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BACTERIAL AND FUNGAL PATHOGENESIS - REVIEW

Caspofungin resistance in *Candida albicans*: genetic factors and synergistic compounds for combination therapies

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Abstract

Caspofungin and other echinocandins have been used for the treatment of human infections by the opportunistic yeast pathogen, *Candida albicans*. There has been an increase in infections by non-*albicans Candida* species such as *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, and *Candida auris* in clinical or hospital settings. This is problematic to public health due to the increasing prevalence of echinocandin resistant species/strains. This review will present a summary on various studies that investigated the inhibitory action of caspofungin on 1,3-β-D-glucan synthesis, on cell wall structure, and biofilm formation of *C. albicans*. It will highlight some of the issues linked to caspofungin resistance or reduced caspofungin sensitivity in various *Candida* species and the potential benefits of antimicrobial peptides and other compounds in synergy with caspofungin.

Keywords Echinocandins · Susceptibility · Biofilms · Antimicrobial/antifungal peptides · β-1,3-glucan

Introduction

Caspofungin ((MK-0991; L-743,872) is a fungicidal, water-soluble semisynthetic echinocandin that inhibits synthesis of β-1,3-D-glucan, a main structural component of the fungal cell wall (Fig. 1) [1]. Apart from caspofungin, micafungin and anidulafungin are two additional echinocandins approved for used by the US Food and Drug Administration (FDA) (Fig. 1) [2, 3]. Though these echinocandins have different side chains, there have three common components essential for their activities [2]. They are as follows: (a) a homotyrosine amino acid residue essential for the antifungal activity and for the inhibition of the glucan synthase enzyme; (b) proline residues which enhances the antifungal potency of the echinocandin drugs; and (c) the hydroxyl groups in the echinocandin B nucleus which improve their stability and increase their water solubility [2]. Currently, a novel echinocandin derived from anidulafungin, rezafungin (also known as CD101), is undergoing phase-III trials [4–6].

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Caspofungin and β-(1,3)-glucan synthase

The model yeast *Saccharomyces cerevisiae*

Saccharomyces cerevisiae cell wall comprises an inner layer containing the polysaccharides β-1,3-glucan, β-1,6-glucan, and chitin and while mannoproteins act as "fillers" affecting cell wall porosity and are in the outer layer the yeast cell wall (Fig. 2) [7, 8]. In *S. cerevisiae*, β-1,3-glucan synthase has been shown to catalyze the formation of a β-1,3-glucan polymer, a major component of the fungal cell wall (Fig. 2). In yeast and many fungal species, the β-1,3-D-glucan chains form a solid three-dimensional matrix, which gives the cell wall its shape and mechanical strength. Kollár et al. [9, 10] demonstrated that chitin (a linear polymer composed of β-(1,4)-linked N-acetylglucosamine subunits) is glycosidically linked to nonreducing branches of the β1,3-glucan and β1,6-glucan in *S. cerevisiae*. Later work by Cabib et al. [11] found that β-1,3-D-glucan formed a noncovalent complex with chitin. β-1,6-glucan plays a role in the organization of the yeast cell wall by interconnecting all other wall components into a lattice by attaching mannoproteins via their glycosylphosphatidylinositol (GPI) glycan remnant to β-1,3-glucan and chitin [12, 13].

β-1,3-glucan synthesis in *S. cerevisiae* involves the integral membrane proteins Fks1p and Fks2p which act

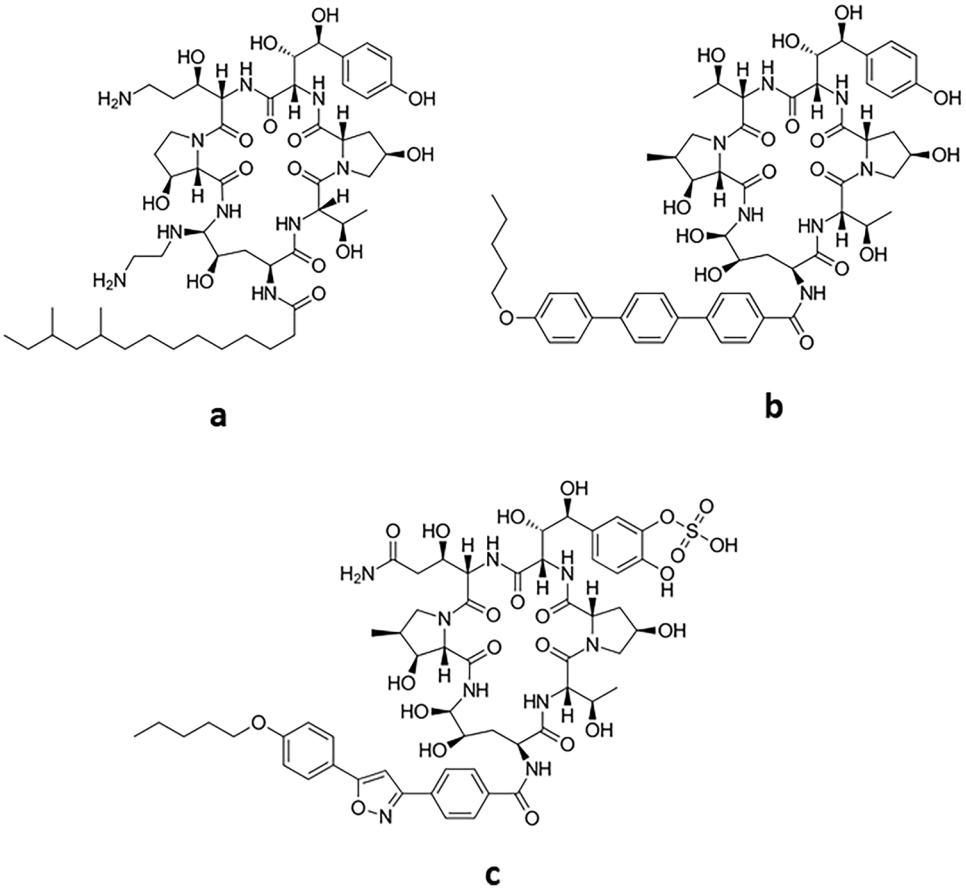


Fig. 1 Chemical structure of three echinocandins. **a** Caspofungin. **b** Anidulafungin. **c** Micafungin

as subunits of the β-1,3 glucan-synthase enzyme complex (Fig. 2) [14–17] and the regulatory subunit encoded by *RHO1* [18–20]. Another FKS homolog, *FKS3*, was required for normal spore wall formation while *FKS2* (*GSC2*) was the primary β-1,3-glucan synthase in *S. cerevisiae* sporulation [21]. Interestingly, work on *FKS1* and *FKS2* by Mazur and colleagues [17] showed that not only *FKS1* was affected by the echinocandin but that *FKS2* was also sensitive to the echinocandin L-733,560, due to the increased sensitivity of fks1 null mutants to this drug. However, work by Douglas et al. [22] and El-Sherbeini and Clemas [23] presented evidence of mutations within the FKS1 gene that could affect the sensitivity to the semisynthetic pneumocandin B, L-733,560 in *S. cerevisiae*. Screening of the *S. cerevisiae* deletion mutant collection for altered sensitivity to the drug found that deletions in 52 genes led to caspofungin hypersensitivity and those in 39 genes to resistance [24]. Use of a genomic approach to identify genes involved in caspofungin susceptibility in *S. cerevisiae* showed that the disruption of 20 genes involved in key functions such as in cell wall and membrane function, chitin and mannan biosynthesis, vacuole, and transport functions led to increased caspofungin sensitivity [25]. For example, the loss of *ERG3*, a C-5 sterol desaturase which catalyzes the introduction of a C-5(6) double bond into episterol, a precursor in ergosterol biosynthesis, led to increased caspofungin resistance in *S. cerevisiae* [25]. Furthermore, Carolus et al. [26], Rybak et al. [27], and Spettel et al. [28] identified that the disruption/mutation in *ERG3* resulted in increased resistance to azole and echinocandin antifungals in *C. albicans*, *Candida auris*, and *Candida parapsilosis*.

Candida albicans

Since identifying the *FKS1* homolog in *C. albicans* [29] and demonstrating that the non-competitive binding ability of echinocandins to *FKS1* gene in *C. albicans* [30], echinocandins including caspofungin have been used in the treatment of *Candida* spp. and other fungal infections [1]. *FKS2* and *FKS3* are also found in *C. albicans* and in *C. albicans* mutants lacking either *FKS2* or *FKS3* that *FKS1* expression was upregulated suggesting that *FKS2* and *FKS3* act as negative regulators of *FKS1* [31].

However, there have been many reports documenting echinocandin resistance in *Candida* species [32–35]. Such echinocandin resistance in *Candida* spp. is due to point mutations in 2 highly conserved "hot spot" regions, i.e., HS1 and HS2 of the *FKS1* gene [32, 33, 36]. Previous studies by

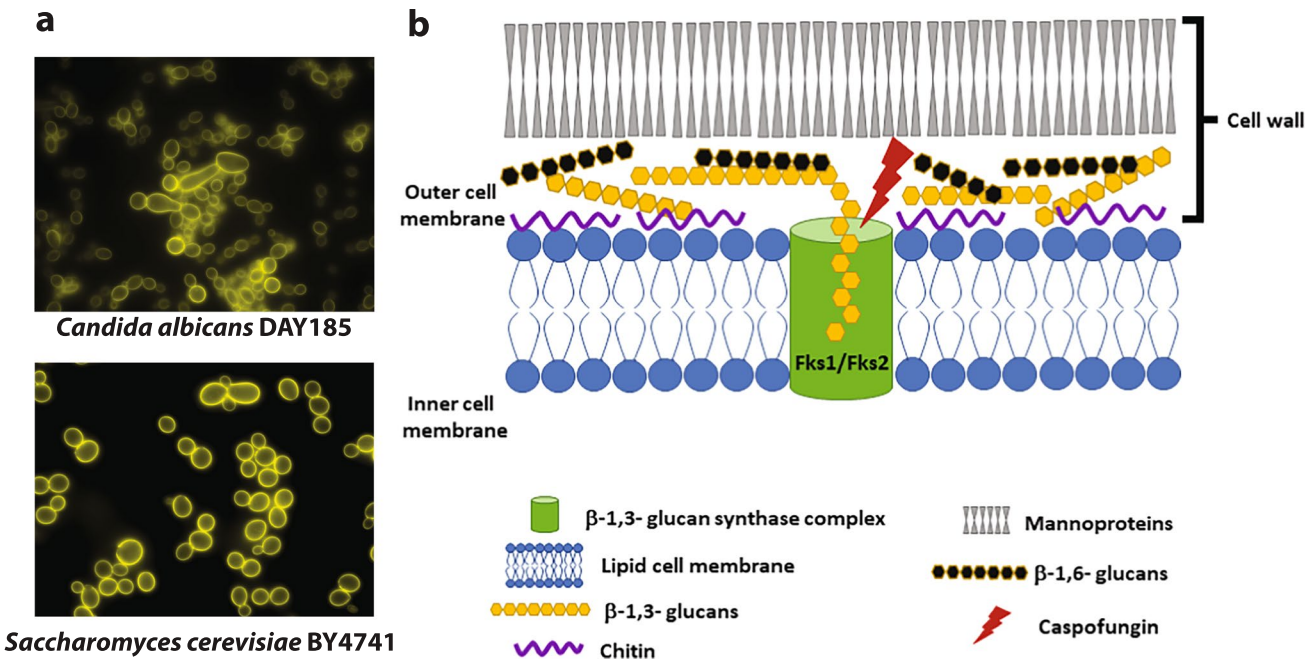


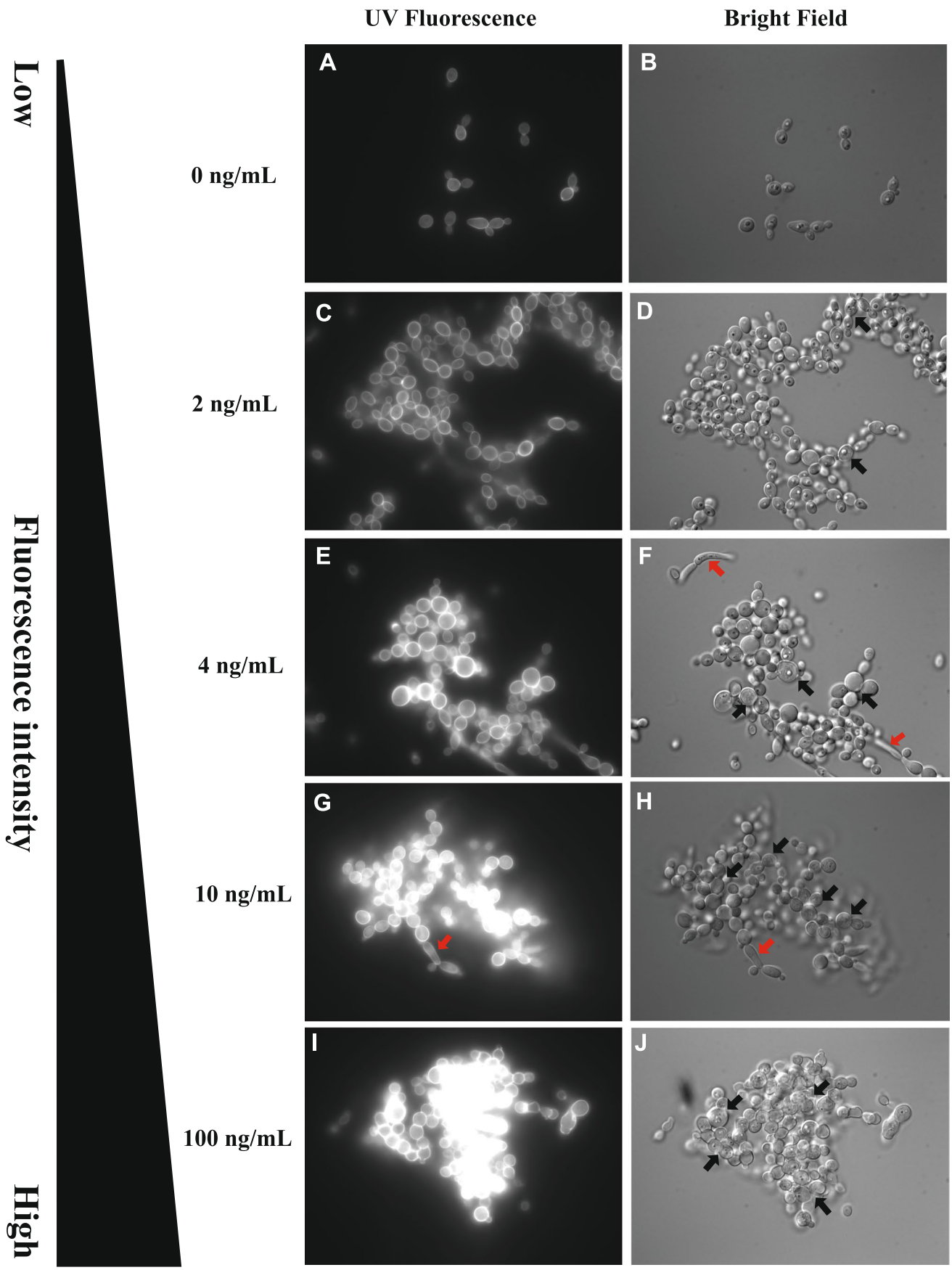
Fig. 2 Caspofungin's target in *Saccharomyces cerevisiae* and *Candida albicans*. **a** Wild-type strains *Saccharomyces cerevisiae* BY4741 and *Candida albicans* DAY185 were stained with fluorochrome Aniline Blue (AB; Biosupplies Australia PTY Ltd) for β-1,3-glucan detection in yeast cell wall (in yellow) under UV fluorescence. **b** Schematic diagram of the yeast cell wall membrane highlighting the inhibitory action of caspofungin on β-1,3-glucan synthesis (red lightning bolt)via noncompetitive inhibition of the β-1,3-glucan synthase complex (Fks1p and Fks2p; green cylinder)

Park and colleagues [37] demonstrated that substitutions in the Fks1p subunit of GS in *S. cerevisiae* and four clinical *C. albicans* isolates and a *Candida krusei* isolate were sufficient to confer reduced susceptibility to echinocandins.

The haploid *Candida glabrata*, an evolutionarily close relative of *S. cerevisiae*, causes mucosal and systemic infections especially in the human immunodeficiency virus-infected population [38]. Its genome has also three GS homologs, *FKS1*, *FKS2*, and *FKS3* [39]. In this particular species, mutations in both *FKS1* and *FKS2* but not *FKS3* have been associated with echinocandin resistance [40, 41]. Clinical *C. glabrata* isolates displaying reduced susceptibility or resistance to anidulafungin, caspofungin, and micafungin were not only due to *FKS1* modification but to point mutations in the *FKS2* [42]. Similarly, FKS1 hot spot 1 (HS1) and FKS2 HS1 have been identified in clinical *C. auris* isolates with reduced caspofungin susceptibility [43]. Genetic engineering for full-length replacement of the *FKS1* gene, containing FKS1 hotspot (HS) regions HS1 or HS2 mutations from *C. albicans*, the F659 deletion in the *FKS2* allele of *C. glabrata* and the naturally occurring P660A substitution in *FKS1* of *C. parapsilosis* respectively into *Candida lusitaniae* confirmed the role of FKS mutations associated with in vitro caspofungin resistance or reduced echinocandin susceptibility [44]. For additional information, Arendrup [45], Arendrup and Perlin [46], and Lackner et al. [47] listed mutations in *FKS1* and *FKS2* known to contribute to resistance in various *Candida* isolates. Another study using fluorescently labeling caspofungin, Jaber and colleagues [48] observed enhanced caspofungin uptake in the vacuoles of echinocandin-resistant *C. albicans* and *C. glabrata* strains with point mutations in the *FKS* genes compared to echinocandin-sensitive isogenic strains.

Other fungal pathogens displaying decreased echinocandin susceptibility have been reported such as *FKS1* mutation (E671Q) in *Aspergillus fumigatus* following anidulafungin exposure [49]. Mutations studies in *FKS1* and *FKS2* have helped in our understanding in the intrinsic resistance to echinocandins in *Scedosporium prolificans* and *Scedosporium apiospermum* [50], and *Fusarium solani* [51].

Another explanation for tolerance to caspofungin independent of *FKS1* (located on chromosome 1; Ch1) in *C. albicans* was observed in strains adapted in vitro to lethal doses of caspofungin [52]. Similarly in the previous study investigating *C. albicans* adaptation to toxic levels of the sugar L-sorbose [53], monosomy of chromosome 5 (Ch5) played a role in tolerance of *C. albicans* to caspofungin [52]. In addition, monosomy of the left arm and trisomy of the right arm of Ch5 were also detected in caspofungin-adapted *C. albicans* cells [52]. In such mutants, there was a downregulation of FKS genes hence a decreased amount of β-1,3-D-glucan content and an increase in chitin content


UV Fluorescence
Bright Field
Low
Fluorescence intensity
High
0 ng/mL
2 ng/mL
4 ng/mL
10 ng/mL
100 ng/mL
A
B
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◂**Fig. 3** Paradoxical growth of *Candida albicans* DAY185 cells treated with caspofungin at different concentrations stained with calcofluor white (CFW). *C. albicans* DAY185 grown in ½ strength PDB liquid 30 °C supplemented with caspofungin at 0, 2, 4, 10, and 100 ng/mL respectively were examined with an Olympus BX50 upright microscope with UPlanApo × 100/1.35 oil objective equipped with a filter cube U-MWU2 (excitation 330–385 nm/emission 420 nm/dichromatic mirror 400 nm). Images were acquired with SPOT Camera using Spot RT analysis software. Black and red arrows highlight the presence of enlarged and elongated yeast cells (putative pseudohyphae). Note the increase in flocculation and CFW fluorescence emitted by cells treated with an increasing concentration of caspofungin. All images were acquired after 8 ms

in the cell wall [52]. Further analyses of Ch5 found genes encoding positive regulators of caspofungin susceptibility, *CNB1* (the regulatory subunit of calcineurin B), and *MID1* (a putative stretch-activated Ca^{2+} channel) [52]. Negative regulators of caspofungin susceptibility *CHT2* (a GPI-dependent chitinase), *PGA4* (a GPI-anchored cell surface 1,3-β-D-glucanosyltransferase), and *CSU51* (a putative GPI-anchored protein) were also found in the same chromosome [52].

Effect of caspofungin on *C. albicans* cell surface

Caspofungin and other echinocandins not only disrupt β-1,3-glucan synthesis in *C. albicans* (Fig. 2), which can compromise cell wall integrity, but can also kill *C. albicans* in a dose-dependent manner [54] by causing metacaspase-dependent apoptosis [55, 56] and necrosis [55]. However, many studies have been focused on cell wall remodeling/mechanical strength and the paradoxical growth (PG) of *C. albicans* and various *Candida* species in response to caspofungin (Fig. 3) and how it could be linked to caspofungin or echinocandin resistance in *Candida* spp.

In four *Candida* species (*C. albicans*, *Candida orthopsilosis*, *C. parapsilosis*, and *Candida tropicalis*) exhibiting PG at approximately 16 μg mL^{-1} of caspofungin, PG cells had a decrease in β-1,3-glucan and an elevated chitin cell wall content compared to control untreated cells [57] (Fig. 3). In the same study, PG cells were altered in their morphology where they formed clumps of enlarged cells, had abnormal septa, and lacked filamentation [57] (Fig. 3). Another feature was that the control (WT) *C. albicans* 1399 cells had the characteristic two layered cell walls, i.e., an electron-dense outer layer and an inner layer of low electron density, while caspofungin-treated *C. albicans* 1399 PG cells had a decreased inner cell wall layer and a predominant more-electron-dense layer [57]. Prior work by Nishiyama and colleagues [58] observed similar morphological changes induced by 0.1 μg mL^{-1} or above of micafungin post 24 h in *C. albicans* ATCC 99,028, a strain known to be susceptible to micafungin. Later work by Rueda et al. [59] suggested that the increase in chitin in caspofungin-treated *C. albicans* may contribute to the protection of the cells from the fungicidal effect of the drug. Interestingly, paradoxical growth can be induced by the application of 4 μg mL^{-1} caspofungin for 24 h in *A. fumigatus* [60]. In this case, Wagener and Loiko [61] proposed that an increase in chitin in fungi in response to echinocandins could facilitate their survival upon the inhibition of β-1,3 glucan synthesis. However, paradoxical growth was not observed in a laboratory *A. fumigatus* strain Af293 and 7 clinical *A. fumigatus* strains in the presence of micafungin or anidulafungin [62].

Candida cell wall remodeling—elevated chitin

An atomic force microscopy (AFM) study investigating the effect of caspofungin (at 50 ng ml^{-1} for 2 h) on *C. albicans* cell wall surface demonstrated that there was a decrease in mechanical cell wall strength, i.e., caspofungin-induced softening of the cell wall due to a decrease in β-1,3 glucan content [63]. This would affect *C. albicans*' cell shape, mechanical rigidity, and resistance to osmotic pressure leading to the induction of osmotically fragile cells or swollen cells [63]. Another study using the AFM approach combined with Fourier transform infrared spectroscopy in attenuated total reflection mode (ATR-FTIR) investigated the effect in caspofungin on *Candida lusitaniae* CBS 6936 and its caspofungin-resistant mutant (bearing the mutation S645P in *FKS1*) [64]. They demonstrated that cell wall stiffening occurred only at low concentration of caspofungin (~0.06 μg mL^{-1}; 0.5 MIC) for WT cells, whereas it was observed at high concentration (~6.25 μg mL^{-1}; 50 MIC) for resistant strains [64].

To restore cell wall integrity, *C. albicans* stimulates chitin synthesis to enable cells to survive lethal concentrations of echinocandins in vitro [65]. Lee et al. [66] observed that high chitin *C. albicans* cells were less susceptible to caspofungin in mice infection and that *C. tropicalis*, *C. parapsilosis*, *Candida guilliermondii*, and *C. krusei* elevated their chitin cell wall content in response to caspofungin treatment [67]. Similar observations were documented in *C. auris* in response to caspofungin [43]. Protein kinase C (PKC), high osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase, and Ca^{2+}/calcineurin signaling pathways have been shown to regulate chitin synthases, *CHS1*, *CHS2*, *CHS3*, and *CHS8* gene expression and chitin synthesis in *C. albicans* and various mutants grown in YPD supplemented with different agents such as caffeine, cyclosporin A, the calcineurin inhibitor, FK506, and A23187 (Calcimycin) [68]. Recent work by Han et al. [69] on *C. albicans* SC5314 and its deleted mutants of β-1,6-glucan synthesis, *KRE6,* and *SKN1* found that cell wall chitin levels increased through the post-transcriptional regulation of the chitin synthase

Chs3 leading to the cell viability maintenance via Ca^{2+}/calcineurin and PKC signaling pathways. Furthermore, β-1,3-glucan had no role in compensating β-1,6-glucan synthesis in *C. albicans kre6*Δ/Δ *skn1*Δ/Δ cells as both the WT and mutants grew on YPD plates containing 0.064 μg mL^{-1} of caspofungin [69].

In *S. cerevisiae*, deletion of *FKS1* induced a compensatory mechanism, i.e., high rates of chitin synthesis due to a significant increase in CHS3 activity [70]. Calcofluor white staining of a β-1,3-glucan synthase knockout *fks1*::URA3 strain of *S. cerevisiae* displayed an elevated fluorescence signal (i.e., elevated chitin content) compared to the wild type [71]. Lesage et al. [24] suggested that there was functional link between chitin and glucan synthesis where an increased in chitin synthesis could compensate for defective β-1,3-glucan assembly for survival in the presence of caspofungin such as the deletion of *CHS3* or *CHS4–7* leading to caspofungin hypersensitivity in *S. cerevisiae*.

Cell–cell interactions

A review by Heredia et al. [72] highlighted that there are three transcription factors Sko1, Rlm1, and Cas5 that coordinate and regulate the caspofungin-induced cell wall damage response in *C. albicans*. The transcription factor Sko1 (ORF 19.1032) and its upstream regulator, the PAS-domain protein, and protein kinase Psk1 (ORF19.7451) were shown to be involved in *C. albicans*' wall regulatory pathway [73]. Deletion mutants *sko1* Δ/Δ and psk1 Δ/Δ were hypersensitive to 125 ng mL^{-1} caspofungin compared to its wild-type strain *C. albicans* DAY185 [73]. The same authors demonstrated that up to 79 caspofungin-responsive genes were regulated by Sko1 including key genes involved in cell wall biosynthesis *CRH11*, *MNN2*, and *SKN1* and in cell wall damage, *PGA13* [73]. The latter encodes a GPI protein and *pga13Δ* mutants exhibited a higher surface hydrophobicity, and increased adherence and flocculation (cell–cell interactions) [74]. Later work by Alonso et al. [75] highlighted that Sko1 mediated the hyphal formation in *C. albicans* by repressing two genes, *HWP1* (Hyphal Wall Protein 1), and *ECE1* (Extent of Cell Elongation 1) known to be involved in yeast-to-hyphal transition [76] as well as oxidative stress response via *HOG1*.

The role of the transcription factor *RLM1* in the maintenance of the cell wall integrity was shown by susceptibility assays of *C. albicans Δ/Δ rlm1* mutants to 30 ng mL^{-1} caspofungin and various compounds such as calcofluor white [77]. In this study, the authors found that the caspofungin susceptible *rlm1* deletion mutants which in the presence of 1 M sorbitol reverted to a wild-type phenotype had an elevated chitin and a reduced mannan cell wall content compared to the wild type [77]. Microarray analysis of the *rlm1* deletion mutants in the absence of stress showed an upregulation of genes linked to cell adhesion like ECE1, HWP1, and two genes that belong to agglutinin-like sequence (ALS) family, *ALS1* and *ALS3* [77]. Within this family, eight genes (*ALS1*, *ALS2*, *ALS3*, *ALS4*, *ALS5*, *ALS6*, *ALS7*, and *ALS9*) encode cell-surface glycoproteins that play a role in adhesion, biofilm formation, hydrophobicity, and pathogenesis in *C. albicans* [78–80]. Interestingly, *ALS1* has been linked to caspofungin-induced cell flocculation/aggregation of *C. albicans* as yeast cells flocculated in growth media supplemented with 10 ng mL^{-1} and 100 ng mL^{-1} caspofungin, respectively [81]. Furthermore, compared to the wild-type strains, the *als1*Δ/Δ mutant cells had diminished flocculation in the presence of 100 ng mL^{-1} caspofungin [81]. In the same study, the authors linked flocculation to the regulator of morphogenesis, EFG1, a *Candida* homolog of PHD1 from *S. cerevisiae* as *efg1* Δ/Δ deletion mutants were susceptible to caspofungin and impaired in flocculation compared to *C. albicans* wild-type strains [81]. Similarly, *efg1* knockouts of *C. parapsilosis* were sensitive to caspofungin compared to the wild-type strain CLIB214 and the *efg1/ACT1-EFG1* complemented strain [82]. EFG1 and various transcription factors (TFs) have been linked to hyphal morphogenesis (the switch from a unicellular budding yeast to multicellular filamentous hyphal growth) thus allowing *C. albicans*' hyphae to attach and to penetrate through the epithelial cell layers of an infected host [83]. Past work by Noffz et al. [84] and Stoldt et al. [85] demonstrated that the *EFG1* overexpression in *C. albicans* led to pseudohyphae development. Another TF is *C. albicans CaSFL1* (suppressor for flocculation gene) which acts as a negative regulator of hyphal development and flocculation in *C. albicans* [86, 87]. Interestingly, a recent study demonstrated a link with *SFL1* and *EFG1* in negatively and positively regulating hyphal morphogenesis and microcolony formations [88].

Moreover, efg1 interacts with cas5, a transcription factor involved in stress responses, cell cycle regulation, and drug resistance [89, 90] in vivo and both regulators are critical for the induction of caspofungin-responsive genes such as *ALS1* in *C. albicans* [91]. Apart from *ALS1* and *PGA13*, additional GPI-anchored proteins were found to be affected by caspofungin in *C. albicans* [92].

One of the consequences of caspofungin inducing cell wall changes in various *Candida* species is that the additional modification to cell wall GPI-anchored proteins and the increase in chitin/glucan exposure decreased phagocytosis *of C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. lusitaniae*, and *C. guilliermondii* by J774 macrophages [93]. In addition, there was no change in phagocytosis by J774 macrophages with *C. glabrata* and *C. parapsilosis* in the presence and absence of caspofungin as there were no changes in glucan exposure in response to caspofungin treatment in these species [93]. It has been shown that chitin blocked the recognition of live *C. albicans* yeast cells by human

peripheral blood mononuclear cells (PBMCs), leading to significant reduction in the stimulation of TNF-α, IL-6, and IL-1β [94]. In contrast, an increased elicitation of TNF-α from macrophages in a Dectin-1-dependent manner has been linked to the unmasking (or exposure) of β-1,3-glucan in *C. albicans* and its *cho1*Δ/Δ mutant which is unable to synthesize phosphatidylserine [95]. Similar observations have been made due to unmasking of β-1,3-glucan in *C. albicans kre5*Δ/Δ to hyperelicit TNFα from macrophages [96]. *KRE5* encodes a UDP-glucose:glycoprotein glucosyltransferase localized in the endoplasmic reticulum in *C. albicans* and *S. cerevisiae* [97]. Work by Herrero et al. [97] demonstrated that the lack of Kre5p in *C. albicans* reduced adherence to human epithelial cells and the *KRE5* homozygous mutant strains were avirulent in a BALB/c mouse model of systemic infection. A recent investigation in *C. glabrata* also identified that a functional homolog of *KRE5*, *CgKRE5*, and *C. glabrata* cells with the tetracycline-dependent system to repress *CgKRE5* in the presence of 20 μg mL^{-1} doxycycline (DOX) had enhanced sensitivity to micafungin (at 3 μg mL^{-1}) compared to those grown in the absence of DOX [98]. AFM work on *C. albicans* SC5314 (WT), the *cho1*Δ/Δ mutant, the *kre5*Δ/Δ mutant, and the caspofungin-treated WT confirmed that by inhibiting key steps in cell wall synthesis increased cell wall roughness and decreased cell wall elasticity [99]. By using AFM tips functionalized with sDectin-1-Fc, which is highly specific for glucans with a pure (1 → 3)-β-linked backbone structure [100], the authors demonstrated that the *kre5*Δ/Δ mutant had the highest frequency of binding (or peak adhesion frequency) followed by caspofungin-treated WT cells, the *cho1*Δ/Δ mutant, and almost no binding with the WT [101]. Thus, the differences in β-1,3-glucan layer exposure could contribute to *Candida*'s pathogenicity, virulence, and the immune system evasion and/or survival.

C. albicans biofilms and caspofungin

Another aspect of *C. albicans* contributing to its pathogenicity and virulence is its ability to form biofilms, i.e., where densely packed communities yeast cells adhere to surfaces [101–103]. In *C. albicans*, biofilm formation involve adherence, the formation of microcolonies, and of hyphae surrounded by an extracellular matrix (ECM) of polysaccharides to the subsequent dispersal of planktonic cells after reaching maturation [104–106]. *C. tropicalis* biofilms are similar in structure to *C. albicans* while *C. parapsilosis* form pseudo-hyphae while *C. auris* and *C. glabrata* biofilms are made up of blastospores within an ECM [104, 105, 107]. Many genes involved in biofilm formation in *C. albicans* and other *Candida* species have been studied (refer to reviews [104, 105, 107–109]). Bachmann et al. [110] spotlighted the benefits of caspofungin against *C. albicans* biofilms in vitro. Later investigations by Ferreira et al. [111] and Melo et al. [112] observed paradoxical growth in caspofungin-treated biofilms, i.e., enlarged, globose cells, and a resurgence of growth at drug concentrations above the MIC in clinical *Candida* species.

Candida spp. biofilm formation poses a clinical problem in transplant, oncology, and intensive care medicine and echinocandins are still used in its management [113–115]. An in vitro study on the novel echinocandin, rezafungin (CD101), on *C. albicans* biofilms suggests that it could be useful in preventing and treating biofilm-associated nosocomial infections [113].

Synergistic activity of caspofungin with other compounds

Due to increased resistance of *C. albicans* to caspofungin, there are have been various studies in the use of combination therapies which would result in synergistic action and greater potency than the constituent drugs used in monotherapy [116, 117].

Work by Troskie et al. [118] have shown the benefits of combining tyrocidines, a type of cationic cyclodecapeptides with potent antibacterial and antimalarial activities from *Bacillus aneurinolyticus*, with caspofungin against *C. albicans* strain SC5314, which is known to form robust biofilms. In combination, the three major tyrocidines, TrcA, TrcB, and TrcC, significantly increased the *C. albicans* biofilm eradication activities of caspofungin [118]. In addition, the fractional inhibitory concentration index (FICI) values of TrcA with caspofungin were more promising than TrcB and TrcC with caspofungin combination respectively against 24-h-old *C. albicans* biofilms [118]. Further testing in the *C. elegans* infection model, 5 days posttreatment of a single dose of 3.0 μM TrcA and 0.19 μM caspofungin almost doubled the nematode survival rate of *C. albicans*-infected nematodes compared to *C. albicans*-infected nematodes treated with a single dose of 0.19 μM caspofungin only [118].

A recent review by Oshiro and colleagues [119] highlighted the benefits of antifungal peptides (AFPs) or antimicrobial peptides (AMPs) such as plant defensins, cathelicidins, and histatins in the inhibition and eradication of *Candida* spp. biofilms. It has been shown that the use of AMPs with caspofungin had an enhanced antifungal activity against *C. albicans* in vitro and in vivo [117]. One AMP of human origin, hMUC7–12 [120], and one of amphibian origin, DsS3(1–16) [121], when combined with caspofungin respectively were shown to improve the survival of wax moth larvae (*Galleria mellonella*) infected with *C. albicans* SC5314 compared to those infected wax moth larvae treated with PBS or hMUC7–12 (25 mg kg^{-1}), DsS3(1–16) (25 mg kg^{-1}), and caspofungin (0.5 mg kg^{-1})

only [117]. The same authors observed similar results in infected wax moth larvae when treated with the cyclic peptide, colistin sulfate (10 mg kg^{-1}) in combination with caspofungin [117].

Plant defensins have also been investigated for their synergistical efficacy with caspofungin against *C. albicans*. Of the two radish defensins, RsAFP1 and RsAFP2, the later induced mislocalization of septins in *C. albicans* CAI4 cells expressing SEP7-GFP-tagged allele and blocked the yeast-to-hypha transition in a dose-dependent manner in *C. albicans* CAI4 cells [122]. Further work by Vriens et al. [123], using the recombinant (r)RsAFP2, heterologously expressed in *Pichia pastoris*, demonstrated that RsAFP2 prevented *C. albicans* biofilm formation and acted synergistically with caspofungin and amphotericin B in the prevention and eradication of *C. albicans* biofilms. Recent work by Cools et al. [124] demonstrated that a truncated peptide variant of the plant defensin HsAFP, isolated from *Heuchera sanguinea*, HsLin06_18, when combined with caspofungin reduced in vitro biofilm formation of *C. albicans* SC5314 WT on polyurethane catheters as well as a caspofungin-resistant *C. albicans* mutant strain M177 and, the use of a subcutaneous rat catheter model in immunosuppressed female Sprague–Dawley rats, the combination reduced biofilm formation of *C. albicans* in vivo. Furthermore, the authors observed that the caspofungin facilitated the internalization and the membrane permabilization of HsLin06_18 into planktonic *C. albicans* SC5314 WT cells [124].

Work by Sun and colleagues [125] demonstrated the benefits of combining polyphenols such as caffeic acid phenethyl ester (CAPE) with caspofungin against *C. albicans*. CAPE not only deprived iron and increased ROS production in *C. albicans* YEM30 cells but when used in combination with caspofungin, there was a significant 16-fold decrease for the minimum inhibitory concentrations (MICs) of CAPE and caspofungin compared to the MIC values of individual drugs [125].

The natural plant metabolite, poacic acid (diferulate, 8–5-DC; PA), was shown to bind to cell wall β-1,3-glucan in *S. cerevisiae* and inhibit β-1,3-glucan synthase activity in vitro [126]. PA also inhibited the growth of plant fungal pathogens *Sclerotinia sclerotiorum* and *Alternaria solani* and the oomycete *Phytophthora sojae* [126]. Work by Lee et al. [127] explored the effects of PA against human pathogenic *Candida* species. *C. guilliermondii*, *C. orthopsilosis*, and *C. parapsilosis* were more sensitive to PA than *C. albicans*, *C. dubliniensis*, *C. glabrata*, and *C. tropicalis* [127]. Furthermore, *C. albicans* strains containing an amino acid substitution in Fks1 Hotspot1 (S645Y or S645P) not only had decreased sensitivity to caspofungin but increased sensitivity to PA suggesting that there is a difference in the mode of action of PA and caspofungin [127].

Conclusion: what else could we learn about caspofungin?

Recent cryo-electron tomography work by Jiménez-Ortigosa et al. [128] has provided a preliminary structure of the putative *C. glabrata* GS complex, i.e., as clusters of hexamers, each subunit with two notable cytosolic domains, the N-terminal and central catalytic domains. The mechanism of action for echinocandins is its ability to inhibit β-1,3-glucan synthesis by non-competitive binding to GS. Fluorescence microscopy work by Utsugi et al. [129] demonstrated with the movement of Fks1p tagged with the green fluorescence protein was colocalized with cortical actin on the *S. cerevisiae* cell surface. Therefore, it would be interesting to see how various mutations in the *FKS1/FKS2* and the application of caspofungin (or other echinocandins) in synergy with other drugs could affect the assembly of GS on the cell plasma membrane and β-1,3-glucan synthesis in *C. albicans* and other pathogenic yeasts.

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Author contribution F.P-W conceptualized the article, performed the literature search and data analysis, and drafted the manuscript and figures.

Declarations

Conflict of interest The author declares no competing interests.

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