.2
Episterol (Ergosta-7,24(28)-dienol)	**16.7**	2.9	**0.6**	0.2	**3.5**	0.4	**0.9**	0.2	**1.0**	0.2	**1.6**	0.3	**1.2**	0.3	**16.9**	1.0	**10.5**	0.7	**0.8**	0.2
Ergosta-7-enol	**2.9**	0.2			**0.3**	0.1			**0.0**	0.0	**0.2**	0.1	**0.1**	0.1	**3.4**	0.1	**1.2**	0.1	**0.0**	0.0
Lanosterol	**0.7**	0.2	**2.5**	0.4	**2.5**	0.4	**2.9**	0.3	**2.2**	0.3	**2.2**	0.4	**1.8**	0.5	**0.7**	0.2	**1.7**	0.2	**2.2**	0.2
4 methyl ergosta-8,24(28)-dienol	**1.6**	0.1	**1.3**	0.1	**1.5**	0.2	**1.8**	0.3	**1.5**	0.2	**1.4**	0.2	**1.4**	0.2	**1.5**	0.1	**1.4**	0.2	**1.4**	0.1
Eburicol	**0.2**	0.0	**0.1**	0.0	**0.1**	0.0	**0.1**	0.0	**0.2**	0.1	**0.1**	0.0	**0.1**	0.0	**0.2**	0.0	**0.1**	0.0	**0.1**	0.0
Total	**100.0**		**100.0**		**100.0**		**100.0**		**100.0**		**100.0**		**100.0**		**100.0**		**100.0**		**100.0**	
Total C5 desaturated sterols	**0.0**		**92.5**		**85.3**		**91.8**		**91.9**		**90.8**		**93.3**		**0.0**		**62.9**		**93.1**	