Sphingolipid Biosynthetic Pathway Genes FEN1 and SUR4 Modulate Amphotericin B Resistance

Running Title: FEN1 and SUR4 Modulate Amphotericin B Resistance

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Deletants of sphingolipid biosynthetic pathway genes FEN1 and SUR4 of Saccharomyces cerevisiae, as well as deletants of their orthologs in Candida albicans, were found to be 2 to 5-fold more sensitive to AmB than parent strains. Inhibition of sphingolipid biosynthesis in parent strains by myriocin sensitized them to AmB, which could be reversed by providing phytosphingosine, an intermediate in sphingolipid pathway. These results indicate that sphingolipids modulate AmB resistance, with implications for mechanisms underlying AmB action and resistance.

Candidiasis is the fourth most common hospital acquired infection, which is associated with high mortality in case of invasive infections (1-5). C. albicans is the main causative agent, but other Candida species like C. glabrata, C. parapsilosis, C. tropicalis, C. krusei and C. lusitaniae are becoming more prevalent (2, 6, 7). Rapid emergence of resistant strains against major antifungal drugs often renders therapy ineffective (8-10). Amphotericin B (AmB) is a commonly used fungicidal polyene drug, and resistance against it is reported mainly in some species of Candida (11). However, severe side effects are associated with high therapeutic doses of AmB (12). Thus, there is an urgent need for AmB analogs that have similar or higher potency than AmB, but with minimal side effects. Another approach is to use AmB in combination with drugs that sensitize fungal cells to AmB, or potentiate AmB action, such that less AmB is needed for effective therapy, thereby reducing AmB toxicity.

The mechanism underlying the fungicidal action of AmB is becoming better understood; a recent report (13) showed that binding of AmB to ergosterol as such is sufficient to kill the cells, and leakage of ions due to pore formation is a secondary effect of AmB. Absence of ergosterol in ergosterol biosynthesis mutants results in AmB resistance (11, 14-18). Sterols
(ergosterol in fungi and cholesterol in higher eukaryotes) and sphingolipids form distinct domains (lipid rafts) in plasma membrane and are required for several cellular processes including maintenance of plasma membrane integrity, protein sorting, endocytosis and proper functioning of certain membrane proteins (19-24) Altered composition or loss of these membrane constituents affects targeting of ATP-binding cassette transporter Cdr1p to lipid rafts and susceptibility of C. albicans and S. cerevisiae to drugs that are substrates of drug efflux pumps (25, 26). Since ergosterol interacts physically as well as functionally with sphingolipids, and biosynthesis of sphingolipids is closely coordinated with that of sterols (27-29), we hypothesized that sphingolipids, like ergosterol, might be involved in modulation of AmB resistance. To test this, first we evaluated AmB resistance of S. cerevisiae deletants impaired in sphingolipid biosynthesis. We also tested the effect of myriocin (a sphingolipid biosynthesis inhibitor), and myriocin in combination with phytosphingosine (an intermediate in sphingolipid pathway) on the AmB resistance of wild-type strains of S. cerevisiae and Candida species.

Wild-type strains of C. albicans, C. glabrata, C. lusitaniae and S. cerevisiae and deletion mutants of S. cerevisiae strain BY4743 and C. albicans strain SN95 were used in this study (Table 1). The yeast strains were regularly revived from frozen glycerol stocks on YPD agar medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose and 2% Bacto agar). For exponential cultures strains were grown in YPD broth at 30°C with agitation (200 rpm).

Synthetic complete (SC) agar medium plates contain 0.17% yeast nitrogen base w/o amino acids and ammonium sulphate (Difco), 0.5% (wt/vol) ammonium sulphate, 2% glucose, 1.92 gm/litre yeast synthetic dropout medium supplement without uracil (Sigma), 76 mg/litre uracil (Sigma) and 2% Bacto agar. RPMI-1640 medium with L-glutamine without sodium...
bicarbonate (Sigma) was buffered with 0.165 M morpholinepropanesulfonic acid (Sigma) to pH of 7.0 (30). Stock solutions of AmB (2 mg/ml; Sigma) myriocin (2 mg/ml; Sigma) and phytosphingosine (10 mM; TCI chemicals) were prepared in dimethyl sulfoxide (Sigma), and stored at -20°C until use.

To test drug susceptibility by dilution spotting, the strains were grown overnight in YPD broth, re-inoculated in fresh YPD medium at a starting OD$_{600nm}$ of 0.1 and incubated for 4 to 5 hrs at 30°C with shaking at 200 rpm until OD$_{600nm}$ of 0.6-0.8 was reached. The cells were harvested, washed with water and normalized to OD$_{600nm}$ of 1.0 (2x10$^7$ cells/ml). These cells were ten-fold serially diluted and 5 µl of each dilution was spotted on SC agar plates containing different concentrations of AmB. Growth was assessed after incubating the plates at 30°C for 2 days. We screened homozygous deletants of all non-essential genes in the sphingolipid pathway of S. cerevisiae, which were constructed as part of the yeast deletion project (31). Strains deleted in SUR4 and FEN1 were found to be hypersensitive to AmB compared to parent strain (BY4743), by 5-fold and 2-fold, respectively (Fig. 1). These strains were confirmed as true deletants for these genes by diagnostic PCR (Fig. S1 and S2).

FEN1 and SUR4 are non-essential genes involved in the synthesis of very long chain fatty acids. Other genes involved in elongation of fatty acids are YBR159w and ELO1, which are nonessential, and PHS1 and TSC13, which are essential genes (32, 33). Strains deleted in YBR159w and ELO1 were not sensitive to AmB (results not shown). It has been reported that sphingolipid and ergosterol biosynthetic pathways interact genetically (27). Thus, to check if perturbation in sphingolipid levels perhaps leads to increased ergosterol level resulting in hypersensitivity to AmB in fen1 and sur4 deletants, total ergosterol content was determined in these strains (34). The ergosterol content of parent strain and fen1 deletant was
0.026±0.001% and 0.031±0.005% wet weight of cells, respectively, and the difference is not statistically significant (p-value 0.0629). On the other hand, the ergosterol content of \textit{sur4} deletant was 0.021±0.001%, and the difference with respect to parent strain is statistically significant (p-value 0.003). If ergosterol is the key determinant of AmB resistance, then its slight decrease in \textit{sur4} deletant would have increased AmB resistance. On the contrary, AmB resistance is 5-fold less than that of wild-type, and thus it is unlikely that ergosterol level modulates this phenotype in this deletant.

To further validate the role of \textit{ScFEN1} and \textit{ScSUR4} in AmB resistance, we have deleted their orthologs, \textit{Caorf19.6343} (\textit{CaFEN1}) and \textit{Caorf19.908} (\textit{CaFEN12}), respectively, in \textit{C. albicans}. Homozygous deletion strains of these genes as well as a strain deleted in both the genes were constructed, starting from \textit{C. albicans} strain SN95 (Table 1). We have used \textit{HAH2} gene deletion cassette (Fig. S3; GenBank accession number KF318042) constructed in our lab for deletion of both alleles of a gene in \textit{C. albicans} after only a single transformation. This cassette is similar to \textit{UAU1} cassette (35), except that instead of \textit{URA3}, \textit{HIS1} marker is used, since virulence phenotypes of deletants are not reliable when \textit{URA3} is used as a marker (36-38). On the other hand, \textit{C. albicans} strains that are auxotrophic for histidine (or arginine) are comparable to prototrophic strains with respect to their virulence (39), and thus are not expected to confound virulence studies when used as selection markers. The \textit{HAH2} cassette has intact \textit{ARG4} gene flanked with partial and non-functional 5’ and 3’ regions of \textit{HIS1} gene. These regions can recombine by virtue of shared direct repeats to yield functional \textit{HIS1} gene. The cassette is flanked with variant \textit{Lox} sites, \textit{LoxLE} on the left and \textit{LoxRE} on the right. These can recombine upon expression of Cre recombinase, yielding a mutant \textit{Lox} site that will not participate in any further recombination (40). These were
introduced to evict the entire cassette after desired gene deletion to recover the auxotrophic markers for reuse in additional deletion experiments.

To delete \textit{CaFEN1} ORF (including the region from -130 to +25 bp of ORF), 254 bp upstream flanking homology region, corresponding to -384 to -131 bp region upstream of \textit{CaFEN1} start codon, was PCR amplified using \textit{CaFEN1-US1} and \textit{CaFEN1-UA1} primers (Table S1). Similarly, downstream \textit{CaFEN1} flanking homology region (443 bp), corresponding to +26 to +468 bp after \textit{CaFEN1} stop codon was PCR amplified with \textit{CaFEN1-DS2} and \textit{CaFEN1-DA2} primers. Both upstream and downstream \textit{CaFEN1} flanks have 18 bp and 24 bp sequences, respectively, homologous to \textit{HAH2} cassette, introduced as part of the primers (Table S1).

The upstream flanking region was fused with \textit{HAH2} cassette by PCR with amplified upstream flanking DNA and \textit{HAH2} as templates and \textit{CaFEN1-US1} forward primer and cassette specific reverse primer \textit{CaARG4-R1130}, to provide upstream split marker. Similarly, downstream \textit{CaFEN1} flanking region was fused with \textit{HAH2} cassette by PCR with amplified downstream flanking DNA and \textit{HAH2} as templates and cassette specific forward primer \textit{CaARG4-F61} and \textit{CaFEN1-DA2} reverse primer, to provide downstream split marker. The PCR amplified upstream and downstream split markers were mixed together and transformed into \textit{C. albicans} strain SN95 (\textit{arg4\Delta/arg4\Delta his1\Delta/his1\Delta}). These would recombine \textit{in vivo} in the 1070 bp region shared between them, and at the target locus by means of flanking homology regions. After selecting for arginine prototrophy, correct integration was confirmed by diagnostic PCRs for expected PCR product on either side of integration. For upstream region, diagnostic PCR with cassette specific primer \textit{CaHIS1-DR} and gene-specific primer \textit{CaFEN1-DG-S}, external (-429 to -411 of ORF) to the upstream flanking homology region (-384 to -131 of ORF) introduced during transformation, yielded a PCR product comparable to the
expected 383 bp product. For downstream region, PCR with cassette specific forward primer CaHIS1-ter and gene-specific reverse primer CaFEN1-DG-R1, external (+520 to +540) to the downstream flanking homology region (+26 to +468 bp of ORF) introduced during transformation, gave a PCR product comparable to the expected 626 bp product (results not shown). Deletion of CaFEN12 was carried out as described for CaFEN1, but using CaFEN12 specific primers (Table S1).

After confirming targeted deletion of one allele each of CaFEN1 or CaFEN12, the heterozygous strains were grown non-selectively in YPD broth, and plated on minimal plates (without arginine or histidine) to select for Arg⁺ His⁺ prototrophs. Arg⁺ His⁺ segregants arise when both alleles become deleted after spontaneous mitotic recombination (or gene conversion), followed by recombination between direct repeats within 5’ and 3’ regions of his1 in one of the alleles. Among the segregants, some will have the wild-type allele of the target gene as well, which are identified by diagnostic PCR and discarded. Routinely, at least two correct segregants derived from two independent transformants are taken for further studies.

To evict the markers (after deleting the first gene), Cre recombinase, codon-optimised for expression in C. albicans was used (41). For this purpose, MAL2-Cre cassette (Fig. S4; GenBank accession number KF318043) for expression of Cre under the control of CaMAL2 promoter with SAT1 as selection marker was constructed starting from a cassette with CaMET3 promoter and URA3 marker (41). Upon transformation and selection for nourseothricin resistance, MAL2-Cre cassette would integrate within the ARG4 of HAH2 present in one of the disrupted alleles, by virtue of flanking homology to ARG4. The Cre recombinase, upon induction with maltose, would recombine LoxLE and LoxRE sites flanking
the markers. The resulting clones that were auxotrophic to histidine and arginine and sensitive to nourseothricin were tested for true deletion of CaFEN1 or CaFEN12 gene by diagnostic PCR (Fig. 2). CaFEN12 was deleted in a Cafen1Δ/Δ strain, as described above, to yield double delete strains (Cafen1Δ/Δ Cafen12Δ/Δ). The selection markers were also evicted from this strain so as to make the auxotrophies comparable in all the strains. The deleted CaFEN1 and CaFEN12 regions were PCR amplified and sequenced to further confirm the deletions and proper eviction of markers after recombination at Lox sites (Fig. S5 and S6).

The sensitivity of Candida deletants to AmB was checked by dilution spotting. The Cafen1Δ/Δ and Cafen12Δ/Δ deletants were 2-fold and 5-fold more sensitive than the parent strain (Fig. 1), similar to the sensitivity of deletants of S. cerevisiae orthologs. We could construct a C. albicans strain deleted in both these genes as well, though double deletion of ScFEN1 and ScSUR4 is synthetic lethal in S. cerevisiae (42). The Cafen1Δ/Δ Cafen12Δ/Δ double deletant was slow growing and at least 10-fold more sensitive than the parent strain SN95 to AmB (Fig. 1), confirming the role of these genes in modulating AmB resistance.

Since deletion of only two genes in the sphingolipid biosynthetic pathway sensitized cells to AmB, we tested the effect of chemical inhibition of sphingolipid biosynthesis in wild-type strains on AmB resistance. Myriocin is a specific inhibitor of serine palmitoyltransferase encoded by the essential genes LCB1 and LCB2, which catalyze the first committed step of sphingolipid biosynthesis (43, 44). We incubated C. albicans (SC5314), C. glabrata (CG462), C. lusitaniae (CL1 and CL6) and S. cerevisiae (FY4 and BY4743) strains at different concentrations of AmB and myriocin, alone and in combination. Myriocin, at sub-lethal concentration (0.4 μg/ml) rendered the cells sensitive to sub-lethal concentration of AmB.
(0.1 μg/ml for *C. albicans*, *S. cerevisiae* and *C. lusitaniae* strain CL1, and 0.25 μg/ml for *C. glabrata* and *C. lusitaniae* strain CL6), indicating that inhibition of sphingolipid biosynthesis sensitizes cells to AmB (Fig. 3). To quantitate this, checkerboard broth microdilution assay using CLSI standard reference method (30) was used. Myriocin sensitized cells to AmB by 4 to 8-fold, and was synergistic with AmB for all the tested strains with FIC index of less than 0.5 (Table 2). However, the synergistic effect of myriocin remains to be verified with other pathogenic *Candida* species. To confirm if AmB sensitivity is due to depletion of sphingolipids, we supplemented AmB-myriocin plates with sub-lethal concentration (10 μM) of phytosphingosine (PHS). PHS is an intermediate downstream of serine palmitoyltransferase in sphingolipids biosynthesis, and it is known to rescue myriocin inhibition of sphingolipid biosynthesis (45). We found that PHS reversed AmB sensitivity of myriocin treated cells, confirming the role of sphingolipids in AmB resistance (Fig. 3).

To the best of our knowledge, this is the first study demonstrating the role of sphingolipid pathway genes in modulating AmB resistance of *S. cerevisiae* and *Candida* species. *FEN1* (also known as *ELO2*, *GNS1* and *VBM2*) and *SUR4* (also known as *ELO3*, *SRE1*, *VBM1* and *APA1*) encode fatty acid elongases. These enzymes are part of very long-chain fatty acid synthetase complex, which are involved in the synthesis of C22 and C24 (Fen1p) or C26 (Sur4p) fatty acids (46, 47). *FEN1* and *SUR4* deletants show several pleiotropic phenotypes, including defects in secretory pathway, lipid rafts and vacuolar ATPase activities (48-51). Detailed comparative lipidomics has shown that deletion of *FEN1* or *SUR4* perturbed the entire yeast lipidome (52). Such changes, thought to be due to unrelated compensatory “ripple effects” (52), may be responsible for the pleiotropic phenotypes seen with the deletion of individual genes. *S. cerevisiae sur4* and *fen1* deletants overexpressing *C.
albicans drug efflux pump Cdr1p were found to be sensitive to azole and other drugs, which are substrates of Cdr1p (26). This sensitivity was attributed to mislocalization and improper functioning of Cdr1p due to membrane lipid imbalance. This is unlikely to explain sensitivity of these deletants to AmB since it is not a substrate of drug efflux pumps. We have checked if AmB sensitivity of these deletants is modulated by any compensatory changes in ergosterol levels, but did not find any correlation with ergosterol content. However, since ergosterol is preferentially associated with sphingolipids in lipid rafts (53, 54), depletion of sphingolipids may render ergosterol more accessible to AmB binding thereby sensitizing cells to AmB. While this appears to be a straightforward possibility, depletion of sphingolipids might also affect AmB resistance indirectly; since ergosterol and sphingolipids are critical for several cellular functions, impairment of one or more of these functions may sensitize cells to AmB. Experiments are underway to unravel the mechanism(s) through which Fen1p/Sur4p and sphingolipids modulate AmB resistance.

Recently, Healey et al (55) reported that strains deleted in FEN1 and SUR4 show reduced susceptibility to caspofungin, but increased susceptibility to micafungin. This is rather paradoxical since both these drugs are echinocandins, and the possible mechanism for differential susceptibility could only be speculated upon (55). Myriocin treatment sensitized cells to both caspofungin and micafungin (55), confirming the importance of sphingolipids in modulating antifungal resistance. Myriocin is an immunosuppressor and quite toxic to humans and thus cannot be used in combination with AmB or echinocandins. Nevertheless, non-toxic analogs of myriocin, if developed, can be used to sensitize pathogenic fungi to these antifungals, thereby enhancing their therapeutic efficacy.
Acknowledgments

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References


Figure legends

Fig. 1. Amphotericin B susceptibility of *S. cerevisiae* (BY4743) and *C. albicans* (SN95) strains deleted in *FEN1* and *SUR4*. All strains are diploids and in deletants both alleles of indicated genes are deleted. *CaFEN1* is ortholog of *ScSUR4*. Serial 10-fold dilutions of cells were spotted on synthetic complete agar plates with indicated concentration of AmB, and incubated at 30°C for two days before being photographed. Comparable results were obtained with *Scfen1* and *Scsur4* deletants in *S. cerevisiae* haploid strain (BY4741) background, and in *Cafen1Δ/Δ, Cafen12Δ/Δ* and double deletant strains independently constructed in *C. albicans* strain SN95.

Fig. 2. Diagnostic PCRs for *CaFEN1* (A) and *CaFEN12* (B) in parent strain (SN95) and in strains deleted in either or both of the genes. Upstream forward and downstream reverse primers, external to flanking regions of homology used for targeted gene deletion, were used for diagnostic PCR. For *CaFEN1* diagnostic PCRs, *CaFEN1-DG-S* and *CaFEN1-DG-R1* were used, and the expected size of amplified products from SN95, *Cafen1Δ/Δ* and *Cafen12Δ/Δ* strains are 1982 bp, 880 bp and 880 bp respectively. For *CaFEN12* diagnostic PCRs, *CaFEN12-DG-S* and *CaFEN12-DG-R1* were used, and the expected size of products from SN95, *Cafen12Δ/Δ* and *Cafen1Δ/Δ* *Cafen12Δ/Δ* strains are 1821 bp, 909 bp and 909 bp respectively.

Fig. 3. Sensitization of wild-type cells to amphotericin B by myriocin and its reversal by phytosphingosine (PHS). *C. albicans* (SC5314), *C. glabrata* (CG462), *C. lusitaniae* (CL1, CL6),
and *S. cerevisiae* (FY4, BY4743) strains were tested for their sensitivity to AmB alone (top panels) or in the presence of myriocin (middle panels) or in the presence of myriocin and PHS (lower panels). Comparable results were obtained from three independent experiments.
### TABLE 1. Strains used in this study

<table>
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<tr>
<th>Strain</th>
<th>Description / genotype</th>
<th>Reference / Source</th>
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<tr>
<td><em>S. cerevisiae</em> FY4</td>
<td>MATa</td>
<td>Winston et al, 1995 (56)</td>
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<td><em>S. cerevisiae</em> BY4743</td>
<td>Mat a/a; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/lys2Δ0; MET15/met15Δ0;ura3Δ0/ura3Δ0</td>
<td>Euroscarf</td>
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<td><em>C. glabrata</em> CG462</td>
<td>BG2, Wild-type clinical isolate</td>
<td>Cormack &amp; Falkow, 1999 (57)</td>
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<td>J. Heitman (58)</td>
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<tr>
<td><em>C. lusitaniae</em> CL6</td>
<td>MATa clinical isolate</td>
<td>J. Heitman (58)</td>
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<td><em>C. albicans</em> SC5314</td>
<td>Wild-type clinical isolate</td>
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<td><em>C. albicans</em> SN95</td>
<td>arg4Δ/arg4Δ his1Δ/his1Δ URA3/ura3::imm^{434} IRO1/iro1::imm^{434}</td>
<td>Noble &amp; Johnson, 2005 (39)</td>
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<td>SN95F12</td>
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### TABLE 2. Synergy testing of amphotericin B and myriocin

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<tr>
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<td>0.125</td>
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<tr>
<td>C. lusitaniae</td>
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<td>0.063</td>
<td>0.313</td>
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<td>(CL6)</td>
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<tr>
<td>S. cerevisiae</td>
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MIC is defined as the concentration at which more than 95% of cells are killed. The synergy testing was done thrice with comparable results.