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Resistance to echinocandin-class antifungal drugs

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Abstract

Invasive fungal infections cause morbidity and mortality in severely ill patients, and limited drug classes restrict treatment choices. The echinocandin drugs are the first new class of antifungal compounds that target the fungal cell wall by blocking β-1,3-D-glucan synthase. Elevated MIC values with occasional treatment failure have been reported for strains of *Candida*. Yet, an uncertain correlation exists between clinical failure and elevated MIC values for the echinocandin drugs. Fungi display several adaptive physiological mechanisms that result in elevated MIC values. However, resistance to echinocandin drugs among clinical isolates is associated with amino acid substitutions in two "hot-spot" regions of Fks1, the major subunit of glucan synthase. The mutations, yielding highly elevated MIC values, are genetically dominant and confer cross-resistance to all echinocandin drugs. Prominent Fks1 mutations decrease the sensitivity of glucan synthase for drug by 1000-fold or more, and strains harboring such mutations may require a concomitant increase in drug to reduce fungal organ burdens in animal infection models. The Fks1-mediated resistance mechanism is conserved in a wide variety of *Candida* spp. and can account for intrinsic reduced susceptibility of certain species. Fks1 mutations confer resistance in both yeasts and moulds suggesting that this mechanism is pervasive in the fungal kingdom.
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1. Introduction

The treatment options for invasive fungal infections are limited since there are relatively few chemical classes and targets represented by existing antifungal drugs. Current drugs target cell wall and membrane components. The most broadly used agents are aimed at ergosterol, a predominant sterol within fungal cell membranes, and are either fungicidal but toxic to the host (polyenes) or fungistatic and more vulnerable to resistance (azoles and triazoles). The newest class of antifungal drugs are the echinocandins, which target biosynthesis of β-1,3-D-glucan, a key fungal cell wall component. These drugs are active against clinically relevant yeasts and moulds (Denning, 2003). Caspofungin, the first class member, received FDA approval in 2002 followed by micafungin in 2005 and anidulafungin in 2006. Clinical resistance appears low with sporadic breakthrough cases reported (Morris and Villmann, 2006; Laverdiere et al., 2006; Miller et al., 2006; Hakki et al., 2006). However, as patient exposure to echinocandin drugs broadens, the number of infecting strains with reduced susceptibility is expected to rise. Unfortunately, the relationship between reduced *in vitro* susceptibility to echinocandin drugs and clinical failure is ambiguous (Kartsonis et al., 2005; Pfaller et al., 2005b). This review will address this critical issue for echinocandin drugs by discussing Fks1 modification as a principal resistance mechanism and will define laboratory-based parameters for resistance that can contribute to clinical failure.

2. Echinocandin antifungal drugs

The echinocandins were the first members of the lipopeptide group to be discovered that inhibit β-1,3-D-glucan synthase, which is responsible for biosynthesis of the major cell wall biopolymer (Kurtz and Douglas, 1997). They are cyclic hexapeptides N-linked to a fatty acyl side chain. Only two chemical classes, the lipopeptides and papulacandins are known to inhibit glucan synthase; although, the latter

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have not developed as antifungal agents. The echinocandin drugs, caspofungin, micafungin, and anidulafungin are the first of a new class of antifungal compounds that target the fungal cell wall (Denning, 2003; Kartsonis et al., 2003; Wiederhold and Lewis, 2003). These drugs have broad-spectrum antifungal activity against *Candida* and *Aspergillus* spp. without cross-resistance to existing antifungal agents, and therefore are effective against azole-resistant yeasts and moulds (Denning, 2003; Morrison, 2006). The echinocandins are fungicidal against yeasts but fungistatic against moulds, where they block the growing tips of hyphae (Bowman et al., 2002; Douglas, 2006). Echinocandin drugs are highly effective on biofilms (Bachmann et al., 2002b) but are less active against *Zygomycetes, Cryptococcus neoformans* or *Fusarium* spp. (Denning, 2003). They are now widely used for antifungal therapy against yeasts and moulds (Morris and Villmann, 2006; Betts et al., 2006; Bennett, 2006; Joseph et al., 2007). Caspofungin and anidulafungin are FDA approved in the US and other countries for the treatment of serious fungal infections including esophageal candidiasis, candidemia, and other *Candida* infections. Caspofungin is also approved for empirical therapy for presumed fungal infections in febrile neutropenic patients, as well as for treatment of patients refractory to standard treatments for *Aspergillus* infections. Micafungin is approved for the treatment of patients with esophageal candidiasis and for prophylaxis of *Candida* infections in patients undergoing hematopoietic stem cell transplantation during the period of neutropenia. Overall, these drugs have excellent safety and tolerability profiles with few drug-related adverse events (Ullmann, 2003; Boucher et al., 2004; Higashiyama and Kohno, 2004; Wagner et al., 2006).

3. Glucan synthase complex

The β-1,3-glucan synthase is a multi-subunit enzyme complex responsible for fungal cell wall construction and division septum deposition. The enzyme catalyses the transfer of sugar moieties from activated donor molecules to specific acceptor molecules forming glycosidic bonds in the reaction UDP-glucose + $\{(1,3)$-β-D-glucosyl$\}(N) \rightarrow$ UDP + $\{(1,3)$-β-D-glucosyl$\}(N+1)$. Most of our understanding of the genetics of glucan synthase has come from studies in yeast (Inoue et al., 1996; Qadota et al., 1996). The UDP glycosyltransferases are one group of enzymes that carry out this reaction and over 100 members of this protein family are known (Ross et al., 2001). The enzyme complex has a minimum of two subunits, Fks1 and Rho. Fks1 appears to be the catalytic subunit, which is supported by cross-linking studies with a photoaffinity analog of the substrate UDP-glucose (Schimoler-O'Rourke et al., 2003). Rho, a GTP-binding protein in the Rho/Rac subfamily of Ras-like GTPases, helps regulate the activity of the glucan synthase (Mazur and Baginsky, 1996). Biochemical studies have implicated other membrane-associated components including Pma1 which appears in close association with the glucan synthase complex (Schimoler-O'Rourke et al., 2003). Pma1's role in maintaining transmembrane electrochemical proton gradients (Monk and Perlin, 1994) may be important to glucan synthase by providing the driving force for translocating product and/or in maintaining an acidic cell wall environment close to the membrane, as glucan polymers are laid down. Additional information about potential interacting proteins has come from labeling studies with the photoactivatable cross-linking echinocandin LY303366. The photoaffinity probe identified two proteins of 40 and 18 kDa in membrane preparations (Radding et al., 1998). It was suggested that the 40 kDa protein could be a homolog of Pil1 and Lsp1, sphingolipid-dependent regulators of cell wall integrity signaling (Edlind and Katiyar, 2004). These gene products may interact with the glucan synthase complex, although genetic/biochemical confirmation is lacking. The mechanistic nature of the interaction between echinocandins and glucan synthase remains ambiguous.

4. Elevated MIC and clinical outcome

Large-scale surveillance studies have documented the outstanding potency of echinocandin drugs against clinical isolates of *Candida* species in routine susceptibility assays (Espinel-Ingroff, 2003; Pfaller et al., 2003b, 2005a, 2006). These studies also reveal the presence of occasional strains from highly susceptible species that display uncharacteristically high MIC values. They further highlight the presence of less-susceptible non-*albicans Candida* spp., such as *C. parapsilosis* and *C. guilliermondii*, which have routine MIC values 4–100-fold greater than those observed for *C. albicans*. The clinical significance of this reduced susceptibility is unclear as infections with these organisms generally respond to current echinocandin therapy (Mora-Duarte et al., 2002; Bennett, 2006). In general, an uncertain correlation exists between clinical failure and elevated *in vitro* MIC values for echinocandin drugs. In two well-documented candidiasis studies, elevated MIC was not a reliable predictor of treatment outcome (Kartsonis et al., 2005; Pfaller et al., 2005b). This discordance may reflect the fact that the majority of elevated MIC values reported in these studies were relatively modest (MIC ≤ 2 μg/ml). Yet, sporadic treatment failures consistent with clinical resistance have been documented with high MIC isolates (Hakki et al., 2006; Laverdiere et al., 2006; Miller et al., 2006). In this context, it is important to distinguish between adaptive mechanisms by fungi that can cause elevated MIC values *in vitro* but do not influence clinical outcome and those that result in treatment failure. As patient exposure to echinocandin drugs broadens, it is anticipated that the number of clinical isolates with elevated MIC values will rise and an increasing number of patients may fail therapy due to resistance. Thus, it is vital to understand the nature of developing resistance mechanisms to this class of drugs.

5. Role of cell wall biosynthesis and integrity pathways in drug adaptation

A key to understanding resistance mechanisms is to fully appreciate the importance of the cell wall as a dynamic structure that is closely coupled to cell physiology. Maintenance of fungal cell wall integrity is essential, as fungi cannot survive without this structure or even if it is markedly altered in some way. The wall is an extracellular matrix with a layered organization consisting of an outer layer of glycoproteins and an inner layer of carbohydrate polymers including glucan, chitin and galactomannan (Latge et al., 2005; Douglas, 2006; Ruiz-Herrera et al., 2006). In saprophytic and pathogenic fungi, the carbohydrate layer is comprised mainly of β-1,3-glucan and α-1,3-glucan, but it also contains some β-1,6-glucan and chitin. Glucans are also released from the fungal cell wall as exopolymers into the blood of patients with fungal infections, and are known to activate a wide range of innate immune responses (Brown and Gordon, 2003). The fungal cell wall undergoes frequent remodeling, as constitutive polymers are constantly being synthesized, chemically modified and rearranged during cell wall biosynthesis. Cell wall biosynthesis and maintenance is a highly dynamic process that is tightly regulated during cell growth and morphogenesis, and it responds to a variety of cell stresses (Lesage and Bussey, 2006). It involves modification of existing biosynthetic machinery through interactions with cell stress and cell integrity pathways, as well as delivery of new enzymes from the Golgi complex through the secretory vesicle system (Fig. 1). Furthermore, Fks1, the presumptive catalytic subunit of the glucan synthase complex is co-localized within cortical actin patches where it moves on the cell surface to sites of cell wall remodeling (Utsugi et al., 2002).

As echinocandins target glucan synthase, the machinery responsible for producing β-1,3-D-glucan the major cell wall biopolymer, it was anticipated that cells exposed to drug would induce cellular mechanisms involved in cell wall biosynthesis and integrity. Resistance could potentially arise from genetic modulation of these pathways. The cell integrity pathway has been well characterized in *S. cerevisiae* (Levin, 2005). It depends on the proper composition of the cell wall, and it is regulated by G-protein Rho1 and MAP kinase signaling cascades, which help coordinate changes to the cell wall during the cell cycle and in response to environmental stress (Errede et al., 1995; Levin, 2005; Lesage and Bussey, 2006) (Fig. 1). Rho1 is also an important regulatory component of glucan synthase where it activates the membrane complex (Mazur and Baginsky, 1996; Roh et al., 2002). Echinocandin drugs create enormous stress on the cell by shutting down glucan synthase resulting in glucan depletion. As expected, genome-wide expression profiling indicated that genes involved in cell wall biosynthesis and integrity were differentially expressed following challenge with caspofungin (Reinoso-Martin et al., 2003). The protein kinase C (PKC) pathway is required for caspofungin tolerance, and modulation of PKC target genes requires the transcription factor

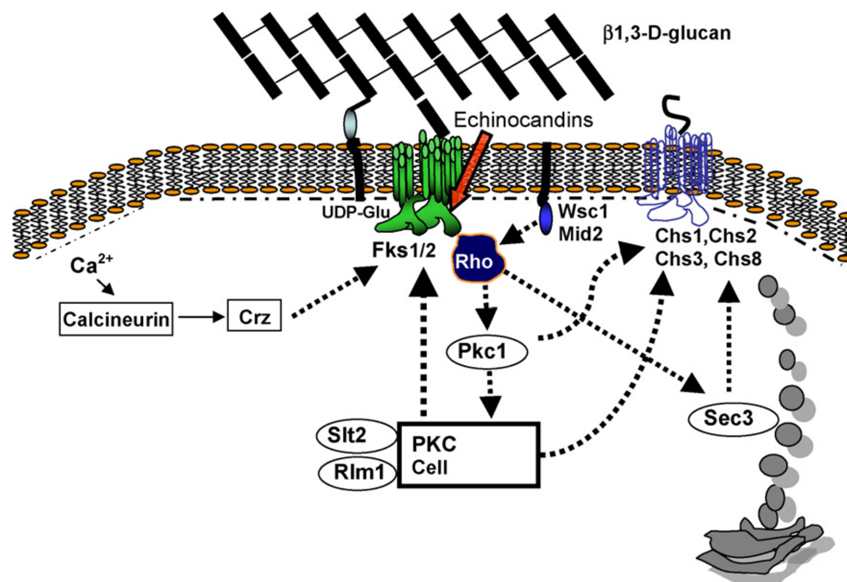

Fig. 1. Schematic diagram depicting interactions between cell wall biosynthesis and cell integrity/stress pathways in yeasts. Glucan synthase (GS), comprised of catalytic subunit Fks1 (or Fks2) and its activator Rho1, is responsible for synthesis of the principal cell wall component β-1,3-glucan. It is the target of inhibition for echinocandin drugs. The GTP binding protein Rho1 plays a central role in cell wall biosynthesis and regulation through a direct activation of GS. It also mediates a wide range of cellular responses through Pkc1 and the protein kinase C (PKC) cell integrity pathway, as well as through the secretary (Sec3) pathway. External stimuli, including cell wall stress due to echinocandin action, are mediated in part through receptors Wsc1 and Mid2, which interact with Rho1 leading to a range of secondary interactions. The responses include activation of the PKC cell integrity pathway and Slt2, as well as modulation of chitin synthase genes Chs1/2/3/8. Feedback of the cell integrity pathway to cell wall biosynthesis and modeling also involves transcription factor Rlm1, which regulates genes encoding enzymes involved in cell wall biosynthesis. Ca^{2+}-calcineurin indirectly affects Fks1 through Crz1. Overall, these complex pathways allow the cells to adapt to echinocandin action and the ensuing cell wall stress via compensatory cell wall biosynthesis and remodelling (schematic adapted from Lesage and Bussey, 2006; Selvaggini et al., 2004; Hahn and Thiele, 2002).

Rlm1, which controls expression of several cell wall biosynthesis and maintenance genes (Reinoso-Martin et al., 2003). Caspofungin-mediated cell wall damage induces Wsc1 as a dedicated sensor and activator of Slt2 stress signaling, which promotes a protective response through the activated salvage pathway for de novo cell wall synthesis (Fig. 1). The assembly and regulation of β-1,3-D-glucan was explored separately with a large collection of *S. cerevisiae* deletion mutants in which synthetic interactions were scored with genes encoding subunits of the β-1,3-D-glucan synthase (*FKS1, FKS2*), the glucan synthesis regulator (*Smi1/Knr4*), and a β-1,3-glucanosyltransferase (*Gas1*). *FKS1, FKS2, GAS1*, and *SMI1* were found to be connected to 135 genes in 195 interactions, including core genes involved in cell wall assembly and polarized growth (Lesage et al., 2004). A complex network exists that has the potential to modulate the cell's response to cell wall stress from echinocandin action.

5.1. Genomic studies provide evidence for altered echinocandin sensitivity

To better understand the potential of complex cellular pathways to influence drug susceptibility, a library of *S. cerevisiae* knockout mutants was analyzed for either hypersensitivity or reduced susceptibility (Markovich et al., 2004). Gene knockouts resulting in increased sensitivity (4–8-fold) included those involved in cell wall and membrane function, notably in the PKC integrity pathway, chitin and mannan biosynthesis, ergosterol biosynthesis, vacuole and transport functions, and general control of transcription. Genes involved in decreased sensitivity (four-fold) included those involved in cell wall function, signal transduction, and vacuole function (Markovich et al., 2004). In a related genome study, deletions in 52 genes led to caspofungin hypersensitivity, while deletion of 39 genes resulted in reduced susceptibility to caspofungin (Lesage et al., 2005). Modulation of susceptibility resulted from an overlapping set of genes involved in FKS regulation, compensatory chitin synthesis, protein mannosylation, and the Pkc1-dependent cell integrity pathway (Fig. 1). Overall, the data suggests that a complex network of pathways have the potential to influence drug susceptibility to echinocandins. The drug resistance observed in these studies was modest, and it could be most aptly described as drug tolerance, as would be expected from adaptive cellular physiology due to environmental stress. Such cellular behavior may account for elevated MIC values for some *Candida* isolates and the observed high-dose paradox in which cells have less susceptibility to high levels of drug (Stevens et al., 2005). Similarly, it was reported that overexpression of Sbe2, which encodes a Golgi protein involved in the transport of cell wall components, confers low level resistance (Osherov et al., 2002). These pathways have the potential to contribute to clinical resistance. Yet they are unlikely by themselves to produce the level of resistance that will result in clinical failure. Thus, it is important to distinguish these lower-level drug tolerance or adaptive mechanisms from bona fide drug resistance that can result in treatment failure.

6. Considerations for a drug resistance mechanism of clinical importance

An understanding of resistance mechanisms that can result in clinical failure are just starting to emerge. Unlike azole class drugs, drug efflux transporters do not appear to be a factor. A report linking caspofungin reduced susceptibility to overexpression of drug pump Cdr2 in *C. albicans* produced only a moderate increase in MIC (Schuetzer-Muehlbauer et al., 2003). The echinocandins are poor substrates for most multidrug efflux transporters. In a comprehensive study, it was found that *C. albicans* and *S. cerevisiae* strains hyper-expressing fungal ATP-binding cassette (ABC) or major facilitator superfamily (MFS) transporters showed only weak changes in echinocandin susceptibility (Niimi et al., 2006). Studies involving fluconazole-resistant strains of *C. albicans* expressing high levels of *CDR1*, *CDR2* and/or *MDR1* were fully susceptible to caspofungin (Bachmann et al., 2002a), and a survey of 351 fluconazole-resistant *Candida* isolates were fully inhibited by caspofungin at conventional MIC90 dose levels (Pfaller et al., 2003a, b). A mechanism for clinically relevant resistance is expected to reflect direct changes in drug–target interactions. Unfortunately, the mechanistic nature of echinocandin inhibition of glucan synthase is poorly understood, as is biochemistry of the glucan synthase complex (Douglas, 2001).

7. Fks1 modification in *Candida*- a mechanism for clinical drug resistance

Early genetic studies by Myra Kurtz and Cameron Douglas (Merck Research Labs) with caspofungin in *S. cerevisiae* (Douglas et al., 1994a, b) and *C. albicans* (Douglas et al., 1997) indicated that Fks1, the major subunit of glucan synthase, is the presumed target of the echinocandins. These genetic studies suggested that target-site modification was a likely cause of reduced susceptibility. Spontaneous *C. albicans* mutants resistant to N_2-PnB0, an analog of caspofungin, were selected that yielded significantly elevated MIC values (>70-fold) and IC_{50} values (>1000-fold) for inhibition of glucan synthase activity in crude microsomal fractions (Kurtz et al., 1996). Furthermore, the mutants were found to require 20-fold higher doses of drug in a mouse model to achieve 99% reduction in kidney burden (ED_{99}) than equivalent infections with wild-type *C. albicans* (Douglas et al., 1997). Mutations that confer reduced echinocandin susceptibility in *S. cerevisiae* and *C. albicans* mapped to *FKS1* (Kurtz et al., 1996; Douglas et al., 1997). Clinical isolates of *C. albicans* displaying highly elevated MIC values for caspofungin were found to contain *fks1* mutations. These strains displayed 2–3 log shifts in ED_{99} values in a murine

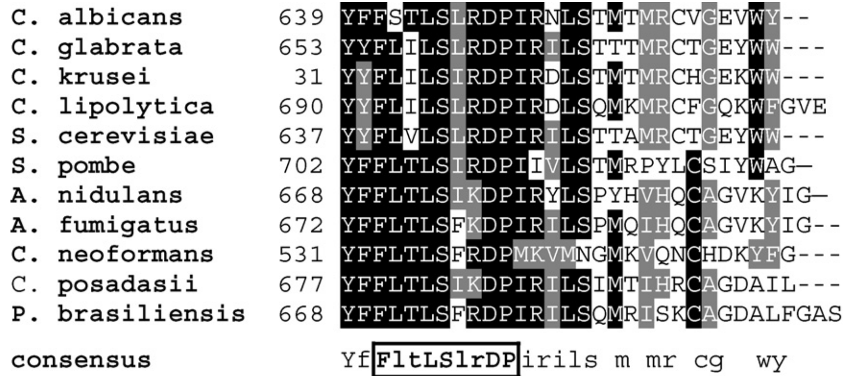

Fig. 2. Amino acid alignment of Fks "hot-spot" 1 regions from 11 different fungi. The dark shading represents identical amino acids, while the gray shading shows homologous residues. NCBI accession numbers for *FKS* genes were as follows: D88815 (Ca), XM446406 (Cg), AF159533 (Cp), AB091349 (Cn v.n), U08459 (Sc), AF198090 (Cl), DQ017894 (Ck), AF148715 (Pb), D78352 (Sp), U51272 (An), and U79728 (Af).

model of disseminated candidiasis, and an equivalent shift in IC50 values for inhibition of glucan synthase (Park et al., 2005). Amino acid substitutions F639I, V641K, D646Y (*S. cerevisiae*) and S645F, S645P, S645Y (*C. albicans*) helped define a region (Ca*Fks1* Phe641-Pro649) termed "hot-spot 1 (HS1)" that confers reduced susceptibility to caspofungin (Park et al., 2005). This region is highly conserved amongst the Fks family (Fig. 2). *Fks1* mutations were confirmed to confer resistance following their introduction into a susceptible tester strain. These findings were consistent with the report of an independent *S. cerevisiae* mutant resistant to the cyclic lipopeptide arborcandin C, which had a L642S substitution in the same conserved region (Ohyama et al., 2004). Genetic studies involving another *S. cerevisiae* mutant containing a R1357S substitution helped identify a separate region conferring reduced susceptibility, which is termed "hot-spot" 2 (HS2) (Park et al., 2005).

Clinical isolates of *C. albicans* obtained from patients who failed or responded poorly to therapy showed amino acid substitutions at Ser645 within HS1 of Fks1. Such strains are still relatively rare, and hot-spot mutations have only been observed in resistant strains (Park et al., 2005). In one patient, multiple *C. albicans* strains were isolated from different sites and were found to have distinct Fks1 substitutions, S645F or S645P, conferring reduced susceptibility (Fig. 3). MLST analysis indicated that the mutant strains were genotypically identical to one another, as well as to susceptible strains from the same patient, suggesting that resistance arose following different exposures (selective pressure) to drug (Park et al., 2005). HS1 mutations conferring resistance have been identified in several *C. albicans* strains from patients (Laverdiere et al., 2006; Miller et al., 2006), as well as in a *C. krusei* strain (Kahn et al., 2007). A HS2 mutation equivalent to R1357S from *S. cerevisiae* was identified in a *C. krusei* strain isolated from a patient refractory to therapy (Park et al., 2005).

7.1. Fks1 mutants display high echinocandin MIC values

A range of mutations have been observed in the hot-spot regions of *FKS1* from clinical isolates of *C. albicans* that show high MIC values for caspofungin. These mutations include non-conservative amino acid substitutions within HS1 (Phe641, Leu642, Thr643, Ser645, Arg647, Asp648, Pro649) and HS2 (Arg1352). Mutations at the C-terminal end of HS1 show the weakest phenotypes (caspofungin MIC ≤2 μg/ml). Despite the wide range of mutations, the highest frequency of substitutions was found at Ser645 (S645P, S645F or S645Y) within HS1 (Park et al., 2005; Balashov et al., 2006). The mutations are dominant, displaying phenotypic resistance when present in both homozygous and heterozygous forms in *C. albicans*. The strains are cross-resistance to micafungin and anidulafungin, indicating that the Fks1 modification mechanism broadly encompasses the

				Glucan Synthesis	Mouse Model (Burden)
Patient	**Isolate**	***Fks1* *Change***	**MIC (μg/ml)**	**IC_{50} (ng/ml)**	**ED_{90} (mg/kg/day)**
A	*C. albicans*/ 16998	None	0.5	0.56	< 0.06
A	*C. albicans*/ 18195	None	0.25	0.91	0.01
A	*C. albicans* 16996	S645F	> 8	162	1.09
A	*C. albicans*/ 16997	S645P	> 8	1997	9.98

Fig. 3. Summary of resistance properties associated with clinical isolates of *C. albicans* from a single patient. Clinical isolates obtained from a single patient were evaluated for MIC values according to CLSI protocol M27A2, mutations in HS1 and HS2, IC50 values for inhibition of glucan synthase, and ED90 values for reduction of kidney burdens in a murine candidiasis model (adapted from Park et al., 2005).

Table 1
Echinocandin cross-resistance in clinical isolates of *Candida albicans*[a]

Strain	Mutation	Caspofungin MIC (μg/ml)	Fold change	Micafungin MIC (μg/ml)	Fold change	Anidulafungin MIC (μg/ml)	Fold change
C. albicans ATCC90028 (WT)	wt	0.125	1	0.016	1	0.016	1
C. albican M85	S645F	4.0	32	2.0	125	0.5	31
C. albicans M86	S645P	16.0	128	2.0	125	0.3	16
C. albicans M89	S645Y	16.0	128	2.0	125	0.5	31
C. albicans NR3	S645Y	16.0	128	2.0	125	0.5	31
C. albicans M195	S645F	4.0	32	1.0	31	0.5	31
C. albicans M196	S645F	4.0	32	1.0	31	0.5	31
C. albican C31	S645P	16.0	128	16.0	1000	2.0	125
C. albicans C41	S645P	16.0	128	16.0	1000	2.0	125

[a] Susceptibility testing performed as per CLSI protocol M27A2.

class of echinocandin drugs (Table 1) (Perlin et al., 2005). The fold-change relative to a fully susceptible wild type strain was consistently greater for micafungin and caspofungin. MIC values for caspofungin (4 to >16 μg/ml) and micafungin (1–16 μg/ml) were higher than anidulafungin, which retained the lowest overall MIC values (MIC ∼0.5–2 μg/ml). The clinical significance of these differences remains to be determined. Strains resistant to echinocandin drugs are fully susceptible to azole and polyene antifungal drugs indicative of class-specific resistance.

7.2. *Fks1 hot-spot mutations alter the kinetic inhibition properties of glucan synthase*

The most compelling evidence for Fks1 modification as a principal mechanism of resistance is the biochemical observation that characteristic hot-spot mutations significantly decrease the drug sensitivity of glucan synthase. In an *in vitro* assay of glucan synthase activity, the caspofungin inhibition profile for mutant enzyme from *C. albicans* containing a S645P substitution was shifted several log orders relative to wild type enzyme (Fig. 4A). In practical terms, the mutant enzyme is 1000-fold less sensitive to drug effectively rendering it drug resistant. The biochemistry also helps explain how mutations in a single *FKS1* allele of *C. albicans* can cause resistance. In this case, both the mutant and wild type enzymes are expressed resulting in biphasic inhibition (Fig. 4B). At low drug concentrations, the wild type enzyme is inhibited but not the mutant enzyme, which requires higher concentrations of drug to achieve inhibition. The behavior depicted by caspofungin is closely mimicked by both anidulafungin and micafungin. Substitutions at the Ser645 locus have the most pronounced affect on inhibition of glucan synthase; weaker biochemical phenotypes are observed at the periphery of HS1 (Fig. 5).

7.3. *fks1 mutants show reduced echinocandin susceptibility in animal models*

Murine infection models have been extremely helpful in assessing the relative susceptibility of *Candida* and *Aspergillus* strains to echinocandin drugs (Kurtz et al., 1996; Bowman et al., 2006). The amount of drug required to reduce kidney fungal burdens by 99% (ED99) in a candidiasis model has been used as a relative measure of *in vivo* susceptibility. The ED99 values were found to increase 100–1000-fold relative to wild type for clinical isolates of *C. albicans* containing S645Y or S645P substitutions in both alleles (Fig. 3) or a lab strain with a S645P in one allele and a disruption of the wild type allele (Park et al., 2005). A heterozygous strain containing both a wild type allele and a S645P allele showed a resistant phenotype in MIC tests and mixed IC50 values for glucan synthase reflecting wild type and mutant enzymes. It required 70-fold more drug to achieve 90% kidney burden reduction (Park et al., 2005). It is apparent that such models provide an important validation for potentially

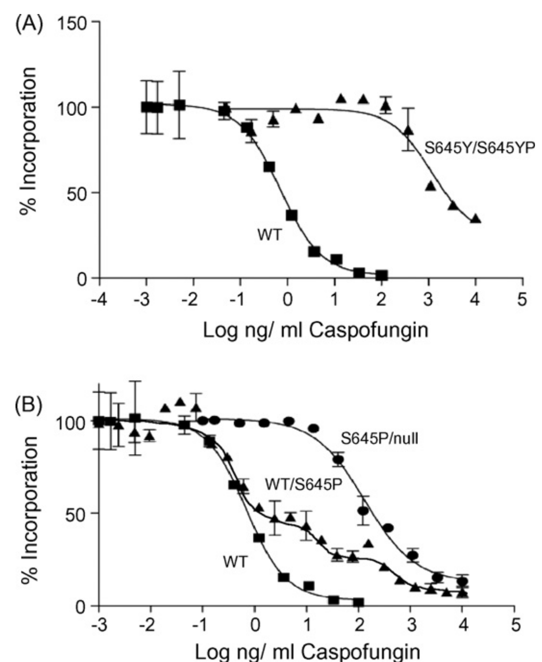

Fig. 4. Inhibition of glucan synthase from *fks1* mutant strains by caspofungin. GS activity was assessed by the incorporation of [^{3}H]-glucose into radiolabeled product. (A) Caspofungin inhibition of wild type (squares) and a homozygous S645Y/S645Y strain (triangles). (B) Caspofungin titration curves for the *C. albicans* wild type (squares), a heterozygous S645P/WT strain (triangles) and homozygotic pseudo-haploid S645P/null strain (circles) (adapted from Park et al., 2005).

Organism	Hot Spot1	Hot Spot 2
C. albicans	FLTLSLRDP	DWIRRYTL
C. kruseii	FLILSIRDP	DWIRRYTL
C. glabrata	FLILSIRDP	DWIRRYTL
C. guilliermondii	FMALSIRDP	DWIRRYTL
C. tropicalis	FLTLSLRDP	DWIRRYTL
C. lypolytica	FLILSIRDP	DWIRRCVL
C. parapsilosis	FLTLSIRDA	DWIRRYTL
C. dubliniensis	FLTLSIRDP	DWIRRYTL

Resistant phenotype   No phenotype   Weak Resistance

Fig. 5. Summary of hot-spot 1 and 2 substitutions associated with resistance in diverse *Candida* spp. Amino acid substitutions in HS1 and HS2 were evaluated following DNA sequence analysis *FKS1* genes from susceptible and resistant *Candida* spp. (Park et al., 2005). MIC values were determined according to CLSI protocol M27A2. Mutations yielding resistant strains (caspofungin MIC > 2 μg/ml) are shown in red; those producing weak resistance (caspofungin MIC = 1–2 μg/ml) are shown in yellow, and mutations with no resistance phenotype are shown in green.

resistant strains. Such an approach can distinguish between bona fide resistance mechanisms that render strains refractory from therapy and those that reflect adaptive mechanisms, which may respond to therapy in the host.

7.4. Fks1, the resistance target

FKS1 encodes a 215-kDa integral membrane protein that is the major subunit of the glucan synthase complex (Mio et al., 1997). It is postulated to be the catalytic subunit, although little is known about the structure and function of glucan synthase (Douglas, 2001). Resistance mutations in HS1 fall within an 89 amino acid domain that is predicted to lie on the cytoplasmic face of the plasma membrane. It is unclear whether this region comprises part of the binding domain for echinocandin drugs or indirectly blocks drug action. A definitive understanding of resistance awaits an elucidation of the molecular structure properties of glucan synthase and its interaction with echinocandin drugs.

8. Fks1 as a universal resistance mechanism

The Fks1 mechanism extends to other non-*C. albicans* spp. including clinical isolates of *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. tropicalis* and *C. dubliniensis*, which have been shown to become resistant through mutations in the highly conserved “hot-spot” regions showing comparable mutations to *C. albicans* (Perlin, unpublished; Katiyar et al., 2006; Kahn et al., 2007) (Fig. 5). In *C. glabrata*, a mutation in HS1 of *FKS2*, a paralog of *FKS1*, was responsible the resistant phenotype (Katiyar et al., 2006). Biochemical studies have confirmed the conserved behavior of hot-spot mutations in altering the sensitivity of glucan synthase for echinocandin drugs (Kahn et al., 2007; Perlin et al., 2005). The universality of Fks1 modification as a mechanism for fungal resistance to echinocandin drugs was demonstrated following the engineering of the equivalent *C. albicans fks1*-S645Y mutation into the mould A. *fumigatus*, which was found to confer reduced susceptibility (Gardiner et al., 2005); such resistance in *Aspergillus* has only been observed in laboratory strains.

9. Amino acid polymorphisms in Fks1 account for intrinsic reduced susceptibility of *C. parapsilosis* and *C. guilliermondii*

Surveillance studies have shown that some *Candida* spp., such as *C. parapsilosis* and C. *guilliermondii*, display higher echinocandin antifungal MIC values (MIC 0.5–8 μg/ml) relative to other *Candida* species (Espinel-Ingroff, 2003; Pfaller et al., 2003a, 2006). The clinical significance of this intrinsic reduced susceptibility is unclear since patients with these infecting strains can be successfully treated with echinocandin drugs at standard dosages (Mora-Duarte et al., 2002; Bennett, 2006). However, the possibility remains that higher baseline levels for susceptibility may predispose such isolates for acquired resistance. The mechanism for this intrinsic reduced susceptibility appears to be a direct reflection of amino acid polymorphisms within the Fks1 HS1/HS2 regions (Fig. 5). In *C. parapsilosis*, a naturally occurring polymorphism occurs that results in the substitution of alanine for proline at Pro649 (*C. albicans* equivalent). A comparable mutation in *C. albicans* Fks1, P649C, confers an equivalent level of reduced susceptibility (Park et al., 2005). *C. orthopsilosis* and *C. metapsilosis*, which are closely-related to *C. parapsilosis* (Tavanti et al., 2005), contain the P649A polymorphism and show reduced susceptibility (Balashov et al., 2006). Furthermore, *C. orthopsilosis* has an additional I1359V polymorphism in HS2, resulting in even slightly higher MIC values (Park et al., 2005). The latter Fks1 polymorphism is also observed in *S. cerevisiae*, which shows higher MIC values relative to highly susceptible *Candida* spp. C. *guilliermondii* shows several amino acid polymorphisms in HS1 (Fig. 5). However, only the conversion of a highly conserved leucine to methionine at Leu642 (*C. albicans* equivalent) appears to be important for reduced susceptibility. Similarly, HS2 mutations in *C. lypolytica* display a weak phenotype. Several species show a polymorphism at the equivalent of *C. albicans* Thr643, but substitutions at this position do not confer reduced susceptibility. Overall, it appears that naturally occurring Fks1 polymorphisms in HS1 and HS2 of certain non-*albicans Candida* spp. can account for reduced susceptibility to echinocandin drugs.

10. Mechanism of inherent resistance in non-*Candida* spp. remains unclear

Several clinically important fungi including *Cryptococcus neoformans*, *Fusarium* spp., *Scedosporium* spp. and members of the Zygomycetes family are resistant to echinocandin

drugs (MIC> 16 μg/ml)(Pfaller et al., 1998; Singh et al., 2005). The mechanism of this inherent resistance is not related to target insensitivity, as glucan synthase activity from *Cryptococcus* and several moulds were strongly inhibited by caspofungin in *in vitro* assays (Maligie et al., 2005; Kahn et al., 2006). However, it should be noted that like *Aspergillus* spp., some moulds (*Curvularia*, *Alternaria*, *Acremonium, Trichoderma*) showed sensitivity to caspofungin in minimum effective concentration (MEC) assays (Kurtz et al., 1994) using morphological endpoints (Kahn et al., 2006). Nevertheless, organisms like *Cryptococcus* are effectively resistant, despite the fact that glucan synthase is fully inhibited by drug (Maligie et al., 2005), indicating the presence of an alternative mechanism impendent of Fks1. As the echinocandins require transport into the cell to their site of action, the surface properties of fungi may contribute to resistance. It has been suggested that melanization can reduce the susceptibilities of *Cryptococcus neoformans* and *Histoplasma capsulatum* to antifungal drugs including caspofungin (van Duin et al., 2002). Other factors such drug modification or metabolism may also play a role, although there is no direct evidence to support these mechanisms.

11. Is the "paradoxical effect" drug resistance?

The "paradoxical effect" refers to the growth of echinocandin-susceptible organisms at highly elevated drug concentrations far in excess of the MIC. The phenomenon was first documented by Stevens et al. (2004) as turbid growth observed with a subset of *C. albicans* isolates at high concentrations of caspofungin. These strains demonstrated a normal susceptibility pattern with a typical low MIC (submicromolar), but then paradoxically at high levels of drug (>16 μg/ml) showed break-through growth. The resistance-like behavior was conditional, as strains undergoing paradoxical growth showed normal susceptibility properties when cultured and re-challenged with drug. Paradoxical growth is not related to *FKS1* mutations or modification of the echinocandin sensitivity of the glucan synthase complex nor to its upregulation in the presence of drug (Stevens et al., 2005). Concentration-dependent drug aggregation resulting in a less active chemical species could contribute to the phenotype. However, the growth behavior was consistent with adaptive stress responses observed in *S. cerevisae* and *C. albicans* that resulted in reduced susceptibility (Lesage et al., 2004, 2005). Biochemical analysis of the cell wall of a *C. albicans* strain undergoing paradoxical growth revealed a large 898% increase in chitin content, which would appear to compensate for a decrease in β-1,3 glucan and β-1,6-glucan following caspofungin action (Stevens et al., 2006). The *in vivo* significance of the paradoxical effect remains unclear, since the drug levels required exceed normal dosing levels. Apparent paradoxical behavior was reported in a murine model of pulmonary aspergillosis (Wiederhold et al., 2004), although it was not reproduced in a systemic candidiasis mouse model utilizing strains displaying paradoxical behavior *in vitro* (Clemons et al., 2006). The relevance to patient therapy has not been observed except for a report indirectly linking an unusual elevation of circulating antigen in a patient with invasive aspergillosis following echinocandin therapy (Klont et al., 2006). For now, the "paradoxical effect" may have more significance under laboratory conditions, but it does highlight the need to assess other potential resistance mechanisms.

12. Criteria for clinical drug resistance

There is now compelling evidence to support the premise that echinocandin resistance in clinical isolates is likely to arise from genetic changes in two defined regions of the highly conserved *FKS1* gene. Elevated MIC alone is an insufficient criterion to define resistance. Rather, a combination of (1) elevated MIC, (2) characteristic mutations in *FKS1* and (3) a several log-fold shift in the drug IC50 value for inhibition of glucan synthase is a more definitive predictor of resistance. While other cell response/adaptive mechanisms can result in elevated MIC values, only the Fks1 mechanism is firmly associated with clinical resistance.

13. Conclusion and perspective

Presently, clinical failures due to resistance are relatively rare events. Yet, as the number of patients exposed to echinocandin therapy broadens, the probability for resistance will increase. However, given our present experience based on the treatment of hundreds of thousands of patients, it is likely that a relatively low incidence of resistance will continue to be observed. Finally, it remains to be seen if there will be a shift toward infections with less susceptible, but rarer species, as was observed following the extensive use of azole drugs.

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