Clinically significant micafungin resistance in *Candida albicans* involves modification of a glucan synthase catalytic subunit GSC1 (*FKS1*) allele followed by loss of heterozygosity


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Received 20 December 2009; returned 27 January 2010; revised 15 February 2010; accepted 16 February 2010

**Objectives:** To determine the mechanism of intermediate- and high-level echinocandin resistance, resulting from heterozygous and homozygous mutations in GSC1 (*FKS1*), in both laboratory-generated and clinical isolates of *Candida albicans*.

**Methods:** The DNA sequences of the entire open reading frames of GSC1, GSL1 (*FKS3*) and RHO1, which may contribute to the β-1,3-glucan synthase of a micafungin-susceptible strain and a resistant clinical isolate, were compared. A spontaneous heterozygous mutant isolated by selection for micafungin resistance, and a panel of laboratory-generated homozygous and heterozygous mutants that possessed combinations of the echinocandin-susceptible and -resistant alleles, or mutants with individual GSC1 alleles deleted, were used to compare levels of echinocandin resistance and inhibition of glucan synthase activity.

**Results:** DNA sequence analysis identified a mutation, S645P, in both alleles of GSC1 from the clinical isolate. GSL1 had two homozygous amino acid changes and five non-synonymous nucleotide polymorphisms due to allelic variation. The predicted amino acid sequence of Rho1p was conserved between strains. Reconstruction of the heterozygous (S645/S645F) and homozygous (S645F/S645F) mutation showed that the homozygous mutation conferred a higher level of micafungin resistance (4 mg/L) than the heterozygous mutation (1 mg/L). Exposure of the heterozygous mutant to micafungin resulted in a loss of heterozygosity. Kinetic analysis of β-1,3-glucan synthase activity showed that the homozygous and heterozygous mutations gave echinocandin susceptibility profiles that correlated with their MIC values.

**Conclusions:** A homozygous hot-spot mutation in GSC1, caused by mutation in one allele and then loss of heterozygosity, is required for high-level echinocandin resistance in *C. albicans*. Both alleles of GSC1 contribute equally and independently to β-1,3-glucan synthase activity.

**Keywords:** *C. albicans*, drug resistance, echinocandins
Introduction

The echinocandin class of antifungal agents non-competitively inhibits β-1,3-glucan synthase (GS) activity and synthesis of β-1,3-glucan, the major and essential component of the fungal cell wall.1 The echinocandins have broad-spectrum antifungal activity against Candida and Aspergillus species,2 including azole-resistant clinical isolates of Candida spp.3–5 Although some Candida species, such as Candida parapsilosis or Candida guilliermondii, are less susceptible to echinocandins,6,7 three common fungal pathogens (Candida albicans, Candida glabrata and Candida tropicalis) are susceptible.8 The β-1,3-GS is a protein complex that consists of at least two components: a catalytic subunit and a regulatory subunit.8,9 Three C. albicans genes, GSC1 (Glucan Synthase Catalytic Subunit 1; orf19.2929; 5694 bp, also known as FKS1), GSL1 (Glucan Synthase Like 1; orf19.2495; 4717 bp, also known as FKS3) and GSL2 (orf19.3269; 4924 bp, orf19.2495; 4717 bp, also known as FKS2), have significant sequence homology with Saccharomyces cerevisiae FKS genes.10 GSC1, an orthologue of S. cerevisiae FKS1, has been previously described as FKS1,11 GSL212 or GSL2.13 Although FKS1 has been commonly used for GSC1 in this paper we use GSC1 in accordance with the nomenclature of the Candida Genome Database (http://www.candidagenome.org/). C. albicans GSL1 has similarity to S. cerevisiae FKS3 (YMR306W), which plays an important role in assembling spore walls in S. cerevisiae and during normal growth conditions expression of GSL1 was lower than that of GSC1.10,15 In addition, GSL2 expression was barely detectable.10 C. albicans GS is thought to include the regulatory subunit Rho1p (orf19.2843),16 a GTP-binding orthologue of S. cerevisiae Rho1p that plays a central role in cell wall biosynthesis, morphogenetic control and osmotic integrity.17

There is a low incidence of echinocandin resistance in clinical isolates of Candida species that are normally susceptible to echinocandins, and in vitro selection of echinocandin-resistant variants of C. albicans11,18 or S. cerevisiae19 have been reported. There are a small number of reports of resistance-related treatment failures in patients with Candida oesophagitis or prosthetic valve endocarditis, with resistant clinical isolates having MICs of ≥100-fold higher than susceptible strains.20–24

Studies on caspofungin-resistant clinical isolates or laboratory strains of C. albicans revealed that single amino acid changes in the echinocandin resistance regions or ‘hot spots’ of the GS catalytic subunit GSC1 are associated with the resistance,11,15,18,25–27 although often only the ‘hot spots’ are sequenced and other mutations elsewhere in the gene may be present. The echinocandin resistance regions are in well-conserved regions of genes encoding the GS catalytic subunit of major fungal pathogens and the model yeast S. cerevisiae.28

Mutation in the echinocandin resistance regions appears to confer echinocandin resistance on fungal species, including C. albicans, Aspergillus fumigatus,9 C. glabrata30 and C. krusei.27 In C. albicans GSC1 there are two echinocandin resistance regions: hot spot 1 (amino acids 641–649); and hot spot 2 (amino acids 1357–1364). Most mutations have been detected in hot spot 1.21,22,24–27,31 Although hot-spot mutations in GSC1 have been implicated in echinocandin resistance in C. albicans, other mechanisms cannot be excluded.

Consideration of the putative functions and reported gene expression levels of the catalytic and regulatory subunits of GS led us to determine DNA sequences for GSC1, GSL1 and RH01 from a micafungin-resistant clinical isolate and a susceptible reference strain of C. albicans. Although previous studies have used focused sequence analysis to identify hot-spot mutations in GSC1, which are the main cause of echinocandin resistance in C. albicans, complete sequencing of the open reading frames (ORFs) for those three genes was used to ensure that no other mutations contributed to the phenotype.

A homozygous hot-spot mutation, S645P, was identified in the GSC1 ORF from the resistant clinical isolate. As a micafungin-susceptible isogenic parental strain of the clinical isolate was not available, we investigated the mechanisms of micafungin resistance in C. albicans by selecting a resistant variant from a susceptible laboratory strain and constructing a panel of isogenic laboratory mutants that possessed a hot-spot mutation in one or both alleles of the GSC1. The echinocandin susceptibility and the kinetics of GS activity of the mutants were then compared.

Materials and methods

Strains and culture conditions

The micafungin-resistant C. albicans clinical isolate, wild-type susceptible strain and laboratory mutants used in this study are listed in Table 1. The resistant C. albicans strain 23002 was isolated from a blood sample of a 25-year-old Japanese male with candidaemia after treatment with micafungin. The patient had myelodysplastic syndrome and underwent bone marrow transplants from an unrelated donor. After developing

<table>
<thead>
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<th>Strain</th>
<th>Parental strain</th>
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<th>Reference/source</th>
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<td>American Type Culture Collection</td>
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<td>23002</td>
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<td></td>
<td>Astellas Pharma Inc., Osaka, Japan</td>
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</tr>
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<td>this work</td>
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</table>

GSC1\^R, micafungin-susceptible wild-type allele S645; GSC1\^R, resistant allele S645F.
candidaemia following the transplant, treatment with amphotericin B (1 mg/kg), and then with a combination of amphotericin B and micafungin (300 mg once daily) for a period of 1 month failed, and the patient died. Cells were routinely maintained on yeast extract peptone dextrose (YPD) agar plates containing 1% (w/v) Bacto yeast extract (Becton, Dickinson and Company, Sparks, MD, USA), 2% (w/v) Bacto peptone (Becton, Dickinson and Company), 2% (w/v) glucose and 2% (w/v) agar at pH 5.5. For growth of the TUA4 host strain, uridine (100 mg/L) was added to YPD (YPDU).

**Chemicals**

Micafungin and caspofungin were supplied by Astellas Pharma Inc. (Osaka, Japan) and Merck & Co., Inc. (Rahway, NJ, USA), respectively. Fluconazole and itraconazole were purchased from Pfizer Laboratories Limited (Auckland, New Zealand) and from Janssen–Kyowa (Tokyo, Japan), respectively. Nidderrumycin Z, uridine and amphotericin B were from Sigma (St Louis, MO, USA). Polyoxin D, 5-fluoro-orotic acid and Calcofluor White were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

**Antifungal susceptibility test**

Liquid microdilution assays were performed in triplicate, in at least three independent experiments, in accordance with the CLSI document M27-A2, with minor modifications. Log-phase yeast cells were used for MIC determination in three different media: YPD; RPMI 1640 (Sigma); or Antibiotic Medium 3 (AM3; Difco) supplemented with 2% glucose. Cell growth was monitored at 590 nm using an EL340 Bio Kinetics plate reader (BioTek Instruments Inc., Winooski, VT, USA). The MICs for C. albicans and construction of GSC1 mutants

**DNA sequence analysis of GSC1, GSL1 and RHO1**

Entire ORFs of GSC1, GSL1 and RHO1 were PCR amplified from genomic DNA using high-fidelity KOD DNA polymerase (Toyobo, Osaka, Japan or Novagen, San Diego, CA, USA), and purified using QIAGEN purification columns (QIAGEN Pty Ltd, Victoria, Australia). Primers used for the PCR amplification of individual DNA fragments are listed in Table 2. DNA sequencing was performed using the BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems Inc., Foster City, CA, USA) and a Capillary ABI3730 Genetic Analyzer (Applied Biosystems Inc.). GenBank accession numbers for GSC1, GSL1 and RHO1 are XM_716336, XM_713421 and XM_710681, respectively.

**Isolation of a spontaneous micafungin-resistant mutant of C. albicans and construction of GSC1 mutants**

The micafungin-susceptible parent strain TUA4 was grown in YPDU liquid medium at 30°C for 48 h. Cells (1 x 10⁷) were plated onto YPD agar containing 0.025 mg/L micafungin and incubated at 30°C for 48 h. The 26 colonies that survived at this concentration of micafungin were incubated on fresh YPDU plates containing 0.025 mg/L micafungin at 30°C for a further 48 h and were then maintained on YPDU agar medium without micafungin. These clones were tested for higher micafungin resistance by patching onto YPDU plates containing 0.05 or 0.1 mg/L micafungin. Fourteen colonies survived 0.05 mg/L micafungin, but only one (strain Y30G) grew on 0.1 mg/L micafungin.

Individual GSC1 alleles were disrupted using a previously described method. Primers used for the following genetic manipulations are listed in Table 2. DNA fragments (500 bp) corresponding to the promoter and the 3′ non-translated regions of the GSC1 ORF were amplified from Y30G genomic DNA using the primer sets GSC1-LFH1 and GSC1-LFH2, and GSC1-LFH3 and GSC1-LFH4, yielding the DNA fragments GSC1-LFH3 and GSC1-LFH4, respectively (Figure 1a). A transformation cassette consisting of two DNA fragments was then constructed. The first fragment, containing the promoter region of the GSC1 and URA3 marker, was

<table>
<thead>
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<th>Name and application</th>
<th>Sequence 5′→3′</th>
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<tr>
<td>GSC1 amplification</td>
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<tr>
<td>PacI GSC1</td>
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</tr>
<tr>
<td>NotI GSC1</td>
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<tr>
<td>GSL1 amplification</td>
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<tr>
<td>PacI GSL1</td>
<td>CACTTAAATAAAAATGCTGCAACTGCTTCATCTCCGA</td>
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<tr>
<td>NotI GSL1</td>
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<td>RHO1 amplification</td>
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<tr>
<td>NotI RHO1</td>
<td>CACGCGGCCGCTTACAGAACAACATTTTCTTCTT</td>
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<td></td>
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<td>GSC1-LFH4</td>
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<td>RV-M</td>
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Sequences that correspond to introduced restriction sites are underlined.
Figure 1. Construction of a panel of C. albicans GSC1 mutants. A panel of homozygous and heterozygous mutants of GSC1 was constructed as described in the Materials and methods section. (a–c) Either the S645 or S645F allele of Y30G was deleted to construct Y30G01 (D/S645F) and Y30G02 (S645/D). (d–f) S645F and S645 were restored in Y30G01 and Y30G02, respectively, to construct the homozygous strains Y30G03 (S645F/S645F) and Y30G04 (S645/S645). (g) Genotypes of the constructs at the GSC1 locus. GSC1<sup>S</sup>, S645 allele; GSC1<sup>R</sup>, S645F allele. 5-FOA, 5-fluoroorotic acid.
PCR amplified using the fragment GSC1-LFH4 and the plasmid pUC19-URA3, plus the primer set GSC1-LFH1 and RV-M. The second fragment containing URA3 and the 3’ non-translated region of GSC1 was PCR amplified using the plasmid pUC19-URA3, the fragment GSC1-LFH8, and the primer set M13-47 and GSC1-LFH4 (Figure 1b). The two resultant PCR amplimers were used together to transform Y30G using the lithium acetate method. Either the susceptible (S645; GSC1<sup>+</sup>) or resistant (S645F; GSC1<sup>F</sup>) GSC1 allele of strain Y30G was disrupted by homologous recombination, as shown in Figure 1(c). The resultant mutants selected on CSM-ura medium (0.67% (w/v) yeast nitrogen base (Difco, Becton Dickinson and Company), 0.077% (w/v) CMU-ura (BIO 101, Irvine, CA, USA) and 2% (w/v) glucose) were designated Y30G01 (susceptible allele disrupted ΔS645F) and Y30G02 (resistant allele disrupted ΔS645F; Figure 1g and d). Deletion of the GSC1 ORF was confirmed by colony PCR and DNA sequence analysis. The disruptants Y30G01 and Y30G02 were then used to construct homoyzogous strains by restoring either a resistant or a susceptible GSC1 allele, respectively, at the GSC1 locus. DNA fragments containing the GSC1<sup>+</sup> and GSC1<sup>F</sup> alleles were PCR amplified from genomic DNA of Y30G02 and Y30G01, respectively, using the GSC1-LFH1 and GSC1-LFH4 primer set. DNA fragments corresponding to the resistant and the susceptible alleles were used to transform Y30G01 and Y30G02 to Y30G03 and Y30G04, respectively (Figure 1e and g), by selection of Ura<sup>+</sup> transformants on CSM medium containing 1 g/l 5-fluoroorotic acid (Figure 1f). Restoration of the GSC1 ORF was confirmed by colony PCR and DNA sequencing of PCR-amplified GSC1 using the primer set GSC1-N and GSC1-C.

β-1,3-GS preparation from C. albicans and GS inhibition assay

Cells grown overnight in YPD were diluted to OD<sub>650</sub> = 0.5 and grown to early stationary phase (OD<sub>650</sub> = 10) with vigorous shaking (200 rpm) at 37°C using a TAITEC Bio Shaker BR-180LF (TAITEC Co. Ltd, Saitama, Japan). The cells were harvested by centrifugation at 5000 g for 10 min and membrane fractions used as the source of GS were prepared using the method described by Hatano et al., with minor modifications. In brief, cells were resuspended in 2 mL of buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol and 1 M sucrose) per g of cell pellet and disrupted with 0.6 mm glass beads using a Kubota Insonator 201 M (Kubota, Tokyo, Japan) at 170 W for 7 min. An equal volume of buffer B (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol and 25 μM GTP) was added to the lysate, and cell debris and glass beads were removed by centrifugation at 3000 g for 10 min. Membrane fractions containing GS activity were washed twice by sedimentation at 100000 g for 45 min using an Optimus XL-80K Ultracentrifuge (Beckman Coulter, Fullerton, CA, USA). The membranes were suspended in buffer C (buffer B containing 50% (v/v) glycerol) at 10 mg of protein/mL and stored at -80°C.

The susceptibility of GS activity to micafungin and caspofungin was measured in a 96-well format according to the method of Hatano et al., with minor modifications. In a final volume of 50 μL, each well contained 100 mM Tris-HCl (pH 7.0), 1 mM EDTA, 10 mM sodium fluoride, 100 μM GTP, 0.25% BSA, 0.2 mg/mL membrane fraction and serial dilutions of micafungin or caspofungin dissolved in 0.5 μL of DMSO. After 20 min of pre-incubation at room temperature, 1 mM UDP-glucose containing 0.35 μCi of [3H]UDP-glucose (GE Healthcare, Bucks, UK) was added and the reaction was incubated for 1 h at 22°C. Reactions were terminated by the addition of 50 μL of 20% trichloroacetic acid (TCA) and the precipitated glucan was collected by filtration on chilled GF/C plates (Unifilter-96, PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA, USA) and washed three times with 200 μL of 5% TCA using a Perkin Elmer Filtermate Harvester. The GF/C plates were dried for 3 h at 50°C and the retained radioactivity was measured using a NXT scintillation counter (Perkin Elmer) in the presence of 20 μL/well MicroScint-0 (Perkin Elmer) scintillation cocktail. Two control reactions were used to calculate the incorporation rate of UDP-glucose: for 0% UDP-glucose incorporation, 50 μL of 20% TCA and radiolabelled UDP-glucose were added to the reaction at time zero; and for 100% incorporation, the GS assay was carried out without micafungin or caspofungin. Inhibition curves and 50% inhibitory concentrations (IC<sub>50</sub>) were obtained with GraphPad Prism, version 4.03, software (GraphPad Software, San Diego, CA, USA) using a two-site fitting algorithm (for strain Y30G) or sigmoidal response curves.

Nucleotide sequence accession numbers

The full nucleotide sequences of the C. albicans GSC1 and GSL1 genes from strains 23002 and ATCC 90028 determined in this study have been deposited in GenBank with the following accession numbers: GSC1 from 23002 (GQ456065) and ATCC 90028 (GQ456066); and GSL1 from 23002 (GQ497115) and ATCC 90028 (GQ497116).

Results

Characterization of a micafungin-resistant clinical isolate

Antifungal susceptibility of the clinical isolate

The liquid MICs of micafungin for the resistant C. albicans clinical isolate 23002 and the susceptible reference strain ATCC 90028 were measured in RPMI 1640 medium. The micafungin MIC for strain 23002 (2 mg/L) was >100-fold higher than that for ATCC 90028 (0.016 mg/L, Table 3). Neither resistant nor susceptible cells showed trailing growth and each 50% endpoint gave a MIC result similar to the corresponding 90% endpoint (data not shown). Strain 23002 developed resistance to micafungin and also caspofungin (data not shown), but neither strain was cross-resistant to any other class of antifungals tested, including azoles (fluconazole and itraconazole) and a polyene (amphotericin B).

DNA sequences of C. albicans GSC1, GSL1 and RHO1 from the reference strain and the resistant clinical isolate

DNA sequences were determined for the entire GSC1 ORF (5694 bp) from both the 23002 and ATCC 90028 strains. There were 12 nucleotide differences between the ORFs. These resulted in one homozogous amino acid change (S645P) in strain 23002, plus two heterozygous amino acid changes at positions 101 and 1838. No single nucleotide polymorphisms (SNPs) were found in GSC1 alleles from strain 23002, but eight synonymous and two non-synonymous SNPs were found in GSC1 alleles from ATCC 90028. The absence of SNPs in the GSC1 alleles of strain 23002 is particularly significant, considering the size of the ORF. This finding could be explained by a recent gene conversion event or because only one allele from this strain was amplified by PCR. To limit the latter possibility, GSC1 was PCR amplified from genomic DNA from strain 23002, and cloned into Pacl and NotI sites in the yeast expression vector pABC3 using Escherichia coli strain XL1-blue. Nucleotide sequencing of the GSC1 from seven individual clones showed that all the GSC1 sequences were identical. Thus, strain 23002 appears to be homozogous for the entire GSC1 ORF. In contrast, the two GSC1 alleles from ATCC 90028 had synonymous and non-synonymous SNPs. A
comparison of nucleotide and amino acid changes in the GSC1 genes for strains 23002, ATCC 90028 and database strains (SC5314, http://www.candidagenome.org/; ATCC 10231, accession number D88815) indicates interstrain variation. The S645P amino acid change in GSC1 is within the echinocandin resistance region hot spot 1 (amino acids 641–648) that was previously reported in caspofungin-resistant clinical isolates and laboratory strains of *C. albicans*.21,26,27,31

The GSL1 gene from strains 23002 and ATCC 90028 contained several SNPs, which indicated that there was both intrastain variation between alleles and interstrain nucleotide changes. There were 56 SNPs between the genes from the two strains, but only two of these caused homozygous amino acid changes (T892Q and D898E). Five other SNPs resulted in heterozygous amino acid changes due to allelic variation (I181/T181, M938/T938, V1274/I1274 and S1331/N1331 in ATCC 90028; M938/T938, V1274/I1274 and S1331/N1331 in ATCC 10231, accession number D88816).16 Several SNPs, which indicated that there was both intrastrain and laboratory strains of *C. albicans*. The amino acids at these positions of both 23002 and ATCC 10231 Gsc1p. The amino acids at these positions of both 23002 and ATCC 10231, accession number D86430).16

DNA sequence analysis of the regulatory subunit RHO1 gene from 23002 and ATCC 90028 found no intrastrain SNPs. The DNA sequences from both strains were identical to the RHO1 sequence in the GenBank database (strains SC5314 and ATCC 10231, accession number D86430).16

### Isolation and characterization of *C. albicans* variant Y30G with reduced susceptibility to micafungin

A spontaneous variant (Y30G) with reduced susceptibility to micafungin was obtained from a susceptible auxotroph (TUA4) by selection for micafungin resistance. The strain Y30G had a ~30-fold higher micafungin MIC (1.0 mg/L) than the susceptible parent (MIC = 0.031 mg/L). However, its resistance was not as high as that of the clinical isolate 23002 (4 mg/L in YPD; Table 3). TUA4 and Y30G were not cross-resistant to other antifungal classes (fluconazole, itraconazole or amphotericin B; data not shown), and there was no difference in the susceptibility of the strains to other compounds such as chitin synthase inhibitors (nikkomycin Z and polyoxin D) or a chitin assembly inhibitor (Calcofluor White; data not shown). These data indicated that Y30G had developed resistance to micafungin only. DNA sequence analysis of the GSC1, GSL1 and RHO1 ORFs identified no nucleotide changes in the entire ORFs from both strains, apart from a single heterozygous amino acid change (S645F/S645P) in GSC1 strain Y30G.

Because Y30G possessed a single amino acid change in the echinocandin resistance region of only one GSC1 allele, and had intermediate resistance to micafungin, we hypothesized that a homoyzgous hot-spot mutation in GSC1 would confer a significantly higher level micafungin resistance than a heterozygous mutation. This hypothesis was tested using a panel of laboratory mutants that had either susceptible wild-type or mutated resistant GSC1 alleles deleted or restored.

### Micafungin susceptibility of homoyzgous and heterozygous mutant strains

The panel of mutant strains (Figure 1g) constructed from strain Y30G was tested for micafungin susceptibility in three different media using the CLSI method (Table 3). The Y30G03 strain, which possesses homoyzgous resistant (S645F) GSC1 alleles, showed micafungin resistance equivalent to that of clinical isolate 23002. The parent TUA4 strain and susceptible reference strain ATCC 90028, which possess homoyzgous susceptible (S645P) alleles, were both highly susceptible to micafungin. Strains possessing either a single S645 allele (Y30G02) or homoyzgous S645 alleles (Y30G04 derived from Y30G02) were also susceptible to micafungin, as expected. The high-level

### Table 3. In vitro susceptibilities of *C. albicans* GSC1 mutants to echinocandins

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<th>Strain</th>
<th>GSC1 allele</th>
<th>YPD MIC (mg/L)</th>
<th>RPMI 1640 MIC (mg/L)</th>
<th>AM3 MIC (mg/L)</th>
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</thead>
<tbody>
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<td>0.016</td>
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<td>Y30G01</td>
<td>Δ/R</td>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Y30G02</td>
<td>S/Δ</td>
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<td>Y30G04</td>
<td>S/S</td>
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MCF, micafungin; CSF, caspofungin.

aMIC was determined after 24 h of incubation at 35°C. Culture media were supplemented with 100 mg/L uridine. RPMI 1640 was further supplemented with 100 mg/L each of arginine and histidine.

bGSC1 allele: S, susceptible allele S645; R, resistant allele S645F; Δ, allele deleted; R*, resistant allele S645P.
micafungin resistance of strain Y30G01 (ΔS645F) showed that a single resistant GSC1 allele was sufficient to confer full resistance to micafungin. All the micafungin-resistant strains were cross-resistant to caspofungin, although the clinical isolate 23002 showed higher caspofungin MIC values (≥16 mg/L) when tested in AM3 (Table 3).

In order to test for the selection for homozygosity of the GSC1 locus upon exposure to micafungin, the heterozygous mutant Y30G was incubated in YPD liquid medium containing micafungin (1, 2 or 4 mg/L) at 30 °C with shaking (200 rpm). Resistant variants grew at each concentration of micafungin after 3 days. These cells were then plated on YPD agar medium and three individual variants, one from each of the three different micafungin concentrations (1, 2 or 4 mg/L) used for selection, were picked for DNA sequencing analysis of the GSC1 ORF. None of the variants were heterozygous at the hot-spot region of GSC1 and both alleles had homozygous nucleotide changes from C to T at position 1934. This mutation is predicted to cause an amino acid change from serine (S) to phenylalanine (F) at amino acid position 645. Liquid MIC values of micafungin for all the variants were 4 mg/L, equivalent to those for strain 23002 and the homozygous mutant Y30G03.

**Inhibition of GS activity by echinocandins**

Kinetic analysis of the GS enzyme in membrane preparations from the micafungin-resistant clinical isolate 23002, the susceptible reference strain ATCC 90028 and the laboratory-generated mutants showed that GS from all the strains had similar $K_m$ values and GS activities (Table 4). *In vitro* inhibition of the GS enzyme from the resistant isolate 23002 and the susceptible strain ATCC 90028 were evaluated using micafungin (Figure 2a) and caspofungin (Figure 2b). The sigmoid inhibition profiles suggested that the enzyme from 23002 with homozygous S645P and from ATCC 90028 with homozygous S645 each contained GS enzyme activity in a single complex. The GS activity from 23002 was less sensitive to both echinocandins than the GS activity from ATCC 90028. The IC50 of micafungin (21 mg/L) and caspofungin (51 mg/L) for the resistant GS was two and three orders of magnitude higher, respectively, than for the sensitive GS (Table 4). The GS inhibition profiles for laboratory-generated GSC1 mutants were also evaluated for micafungin (Figure 2c) and caspofungin (Figure 2d). The GS activities of the single allele S645F strain Y30G01 and the homozygous S645F strain Y30G03 were highly resistant to both echinocandins compared with the parental strain TUA4. The sigmoid inhibition curves for each GS activity suggest that all three strains had a single species of enzyme. The GS from Y30G01 and Y30G03 had a similar IC50 for micafungin (138 and 385 mg/L, respectively) and for caspofungin (29 and 26 mg/L, respectively); these values were more than three log orders higher than those of the GS from TUA4 (Table 4). The inhibition curve for GS from the heterozygous Y30G strain was biphasic. It produced two distinct IC50 values (0.07 and 173 mg/L for micafungin) that reflected the presence of sensitive and resistant GS enzymes. The intermediate micafungin and caspofungin inhibition profiles correlated well with in vivo MIC values and indicated that both the wild-type and mutant enzymes contributed equally and independently to overall enzyme activity. However, as the enzymes specified by the Gsc1p alleles were not separable in the membrane fractions from strain Y30G, values for the individual $K_m$s of the wild-type and mutant proteins were not obtained. There were also differences between micafungin and caspofungin in the inhibition profiles of GS activities. The GS enzymes from all the strains tested, including 23002 and ATCC 90028, were completely inhibited by caspofungin (Figure 2b and d), whereas the GS enzymes from only the susceptible TUA4 and ATCC 90028 strains were inhibited completely by micafungin. Micafungin inhibited GS enzymes from resistant and intermediate resistant strains strongly, but incompletely (Figure 2a and c).

**Discussion**

The clinical isolate 23002 had micafungin and caspofungin MICs that were at least 125- and 16-fold higher, respectively, than the susceptible strain ATCC 90028. Although the MIC values of the two echinocandins for both strains were affected to some extent by the medium used in the assay, micafungin was less affected than caspofungin. In particular, the susceptibility of susceptible and resistant strains to caspofungin occurred over a narrower window in RPMI 1640 medium than in YPD medium or AM3. In agreement with our results for strains 23002 and ATCC 90028 with caspofungin, studies of the effect of medium composition on caspofungin MICs for *Candida* species showed that AM3 distinguishes between resistant and susceptible strains better.

**Table 4.** Kinetic properties of β-1,3-glucan synthase from *C. albicans* GSC1 mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>GSC1 allele</th>
<th>IC50 (mg/L)</th>
<th>Kinetics</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MCF</td>
<td>CSF</td>
</tr>
<tr>
<td>ATCC 90028</td>
<td>S/S</td>
<td>0.09±0.04</td>
<td>0.019±0.004</td>
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<tr>
<td>23002</td>
<td>R*/R*</td>
<td>21±12</td>
<td>51±4</td>
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<tr>
<td>TUA4</td>
<td>S/S</td>
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<td>0.016±0.005</td>
</tr>
<tr>
<td>Y30G</td>
<td>S/R</td>
<td>0.07±0.01 and 173±55</td>
<td>0.015±0.012 and 21±2</td>
</tr>
<tr>
<td>Y30G01</td>
<td>Δ/R</td>
<td>138±29</td>
<td>29±2</td>
</tr>
<tr>
<td>Y30G03</td>
<td>R/R</td>
<td>385±80</td>
<td>26±2</td>
</tr>
</tbody>
</table>

MCF, micafungin; CSF, caspofungin.
aGSC1 allele: S, susceptible allele S645; R, resistant allele S645F; Δ, allele deleted; R*, resistant allele S645P.
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Figure 2. Echinocandin inhibition profiles of glucan synthase complexes from *C. albicans* GSC1 mutants. (a) Micafungin (MCF) inhibition curves for clinical isolate 23002 (homozygous resistant alleles with S645P) and ATCC 90028 (homozygous wild-type alleles). (b) Caspofungin (CSF) inhibition curves for clinical isolate 23002 and ATCC 90028. (c) MCF inhibition curves for GSC1 mutants: parental TUA4 (homozygous wild-type alleles); Y30G (heterozygous S645/S645F); Y30G01 (single GSC1 allele; ΔS645F); and Y30G03 (homozygous S645F). (d) CSF inhibition curves for the GSC1 mutants; strains are the same as for (c). Bars represent the standard errors of the mean of three independent experiments.

C. *albicans* has three *S. cerevisiae* FKS homologues (GSC1, GSL1, and GSL2) and since GSL2 expression is negligible, DNA sequences were analysed for GSC1 and GSL1 only. Several inter- and intrastrain SNPs were detected in GSL1, including two amino acid changes (T893Q and D898E). However, neither amino acid change was in an echinocandin resistance region hot spot. Furthermore, there were no inter- or intrastrain SNPs in the RHO1 genes from resistant and susceptible strains. Thus, the regulatory subunit of the GS complex is not likely to be a primary target of echinocandins. The S645P amino acid substitution detected in the 23002 Gsc1p echinocandin resistance region hot spot was therefore considered to be responsible for the low susceptibility of strain 23002 to micafungin. Although caspofungin and pneumocandin L-733,560 resistance mutations in the *C. albicans* echinocandin resistance region hot spot have been reported previously, and FKS mutations have been reported in *C. glabrata* after treatment with micafungin, this is the first report of such a mutation in a *C. albicans* clinical isolate from a patient treated with micafungin and not other echinocandins. It also presents the most comprehensive sequence analysis of the ORFs of genes that can be expected to contribute to clinically significant echinocandin resistance. Our results also imply that GSC1 alone encodes the functional GS catalytic subunit in *C. albicans* under normal growth conditions, as suggested previously. This interpretation is consistent with the inference that GSC1 is essential for *C. albicans* growth, because the homozygous deletion is lethal. Although the GSL1 and GSL2 genes are homologues of GSC1, their failure to support a GSC1 null mutant suggests that they are not functionally equivalent. This situation is different in *S. cerevisiae*, which expresses the homologues FKS1 and FKS2. These genes encode catalytic subunits of GS that have overlapping functions, with FKS1 being predominant. *C. glabrata* also expresses FKS1 and FKS2 homologues that may more closely mimic the situation in *S. cerevisiae*, although FKS1 and FKS2 contribute equally (Garcia-Effron et al. and K. Niimi, M. A. Woods, K. Maki, K. Hatakenaka, H. Nakayama, H. Chibana, K. Ueno, F. Ikeda, M. Niimi, R. D. Cannon, B. C. Monk, unpublished data).
We have demonstrated that the stepwise exposure of *C. albicans* cells to micafungin yielded the heterozygous Y30G resistant strain. An initial micafungin concentration of 0.025 mg/L was chosen to isolate variants with lower susceptibility than the parent TU4 (MIC=0.031 mg/L in YPDU). This modest concentration was used because solid medium gives lower micafungin MICs than liquid medium, and TU4 failed to grow at 0.025 mg/L on YPDU. The heterozygous amino acid change of S645F found in strain Y30G is identical to that reported in other clinical isolates of *C. albicans*, but not to the homozygous S645P of clinical isolate 23002. The echinocandin resistance of the laboratory isolate appears similar to that of other caspofungin-resistant isolates with the S645P mutation. Because cells with a heterozygous GSC1 deletion are viable, Y30G was used to construct isotonic GSC1 mutants, homozygous susceptible and resistant strains plus single-allele deletants that could be analysed in whole cells and at the level of GS enzyme.

MIC assays for micafungin and caspofungin clearly distinguished between homozygous (Y30G03) and heterozygous (Y30G) GSC1 mutants in all media tested. This finding differs from another study, where strains possessing either homozygous or heterozygous substitution that were otherwise isogenic had comparable susceptibilities. It is not known if there were other genetic differences.

*C. albicans* has natural heterozygosity, but loss of heterozygosity (LOH) is common. LOH can be due to gene conversion, allelic recombination or chromosomal loss and replication. LOH has been described for *C. albicans* clinical isolates recovered from patients withazole antifungal treatment. The strains showed azole resistance that was associated with LOH in TAC1 and MRN1, the transcription factors for ABC and MFS transporters, respectively. Like UV irradiation, antifungal pressure may promote genome modification, including LOH. When exposed to micafungin, the heterozygous mutant Y30G acquired a homozygous mutation of S645F that conferred full resistance. No other amino acid substitutions, such as S645P or S645Y, were found in the homozygous variants of Y30G, indicating that LOH occurred in this strain. We therefore suggest that clinical strain 23002 has acquired the GSC1 mutations in a stepwise manner: a spontaneous heterozygous mutation with intermediate resistance was converted to a homozygous mutation with full resistance upon prolonged exposure to micafungin, probably via LOH. This interpretation is supported by the complete absence of SNPs in the large GSC1 ORF of strain 23002 and their presence in the comparable region of ATCC 90028. The finding is relevant to the clinical use of micafungin for the treatment of *C. albicans* infections. When a concentration of micafungin that is fungicidal for *C. albicans* is present in the blood stream or tissue fluid, the yeast is unlikely to undergo stepwise development of micafungin resistance. The requirement for a mutation in one allele followed by LOH in the GSC1 gene may explain why the incidence of micafungin resistance is low.

The present study indicates that the GSC1 resistant allele appears to be semidominant. Semidominance of the heterozygous resistant marker of GSC1 (FKS1) has been observed in *C. albicans* and in *S. cerevisiae*. It has also been reported for *S. cerevisiae* MRN1, which shows intermediate resistance to manganese when susceptible and resistant alleles are present. Mutants with ΔGSC1 alleles (Y30G01) and with homozygous GSC1/GSC1 alleles (Y30G03) had the same micafungin MIC (e.g. 2 mg/L in RPMI). In the ΔGSC1 strain, the GS catalytic subunit is encoded by the resistant gene only and, hence, a single GSC1 allele confers full resistance to echinocandins. The contributions of the echinocandin-resistant and -susceptible binding sites to echinocandin susceptibility in this strain indicate that the GSC1 alleles contribute equally to GS expression and that the GS subunits operate independently.

The response of GS activity to micafungin and caspofungin gave inhibition profile curve-fits that suggested that the sensitive and fully resistant GS source/stains each contained a single form of Gsc1p. This interpretation is consistent with the genetic findings that the homozygous deletion of GSC1 is lethal, and that the GSC1 homologues GSL1 and GSL2 do not compensate for the deletion of GSC1. The significantly different responses of GS activities of the 23002 strain to micafungin and caspofungin may be due to these echinocandins interacting differently with the mutant Gsc1p. Alternatively, other yeast components might affect drug susceptibility to micafungin, because the GS samples used in the assays were crude membranes and were not purified using a detergent such as CHAPS (3-[3-cholamidopropyl]dimethylammonion)-1-propanesulfonate. Although the isolated GS fraction gave higher specific activity than the plasma membrane fractions, the extraction of GS with detergent might affect the GS structure and function. We avoided this step in order to retain the native GS complex. The enzymes from mutants Y30G01 and Y30G03 behaved similarly for both micafungin and caspofungin. The mutations at S645 may differently affect the GS pharmacophores that bind micafungin and caspofungin. Incomplete inhibition of the mutant GS activity by micafungin, even at the highest concentration used (512 mg/L), is also consistent with micafungin and caspofungin affecting the GS catalytic subunit differently. Neither the S645F nor the S645P mutation in the Gsc1p echinocandin resistance region altered the Km values of the resistant enzyme compared with that of the wild-type enzyme. This indicates that the amino acid changes that caused reduced susceptibility to echinocandins did not affect substrate (UDP-glucose) binding. The homozygous S645F amino acid change in Gsc1p from strain Y30G03 did not affect GS activity significantly.

Our collective findings indicate that the exposure of susceptible *C. albicans* to micafungin can select resistant variants with the hot-spot mutation of Gsc1p. Such variants may also acquire additional extragenic mutations or modulate the effects of GSC1 regulatory elements upon exposure to echinocandins, since GS expression appears to be closely regulated by a complex network of genes involved in cell wall biosynthesis or stress response in the model yeast *S. cerevisiae*. Although strain Y30G was selected for micafungin resistance, and it may have mutations or alterations in addition to GSC1, the panel of *C. albicans* GSC1 mutants derived from this strain appear genetically identical apart from the amino acid change in the GSC1 alleles. The differences in echinocandin susceptibilities between the mutants in whole cell assays and in vitro GS inhibition assays are comparable and unlikely to be affected by unidentified genetic features. There is no indication of an enzyme complex that contains both resistant and susceptible GS subunits and that operates in a mutually dependent fashion. We conclude that the two alleles of GSC1 contribute equally and independently to GS activity.
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**Acknowledgements**

This work has been presented in part at the Seventeenth Congress of the International Society for Human and Animal Mycology, Tokyo, Japan, 2009 (Abstract PP-03-32) and at the Fifty-second Annual Meeting of the Japanese Society for Medical Mycology, Nagasaki, Japan, 2008 (Abstract SY-6-1).

We are grateful to Y. Takano (National Institute of Infectious Diseases, Tokyo, Japan) for technical assistance.

**Funding**

This work was supported by Astellas Pharma Inc. and the Health Science Research Grants for Research on Emerging and Re-emerging Infectious Diseases (H16-Shinko-6 and H19-Shinko-8) from the Ministry of Health, Labour and Welfare of Japan. E. L. and A. H. had fellowships from the Japan Society of Promotion of Sciences.

**Transparency declarations**

K. N. has received grant support and support for conference attendance from Astellas. K. H., K. M. and F. I. contributed to this research as Astellas employees. K. M. declares that he has shares in Astellas. Other authors: none to declare.

**References**


