Medical Mycology September 2006, **44**, S95–S99

Taylor & Francis
Taylor & Francis Group

Understanding the microbiology of the *Aspergillus* cell wall and the efficacy of caspofungin

CAMERON M. DOUGLAS
Infectious Disease Research, Merck Research Laboratories, Rahway, New Jersey, USA

The efficacy of echinocandins against *Aspergillus* species has been established through *in vitro* assays, in animal models of infection, and in clinical practice. Caspofungin is an inhibitor of 1,3-β-D glucan synthesis (GS) that produces dramatic morphological changes, but incomplete clearing, in cultures of growing hyphae. Despite the apparent fungistatic *in vitro* activity against *Aspergillus* species, compounds in this class have strong efficacy *in vivo*. For example, caspofungin prolonged survival in chronically immunosuppressed mice with induced disseminated aspergillosis, even when neutropenia was maintained for weeks after a short dosing regimen. Kidneys of these mice showed no evidence of recrudescent *Aspergilluis fumigatus* burden after the infection had been treated. One possible explanation for echinocandin-mediated clearance of *A. fumigatus* in vivo stems from the newly-discovered role of β-glucan in the inflammatory response. Binding of cell wall β-glucan to the dectin-1 receptor of macrophages leads to production of proinflammatory cytokines, which augment the innate immune response to swollen conidia and germlings. Changes in *A. fumigatus* cell wall structure, such as those produced by exposure to an echinocandin like caspofungin, may increase the opportunity for interactions between 1,3-β-D glucan and dectin-1, and lead to a heightened response to 'wounded' hyphae.

Keywords caspofungin, echinocandin, glucan, dectin-1

Introduction

Invasive aspergillosis (IA) is an important cause of morbidity and mortality among immunocompromised patients. The antifungal drugs currently indicated for use as primary therapy for these infections include polyenes such as amphotericin B (AMB), which disrupt fungal membrane potential, or azoles such as voriconazole, which inhibit the synthesis of ergosterol [1]. Because there is significant mortality despite the widespread use of these drugs, new agents to treat IA are needed.

The echinocandins are a new class of antifungal drugs that inhibit the synthesis of 1,3-β-D-glucan, which is a key component of the fungal cell wall. In 2001 the first agent in this class (caspofungin; CAS) was approved for the treatment of invasive aspergillosis in patients refractory to or intolerant of other therapies [2]. Caspofungin is also indicated for esophageal candidiasis, candidemia, and other Candida infections (including intra-abdominal abscesses, peritonitis and pleural space infections), and for empirical therapy of suspected fungal infections in patients with persistent fever and neutropenia [3].

Micafungin and anidulafungin are two other echinocandins that have been approved for treatment of *Candida* infections [4–6]. Neither agent is currently indicated in the US for clinical use in patients with *Aspergillus* infections, although evidence from preclinical studies and/or clinical trials suggests that both drugs are active against *Aspergillus* spp. Few side-by-side comparisons between caspofungin, micafungin, and anidulafungin have been reported, which makes it difficult to discern which features of the three echinocandins are shared and which are compound-specific.

Correspondence: Cameron M. Douglas, Infectious Disease Research, Merck Research Laboratories, Mail Drop R80Y-260, PO Box 2000, Rahway, NJ 07065-0900, USA. Tel: +1 732 594 5646; fax: +1 732 594 6708; E-mail: cameron_douglas@merck.com

DOI: 10.1080/13693780600981684

Nonetheless, the approval of caspofungin as salvage therapy for IA [1] has stimulated significant interest in understanding and further defining the consequences of echinocandin inhibition of *Aspergillus* cell wall synthesis.

What makes 1,3-β-D-glucan synthesis a good antifungal target in *Aspergillus* spp.?

One of the principal polysaccharides within the *Aspergillus* cell wall is 1,3-β-D-glucan, which takes the form of linear fibrils within the core structure and short cross-links within a mesh-like network that is essential for both plasticity and strength [7]. A multi-subunit enzyme complex that minimally contains two proteins, Fks1p and Rho1p, is responsible for the synthesis of 1,3-β-D-glucan [8]. Several lines of evidence suggest that synthesis of 1,3-β-D-glucan is a critical function for *Aspergillus* spp. and a valid antifungal target. First, genetic studies with *A. fumigatus* have demonstrated that the *FKS1* gene is essential for viability [8,9]. Second, inhibition of GS activity by the echinocandins leads to profound inhibition of *A. fumigatus* growth *in vitro*; hyphae are shorter, thickened, and highly branched, and cells at mycelial tips and branch points lyse [10,11]. Third, other *Aspergillus* species (*A. nidulans*, *A. terreus*, and *A. flavus*, for example) have highly conserved *FKS1* genes and equivalent susceptibility (both enzyme and whole-cell) to the most potent echinocandins [12].

Are echinocandins effective against *Aspergillus in vivo*?

Caspofungin and other echinocandins have been studied in animal models of pulmonary, central nervous system, and disseminated aspergillosis, using prolongation of survival and/or organ burden reduction as primary endpoints for efficacy [13–16]. In all of these models, caspofungin was effective as monotherapy in prolonging survival; however, the effect on *Aspergillus* colony forming units in tissues of infected, treated animals has been less clear. This method of quantifying burden does not accurately reflect the number of viable cells for a filamentous fungus such as *A. fumigatus*. Due to the hyphal morphology of these organisms, a large fungal mass is often indistinguishable from single-cell conidial forms when spread on agar plates, since both will usually yield one colony. Accordingly, results from experiments employing colony forming unit (CFU) measurement to quantify in vivo efficacy against *A. fumigatus* have been mixed. Caspofungin treatment often produces small, insignificant reductions in CFU burden [16]; in at least one model, the number of CFU for CAS-treated animals is actually higher than the value for animals given vehicle alone [13]! The level of galactomannan (GM; a biomarker for *Aspergillus* load) in this study was 'hyper-elevated' in animals receiving caspofungin therapy, while the comparator drug (AMB) produced clear reductions in both CFU and GM, which is consistent with its well-defined fungicidal activity [13]. There are other studies where caspofungin therapy led to reductions in *Aspergillus* CFU [17,18] or GM signal [19]. Quantitative PCR (qPCR) has also been used to measure organ burden and echinocandin efficacy in *A. fumigatus*-infected animals [15,20–22]. The overall conclusion seems to be that caspofungin and other echinocandins prolong survival in a variety of models of aspergillosis, but the impact on burden is more difficult to characterize. The best strategy may require the evaluation of multiple biomarkers (i.e., CFU, qPCR and GM values, infarct scores, etc).

Do *in vitro* susceptibility results predict *in vivo* efficacy?

The *in vitro* activity of echinocandins against *Aspergillus* spp. can not be described as fungicidal. Even though cells at hyphal tips and branch points lyse when exposed to caspofungin or micafungin, the number of CFU in cultures incubated with an echinocandin do not diminish over time [11,23]. The use of liquid microbroth dilution assays to measure the echinocandin susceptibility of *Aspergillus* spp. has been challenging – although growth is significantly reduced, and morphology is dramatically altered, the wells are not clear. A novel endpoint defined as the MEC (minimum effective concentration) has been proposed [10], and several investigators have used the MEC to measure the susceptibility of moulds to echinocandins [24,25]. Validation of MEC values has been hampered by the controversy over burden quantitation, differences in outcome across the various animal models, and the rarity of well-characterized echinocandin-resistant *Aspergillus* mutants which could be used to interrogate the relationship between MEC and *in vivo* efficacy.

The unusual *in vitro* response of *Aspergillus* spp. to echinocandins led to a prediction that these compounds should be fungistatic *in vivo*. In the face of sustained immunosuppression and a short course of caspofungin therapy, an *A. fumigatus* infection in mice would be expected to reemerge, with a resulting rise in burden and mortality. However, at least two published reports suggest this is not the case [22,26]. In the first study,

mice with an induced disseminated *A. fumigatus* infection were maintained chronically pancytopenic and only given caspofungin for seven days, beginning 24 h after infection. There were an insignificant number of additional deaths for at least 21 days after therapy was suspended, even in groups given the modest caspofungin dose of 0.25 milligram per kilogram per day (mpk/day). In fact, caspofungin was at least as effective in this model as AMB [26]. In a second study, caspofungin (dosed at 1 or 2 mpk/day) was given to chronically immunosuppressed mice for 14 days, and kidney burden was monitored by both qPCR and histology [22]. The infection did not recrudesce over the 42 day observation period (including 28 days beyond the end of caspofungin dosing).

The fact that echinocandins are not fungicidal against *Aspergillus in vitro*, but infections in mice fail to re-emerge for several weeks after therapy is withdrawn, creates an apparent conundrum. Either caspofungin is more effective *in vivo* than predicted from *in vitro* susceptibility studies, or some interaction(s) between echinocandins and host factors may be involved in clearing *Aspergillus* infections. Alternative explanations based on persistence of caspofungin in tissues after a finite period of dosing [27] or a postantifungal effect [28] have some precedent in the literature, but these phenomena are likely too short-lived to account for the prolonged effect seen in the animal studies described above. There are potential clinical implications to resolving this question.

What factors might influence the potential for clearance of *A. fumigatus* during echinocandin therapy?

Several years ago Chiller *et al*. [29] reported a cooperative interaction between human monocytes or macrophages and caspofungin in the killing of *A. fumigatus* hyphae. The mechanism behind this enhancement was not defined at the time, but recent results suggest that interactions between β-glucan on the surface of *A. fumigatus* germlings and a toll-like receptor on monocytes and macrophages could play a role. Two recent papers [30,31] explore the dynamics of 1,3-β-D-glucan exposure on *A. fumigatus* hyphae, and how this can promote cytokine production by innate immune cells through signaling mediated by direct binding to the β-glucan-specific dectin-1 receptor. The data illustrate that 1,3-β-D-glucan appears on the cell surface in a growth phase-dependent manner; the polysaccharide is undetectable on the outside of resting spores, but once conidia swell and hyphae emerge, there is a strong signal [30]. In response, monocytes produce the cytokine tumor necrosis factor α (TNF-α) in a dectin-1-dependent manner. Exposure of 1,3-β-D-glucan and the coincident production of TNF-α was reduced when mature *A. fumigatus* mycelium was used in place of swollen conidia or young germlings [31].

What does this have to do with caspofungin, *Aspergillus*, and the conundrum between *in vitro* activity and *in vivo* efficacy? It may be that caspofungin therapy produces dramatic changes in *Aspergillus* cell wall ultrastructure, which leads to hyper- exposure of 1,3-β-D-glucan on the surface of these hyphae. The stunted, swollen, highly-branched hyphae seen *in vitro* have also been observed in tissue sections of caspofungin-treated animals [13,21]. Perhaps cell wall glucan that was already present before the initiation of caspofungin therapy, or a small amount of 1,3-β-D-glucan synthesized in the presence of caspofungin, is exposed on the malformed hyphal surface and available to interact with the dectin-1 receptor. In contrast, the 1,3-β-D-glucan of mature, untreated hyphae probably remains obscured, such that little to no cytokine response by innate immune cells is provoked [31]. The leukocyte activation in response to caspofungin-treated *Aspergillus* cells could account for clearance of hyphae that were not killed during caspofungin therapy. There would be little opportunity for recrudescence of the infection, and few hyphal elements would persist for detection by histology or qPCR.

There is a precedent for cell-surface exposure of 1, 3-β-D-glucan in caspofungin-treated fungi [32]. In *Candida albicans*, β-glucan comprises a significant fraction of the total cell wall, but this polysaccharide is masked by mannoprotein and is invisible to the immune system. When the underlying β-glucan is exposed by subinhibitory doses of caspofungin, the amount of TNF-α produced by bone-marrow-derived macrophages is elevated 3- to 4-fold compared to levels of this cytokine elicited by UV-irradiated *C. albicans* cells. This macrophage response is dependent on the dectin-1 receptor and can be muted by pretreatment with laminarin, a soluble 1,3-β-D-glucan. Caspofungin appears to 'unmask' *C. albicans* β-glucan and promote a proinflammatory response to the 'wounded' cells.

Conclusions

Caspofungin is currently indicated for the treatment of invasive aspergillosis in patients refractory to or intolerant of other therapies [2]. Genetic and biochemical data have established that the target (1,3-β-D-glucan synthase) is: (i) conserved across several *Aspergillus* species; (ii) essential for viability; and (iii) susceptible to caspofungin inhibition at concentrations that are

effective against the *C. albicans* enzyme [33]. The drug prolongs survival in numerous animal models of aspergillosis and has demonstrated efficacy in clinical use [1,34,35]. *A. fumigatus* cells at hyphal tips and branch points lyse when exposed to caspofungin, but growth inhibition *in vitro* is not complete and the drug is not considered fungicidal against *Aspergillus* spp. However, *Aspergillus* infections in animals do not recrudesce after caspofungin therapy is withdrawn, even during periods of sustained neutropenia. A caspofungin-dependent exposure of β-glucan, with corresponding stimulation of cytokine production by cells of the innate immune system, may play a role in the *in vivo* clearance of *Aspergillus* from infected organs. Other antifungal agents such as the azoles and polyenes, which do not target 1,3-β-D-glucan synthesis, are unlikely to produce similar ultrastructural changes in the cell wall of *Aspergillus* spp.; as a result, enhanced cytokine production through stimulation of the dectin-1 receptor may be induced by echinocandin-treated germlings but not by hyphae exposed to other classes of antifungal agents.

Acknowledgements

I would like to thank my colleagues at Merck for many helpful discussions.

References

1 Maertens J, Raad I, Petrikkos G, *et al*. Efficacy and safety of caspofungin for treatment of invasive aspergillosis in patients refractory to or intolerant of conventional antifungal therapy. *Clin Infect Dis* 2004; **39**: 1563–1571.
2 Deresinski SC, Stevens DA. Caspofungin. *Clin Infect Dis* 2003; **36**: 1445–1457.
3 Morrison VA. Caspofungin: an overview. *Expert Rev Anti Infect Ther* 2005; **3**: 697–705.
4 Vazquez JA, Sobel JD. Anidulafungin: a novel echinocandin. *Clin Infect Dis* 2006; **43**: 215–222.
5 Chandrasekar PH, Sobel JD. Micafungin: a new echinocandin. *Clin Infect Dis* 2006; **42**: 1171–1178.
6 Zaas AK, Steinbach WJ. Micafungin: the US perspective. *Expert Rev Anti Infect Ther* 2005; **3**: 183–190.
7 Latge JP. *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* 1999; **12**: 310–350.
8 Beauvais A, Bruneau JM, Mol PC, *et al*. Glucan synthase complex of *Aspergillus fumigatus*. *J Bacteriol* 2001; **183**: 2273–2279.
9 Mouyna I, Henry C, Doering TL, Latge JP. Gene silencing with RNA interference in the human pathogenic fungus *Aspergillus fumigatus*. *FEMS Microbiol Lett* 2004; **237**: 317–324.
10 Kurtz MB, Heath IB, Marrinan J, *et al*. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-beta-D-glucan synthase. *Antimicrob Agents Chemother* 1994; **38**: 1480–1489.
11 Bowman JC, Hicks PS, Kurtz MB, *et al*. The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus* in vitro. *Antimicrob Agents Chemother* 2002; **46**: 3001–3012.
12 Bowman JC, Abruzzo GK, Flattery AM, *et al*. Efficacy of caspofungin against *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus nidulans*. *Antimicrob Agents Chemother* in Press.
13 Petraitiene R, Petraitis V, Groll AH, *et al*. Antifungal efficacy of caspofungin (MK-0991) in experimental pulmonary aspergillosis in persistently neutropenic rabbits: pharmacokinetics, drug disposition, and relationship to galactomannan antigenemia. *Antimicrob Agents Chemother* 2002; **46**: 12–23.
14 Kirkpatrick WR, Perea S, Coco BJ, Patterson TF. Efficacy of caspofungin alone and in combination with voriconazole in a guinea pig model of invasive aspergillosis. *Antimicrob Agents Chemother* 2002; **46**: 2564–2568.
15 Bowman JC, Abruzzo GK, Anderson JW, *et al*. Quantitative PCR assay to measure *Aspergillus fumigatus* burden in a murine model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate. *Antimicrob Agents Chemother* 2001; **45**: 3474–3481.
16 Clemons KV, Espiritu M, Parmar R, Stevens DA. Comparative efficacies of conventional amphotericin B, liposomal amphotericin B (AmBisome), caspofungin, micafungin, and voriconazole alone and in combination against experimental murine central nervous system aspergillosis. *Antimicrob Agents Chemother* 2005; **49**: 4867–4875.
17 Barchiesi F, Spreghini E, Santinelli A, *et al*. Efficacy of caspofungin against *Aspergillus terreus*. *Antimicrob Agents Chemother* 2005; **49**: 5133–5135.
18 Sivak O, Bartlett K, Risovic V, *et al*. Assessing the antifungal activity and toxicity profile of amphotericin B lipid complex (ABLC; Abelcet) in combination with caspofungin in experimental systemic aspergillosis. *J Pharm Sci* 2004; **93**: 1382–1389.
19 van Vianen W, de Marie S, ten Kate MT, Mathot RA, Bakker-Woudenberg IA. Caspofungin: antifungal activity in vitro, pharmacokinetics, and effects on fungal load and animal survival in neutropenic rats with invasive pulmonary aspergillosis. *J Antimicrob Chemother* 2006; **57**: 732–740.
20 Singh G, Imai J, Clemons KV, Stevens DA. Efficacy of caspofungin against central nervous system *Aspergillus fumigatus* infection in mice determined by TaqMan PCR and CFU methods. *Antimicrob Agents Chemother* 2005; **49**: 1369–1376.
21 Wiederhold NP, Kontoyiannis DP, Chi J, *et al*. Pharmacodynamics of caspofungin in a murine model of invasive pulmonary aspergillosis: evidence of concentration-dependent activity. *J Infect Dis* 2004; **190**: 1464–1471.
22 Hickey E, Abruzzo G, Bowman JC, *et al*. Caspofungin versus Amphotericin B treatment of *Aspergillus fumigatus* in kidneys of chronically immunosuppressed infected mice. *Therapy* 2005; **4**: 615–621.
23 Watabe E, Nakai T, Matsumoto S, Ikeda F, Hatano K. Killing activity of micafungin against *Aspergillus fumigatus* hyphae assessed by specific fluorescent staining for cell viability. *Antimicrob Agents Chemother* 2003; **47**: 1995–1998.
24 Espinel-Ingroff A. Comparison of *In vitro* activities of the new triazole SCH56592 and the echinocandins MK-0991 (L-743,872) and LY303366 against opportunistic filamentous and dimorphic fungi and yeasts. *J Clin Microbiol* 1998; **36**: 2950–2956.
25 Pfaller MA, Marco F, Messer SA, Jones RN. *In vitro* activity of two echinocandin derivatives, LY303366 and MK-0991 (L-743,792), against clinical isolates of *Aspergillus, Fusarium, Rhizopus*, and other filamentous fungi. *Diagn Microbiol Infect Dis* 1998; **30**: 251–255.

26 Abruzzo GK, Gill CJ, Flattery AM, *et al*. Efficacy of the echinocandin caspofungin against disseminated aspergillosis and candidiasis in cyclophosphamide-induced immunosuppressed mice. *Antimicrob Agents Chemother* 2000; **44**: 2310–2318.
27 Louie A, Deziel M, Liu W, *et al*. Pharmacodynamics of caspofungin in a murine model of systemic candidiasis: importance of persistence of caspofungin in tissues to understanding drug activity. *Antimicrob Agents Chemother* 2005; **49**: 5058–5068.
28 Clancy CJ, Huang H, Cheng S, Derendorf H, Nguyen MH. Characterizing the effects of caspofungin on *Candida albicans*, *Candida parapsilosis*, and *Candida glabrata* isolates by simultaneous time-kill and postantifungal-effect experiments. *Antimicrob Agents Chemother* 2006; **50**: 2569–2572.
29 Chiller T, Farrokhshad K, Brummer E, Stevens DA. The interaction of human monocytes, monocyte-derived macrophages, and polymorphonuclear neutrophils with caspofungin (MK-0991), an echinocandin, for antifungal activity against *Aspergillus fumigatus*. *Diagn Microbiol Infect Dis* 2001; **39**: 99–103.
30 Hohl TM, Van Epps HL, Rivera A, *et al*. *Aspergillus fumigatus* triggers inflammatory responses by stage-specific beta-glucan display. *PLoS Pathog* 2005; **1**: e30.
31 Steele C, Rapaka RR, Metz A, *et al*. The beta-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. *PLoS Pathog* 2005; **1**: e42.
32 Wheeler RT, Fink GR. A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog* 2006; **2**: e35.
33 Kahn JN, Hsu MJ, Racine F, Giacobbe R, Motyl M. Caspofungin susceptibility in *Aspergillus* and non-*Aspergillus* molds: inhibition of glucan synthase and reduction of beta-D-1,3 glucan levels in culture. *Antimicrob Agents Chemother* 2006; **50**: 2214–2216.
34 Betts R, Glasmacher A, Maertens J, *et al*. Efficacy of caspofungin against invasive *Candida* or invasive *Aspergillus* infections in neutropenic patients. *Cancer* 2006; **106**: 466–473.
35 Kartsonis NA, Saah AJ, Joy Lipka C, Taylor AF, Sable CA. Salvage therapy with caspofungin for invasive aspergillosis: results from the caspofungin compassionate use study. *J Infect* 2005; **50**: 196–205.