SUPPLEMENT ARTICLE

Potential Microbiological Effects of Higher Dosing of Echinocandins

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The antifungal "paradoxical effect" has been described as the reversal of growth inhibition at high doses of echinocandins, most usually caspofungin. This microbiological effect appears to be a cellular compensatory response to cell wall damage, resulting in alteration of cell wall content and structure as well as fungal morphology and growth. In vitro studies demonstrate this reproducible effect in a certain percentage of fungal isolates, but animal model and clinical studies are less consistent. The calcineurin and Hsp90 cell signaling pathways appear to play a major role in regulating these cellular and structural changes. Regardless of the clinical relevance of this paradoxical growth effect, understanding the specific actions of echinocandins is paramount to optimizing their use at either standard or higher dosing schemes, as well as developing future improvements in our antifungal arsenal.

Keywords. echinocandin; paradoxical effect; cell wall; calcineurin; Hsp90.

Echinocandin antifungals play a vital role in the clinical armamentarium against invasive fungal infections [1, 2]. This class of antifungals exerts concentration-dependent effects through noncompetitive inhibition of β-1,3-glucan synthase, leading to damaged fungal cell walls. Due to the nontoxic nature of these agents, many clinicians have attempted to utilize higher than standard recommended doses, especially for difficult-to-treat infections. Although there seem to be few toxicity issues with this elevated dosing strategy, there are potential microbiological concerns that could reduce antifungal activity. These worries are relevant not only for daily high-dose echinocandin use, but also for future schemes that could employ pulse intermittent (eg, once weekly) higher dosing. Understanding these important microbiological effects following high-dose echinocandin use is crucial before newer dosing strategies are adopted into clinical practice.

THE ANTIFUNGAL PARADOXICAL EFFECT

In 1948, Harry Eagle first described what was later called the Eagle effect, whereby penicillin added to media at ascending doses expectedly killed more bacteria, but after increasing beyond a critical concentration the number of bacteria would paradoxically increase, revealing a decreased antibacterial effect at higher drug concentrations [3]. This microbiological phenomenon was first extended to antifungals in a 1988 in vitro study comparing the echinocandin cilofungin (LY121019; no longer available) against various *Candida albicans* and *Candida tropicalis* isolates [4]. In that report, higher doses of the echinocandin paradoxically led to reduced fungal killing. The authors describe that ". . . a paradoxical effect was noted with cilofungin, in that the inhibitory effect observed at lower concentrations was followed by growth at higher concentrations often equivalent to that in the growth control well." A study the next year found that 58% of *C. albicans*

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isolates and 27% of *C. tropicalis* isolates demonstrated the Eagle effect at higher concentrations of cilofungin [5].

Although this phenomenon was subsequently mentioned in the literature [6, 7], the seminal paper describing this microbiological finding in detail was not published until 2004, where the authors outlined resistance to growth inhibition and killing among various clinical *Candida* isolates following exposure to high concentrations of caspofungin [8]. This "paradoxical effect" occurred in 16% (4/25) of *C. albicans* clinical isolates initially tested, and then in 18% (3/17) in another group of isolates examined; in each strain with the paradoxical effect it was consistently reproducible. These isolates all displayed caspofungin mean inhibitory concentrations (MICs) that were considered highly susceptible by routine in vitro testing (0.025–0.09 μg/mL), and therefore the suggestion of growth inhibition might not have been detected using only conventional approaches. Additional *Candida* isolates originally without the paradoxical effect were retested at much higher concentrations (up to 50 μg/mL) and did not develop this condition even at these super-therapeutic levels. However, whereas 52% of those isolates showed nonvisible growth at higher caspofungin concentrations, they possessed a small number of viable colonies on subculture testing, a term coined the "mini-paradoxical effect." This complex dynamic relationship was postulated to be quadriphasic: There was growth below the caspofungin MIC, growth inhibition above the caspofungin MIC for several dilutions, release of growth inhibition at higher caspofungin concentrations in those isolates with the paradoxical growth, and finally growth inhibition again at the highest caspofungin concentrations [8].

Subculture of these paradoxical isolates did not demonstrate progeny colonies with total resistance, but instead yielded the reproducible paradoxical effect of the parent strain. This suggested that this paradoxical phenomenon was not due to selection of a truly resistant subpopulation. Evaluation of caspofungin concentration in the testing wells revealed no destruction of the drug at these higher concentrations to explain the lack of an inhibitory effect [8], ruling out the possibility of drug inactivity as a source for the unexpected growth.

Several additional possibilities were explored related to the mechanism of this paradoxical effect. Most *Candida* strains tested were isolated well before echinocandins were introduced into clinical use, so the emergence of this as a consequence of drug use is not supported. The possibility of this paradoxical effect being due to the physical aggregated state of the drug seems less likely, as that would not explain the effect in some isolates and not in others, nor the growth suppression at very high concentrations and the persistence of most of the caspofungin in active form at the end of incubation [8].

In subsequently examining 17 *Candida* isolates, both susceptible and resistant to azoles, the caspofungin paradoxical effect was seen in 18% (3/17) of isolates, and revealed was no correlation between genetically known azole resistance mechanisms and the paradoxical effect phenotype [9]. Similarly, sequencing of mutations in known resistance-associated areas of the gene encoding the target of the echinocandins, β-1,3-glucan synthase (*FKS1*), showed no correlation. Glucan synthase enzymatic assays also revealed no upregulation of glucan synthase activity in the cells demonstrating paradoxical growth.

Brief exposures to caspofungin eliminated the paradoxical effect with caspofungin (8 μg/mL) on *C. albicans*, yet sustained a long postantifungal effect [10]. It is possible that this short exposure to drug was sufficient to inhibit the target enzyme, and that prolonged exposure at higher concentrations led to adaptive and compensatory responses that limit further killing and promote growth. Paradoxical growth may also be affected by other growth conditions, as 4 different *Candida* species all exhibited paradoxical growth more frequently when grown in biofilm (80%) compared with planktonic cells (40%) [11]. This preponderance of strains in biofilm yielding a paradoxical effect was reproduced in another study with *C. albicans* echinocandin-resistant strains due to *FKS1* hotspot mutations [12].

The paradoxical effect has been demonstrated in *Aspergillus* species as well. The in vitro concentration-dependent effects of each echinocandin against germinated and nongerminated *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus flavus* conidia revealed paradoxical increases in metabolic activity at higher concentrations [13], and each of the 3 echinocandins increased metabolic activity by approximately the same percentage. Caspofungin triggered the greatest number of strains to display paradoxical growth (12/27 isolates), followed by anidulafungin (7/27 isolates) and micafungin (3/27 isolates). Additionally, the mean concentration that yielded paradoxical growth was lowest for caspofungin (4.2 μg/mL), compared with micafungin (11.1 μg/mL) and anidulafungin (10.8 μg/mL).

PARADOXICAL EFFECT INFLUENCE ON CELL WALL BIOSYNTHESIS MECHANISM

As echinocandins specifically target cell wall β-1,3-glucan synthesis, a mechanistic focus for the paradoxical effect has centered on detailed examination of fungal cell wall content for evidence of compensatory upregulation of other cell wall components. By examining the fractionated cell wall of a *C. albicans* strain previously reported to produce the paradoxical effect under both the presence and absence of high doses of caspofungin, β-1,3-glucan and, interestingly, β-1,6-glucan, were decreased, whereas chitin content was significantly elevated [14]. This suggested a likely mechanism for this microbiological phenomenon, whereby caspofungin retains its β-1,3-glucan inhibition even at high concentrations so that the enzyme itself is not the focus, but chitin synthesis upregulation was compensatory, leading to increased overall fungal growth.

Examining cell wall ultrastructure by both scanning and transmission electron microscopy of 4 different *Candida* species revealed that paradoxical growth cells displayed budding cells without clear rings around constrictions between mother and daughter cells, enlarged yeasts forming clumps, and the absence of filamentation, all opposite control cells without high-dose caspofungin exposure [15]. Transmission electron microscopy studies revealed morphological changes, including a thinner inner cell wall with the predominance of a more electron-dense layer, as well as an abnormal thickness of the septa between mother and daughter cells with no clear septation between the cells [15]. Validating the earlier report, this study also demonstrated a 4.0- to 6.6-fold increase in chitin content during the paradoxical effect.

Another study with *C. albicans* validated that paradoxical growth is not due to drug degradation or instability, as media from high caspofungin concentration wells, where the fungus demonstrated paradoxical growth, also inhibited the growth of additional strains [16]. Cells exhibiting paradoxical growth showed the same enlarged size, abnormal septation, and the absence of filamentation as reported earlier. As previously reported [14, 15, 17–19], chitin content increased following caspofungin exposure; however, this study uncovered that 23% of dead cells following caspofungin treatment also had increased chitin, suggesting that this increase in chitin was not sufficient to escape caspofungin killing in all cells. β-1,3-glucan detection via fluorescent antibody revealed that cells exhibiting the paradoxical effect displayed β-1,3-glucan along the cell wall, similar to heat-treated cells and opposite control untreated cells where the β-1,3-glucan is isolated to the inner cell wall and inaccessible on the cell surface.

The addition of 50% human serum eliminated the caspofungin paradoxical effect at concentrations up to 64 μg/mL, and with 10% human sera the effect was shifted to requiring higher concentrations of caspofungin, suggesting a possible role for protein binding or some other sera factor [17]. Combination with the chitin synthesis inhibitor nikkomycin Z [17] eliminated paradoxical growth. Additionally, the paradoxical effect was eliminated in *C. albicans irs4* and *inp51* mutants, which lack phosphatidylinositol-(4,5)-bisphosphate 5′-phosphatase, and the paradoxical effect was reestablished in the reintegration strains. This is likely due to regulation of the Mkc1–cell wall integrity pathway, which targets chitin synthase and other cell wall stress responses [17]. In another study, nikkomycin Z, combined with caspofungin, also eliminated the paradoxical effect of various *Candida* species [20], even when used at sub-MIC concentrations.

CANDIDA SPECIES–SPECIFIC AND ECHINOCANDIN-SPECIFIC FINDINGS

The paradoxical effect has been demonstrated in approximately 15%–30% of *Candida* isolates tested in numerous studies. To evaluate echinocandin specificity surrounding the paradoxical effect, a total of 60 isolates were tested against both micafungin and anidulafungin, and no paradoxical or mini-paradoxical effect was seen against those 2 echinocandins [8].

One analysis of isolates from cancer patients found a much greater percentage of isolates displaying the paradoxical effect than previously reported, including 90% of 20 *C. albicans* isolates with caspofungin [21]. Only 2 species, *C. tropicalis* (7 of 10 isolates) and *Candida krusei* (6 of 10 isolates), showed a paradoxical effect with micafungin. Anidulafungin triggered the paradoxical effect in only 40% of *C. albicans* isolates and only 20% of *C. tropicalis* isolates. *Candida glabrata* did not have the paradoxical effect with any of the 3 echinocandins.

Another study of 101 isolates of *C. albicans* found 14% with paradoxical growth to caspofungin [22]. However, in that study there was no paradoxical effect with micafungin nor with anidulafungin. Interestingly, against *Candida dubliniensis*, the paradoxical effect following caspofungin exposure was seen in 90% of 124 isolates, and micafungin induced the effect in 63% of 126 isolates. Another study found 38% of *C. albicans* strains with paradoxical effect [16], highlighting the reproducible nature of the paradoxical effect, yet no uniformly consistent incidence.

One particular fungal genotype does not seem to predispose to the paradoxical effect. Multilocus microsatellite genotyping found a high genetic diversity in *C. albicans* isolates with the paradoxical effect, and therefore could not predict the paradoxical effect phenotype, suggesting it was not the result of some inheritable trait among strains [23]. Similarly, examination of 37 *A. flavus* isolates collected from 14 patients with invasive aspergillosis found that 6 (16%) had paradoxical growth following caspofungin exposure [24]. However, the paradoxical growth phenotype was again independent of microsatellite genotype.

PARADOXICAL EFFECT WITH ANIMAL MODEL DATA

The in vitro microbiologic effect has been repeatedly seen, and the cellular mechanism explored, but the real test is if the effect carries over to animal models and, ultimately, to infected patients. One of the first animal model studies tested 4 *C. albicans* isolates that displayed the paradoxical effect and 1 isolate that did not in a murine model of invasive candidiasis [25]. This examination was predicated on previous observations from multiple investigators that there was a perceived lack of caspofungin dose-responsiveness above a certain dose [26, 27]. In this murine candidiasis study, there was a flat dose response above 0.5 mg/kg of caspofungin, but there was not a consistently demonstrated paradoxical effect in vivo by either survival or kidney colony forming unit recovery [25]. It is of course conceivable that the plateau response could be attributed to caspofungin pharmacodynamics and is not necessarily a possible in vivo paradoxical effect.

A *C. albicans* strain, after growth in the presence of high-dose caspofungin, was infected in the wax moth larvae *Galleria mellonella* and showed approximately 40% less virulence compared with *C. albicans* not previously exposed to high-dose caspofungin [16]. Additionally, those larvae infected with high-dose caspofungin-exposed cells developed rapid melanization, confirmed by histopathology with melanotic capsules and a significantly decreased number of hyphae in tissue. Study of those cells exposed to high-dose caspofungin revealed a greater proinflammatory response of tumor necrosis factor–α, interleukin 17, interleukin 12, and interferon-γ when exposed to primary murine peritoneal macrophages [16]. However, in a separate *Drosophila* model of candidiasis, *C. albicans* and *C. tropicalis* strains, with and without the paradoxical effect, showed no difference in virulence [28].

The first mention of the paradoxical effect in *Aspergillus* species was in a rabbit model of invasive aspergillosis [29] with no decrease in survival at higher caspofungin dosing. In another murine model of invasive aspergillosis [30], caspofungin and micafungin each exhibited dose-dependent pharmacodynamic activity and reduction in fungal burden, but caspofungin had a steeper dose-response curve and a modest ($P = .35$) paradoxical increase in fungal burden that was not seen in micafungin-treated animals. In 3 subsequent and different murine models of invasive aspergillosis utilizing escalating doses of caspofungin, therapeutic efficacy was dose-dependent at 0.1 mg/kg/day and 1 mg/kg/day, but there was a paradoxical increase in pulmonary fungal burden and inflammation at 5 mg/kg/day [31]. Additionally, at the higher caspofungin dose, *A. fumigatus* morphology appeared comparable to untreated controls vs the impaired hyphae evident at lower caspofungin doses. Immunologically, the higher dose of caspofungin resulted in massive recruitment of neutrophils, compared to the decreased recruitment and inflammatory pathology seen with the lower doses. To evaluate a mechanism for the inflammatory findings, an additional strain that did not exhibit the paradoxical effect in vitro was infected in mice and those animals treated with 5 mg/kg/day of caspofungin. The animals had the noticeable proinflammatory response, which was therefore thought to be unrelated to the paradoxical effect.

A dose-fractionation study of caspofungin (0.25, 1.0, and 4.0 mg/kg) divided into 3 different dosing intervals in a murine model of invasive aspergillosis revealed concentration-dependent reduction in mean pulmonary fungal burden in mice in the 1 mg/kg dosage group [32]. A paradoxical increase in fungal burden was seen for each dosing interval in the highest (4 mg/kg) dosage-fractionation group, yet survival rates did not differ significantly from those in the lower dosage-fractionation group.

A neutropenic murine model of disseminated *C. glabrata* infection treated with a single dose of micafungin was more successful in reducing fungal burden than smaller, more frequent dosing schemes [33]. Therefore, no paradoxical effect was observed with high micafungin doses. In a murine model of mucormycosis due to *Rhizopus oryzae*, low-dose (0.5 mg/kg/day), but not high-dose, caspofungin (2.5 or 5 mg/kg/day) improved survival [34], suggesting the possibility of a paradoxical effect for this organism.

PARADOXICAL EFFECT CLINICAL DATA

A multicenter, randomized, double-blind clinical trial of standard-dose caspofungin (50 mg/day) vs high-dose caspofungin (150 mg/day) included 204 adult patients with invasive candidiasis [35]. Favorable responses were similar between the treatment arms, and the high-dose regimen was well-tolerated, suggesting no paradoxical effect in regards to efficacy. A second multicenter, randomized, double-blind clinical trial evaluated micafungin at 2 doses (100 mg and 150 mg) vs caspofungin at standard maintenance dosing (50 mg/day) and included 595 patients with invasive candidiasis [36]. Favorable responses were again similar in all 3 echinocandin dosing groups, including no differences in adverse events. This study noted a lower than expected rate of success (53% vs 78%) among patients with noncandidemic invasive infections in the higher-dose micafungin arm vs the lower-dose micafungin arm. It is possible that the paradoxical effect played a role, as this clinical trial was substantially larger than the earlier caspofungin trial, and perhaps this larger sample size was able to detect this difference.

A retrospective analysis of 97 total patients with invasive fungal infection from a single center included 63 patients who received standard-dose caspofungin (50 mg/day) and 34 patients who received high-dose caspofungin (100 mg/day), each combined with another antifungal for treatment [37]. Multivariate analysis showed no significant differences in response between the treatment groups, and high-dose caspofungin was well tolerated, suggesting no clinical impact of the higher dose. An updated report from the same center on 91 patients who received high-dose caspofungin showed again that the dose was well tolerated [38]. In another study, 26 patients who received high-dose micafungin (300 mg/day) were compared to 58 patients who received standard-dose micafungin (150 mg/day) in a retrospective review of adult patients with hematologic malignancies that demonstrated adverse events, and outcomes were similar in each group [39]. There was no obvious evidence of a paradoxical effect in the limited number of patients who had invasive fungal infections.

CALCINEURIN PLAYS KEY ROLES IN PARADOXICAL GROWTH IN *ASPERGILLUS FUMIGATUS*

Putative roles for calcineurin and protein kinase C in attenuation of caspofungin activity at higher concentrations in *C. albicans* were first reported in 2005 [19, 40]. The in vitro positive

interaction of caspofungin with calcineurin inhibitors (tacrolimus/FK506, cyclosporine/CsA) in *A. fumigatus* was then shown to exacerbate the delayed filamentation caused by calcineurin inhibition and also result in fungicidal activity [41]. Furthermore, the potential of calcineurin pathway inhibition without the emergence of drug resistance was demonstrated by examining the in vitro antifungal activity of calcineurin inhibitors against *A. fumigatus* clinical isolates [42]. In addition, the direct role for calcineurin in the regulation of the caspofungin-mediated paradoxical growth was demonstrated by deletion of the gene encoding the catalytic subunit of calcineurin (*cnaA*) [43, 44]. While the cell wall inhibitors caspofungin and nikkomycin Z exhibited enhanced morphological defects in the *cnaA* deletion strain, the *cnaA* deletion strain also revealed decreased β-1,3-glucan content of the cell wall, implicating calcineurin inhibition or mutation as an excellent adjunct therapeutic target in combination with the anti–cell wall drugs [45].

Efforts to examine the calcineurin-mediated downstream regulatory cascade important for cell wall biosynthesis identified *crzA*, the fungal ortholog of mammalian NFAT, as the key transcription factor involved in hyphal growth and disease dissemination [46]. Both the *A. fumigatus cnaA* and the *crzA* deletion strains exhibited defects in proper incorporation of chitin and β-1,3-glucan, the major components of the cell wall [47], and abolished caspofungin-mediated paradoxical growth at higher concentrations [48]. Compensatory transcriptional upregulation of chitin synthases and increased chitin content following caspofungin treatment was also evident [48]. The increased sensitivity of the *A. fumigatus cnaA* and *crzA* deletion strains to caspofungin was not recapitulated with the cell membrane–active antifungals amphotericin B and voriconazole, confirming that the observed effects are specific to echinocandin [48].

The molecular role for calcineurin in the activation of chitin biosynthesis process under high-dose caspofungin treatment was revealed by enhanced expression of 2 of the *A. fumigatus* chitin synthase genes, *chsA* and *chsC*, leading to a calcineurin-dependent increase in chitin synthase activity [48], presumably as a compensatory response to inhibition of β-1,3-glucan synthesis. Deletion of *A. fumigatus chsA* and *chsC* indicated that the calcineurin pathway may also play roles in the transcriptional regulation of other gene subsets essential for survival in the presence of caspofungin or involves a more complicated posttranscriptional regulation of chitin synthesis [49]. Adding to this complexity, a recent study deciphering the role of all the 8 chitin synthase genes belonging to the 2 families, 1 and 2, indicated that even quadruple deletion of family 1 chitin synthase genes did not affect chitin content, albeit with morphological abnormalities [50]. However, quadruple deletion of family 2 chitin synthase genes resulted in disorganized mycelial structure without any effect on chitin contents [50].

Although the exact underlying mechanism responsible for the calcineurin-mediated paradoxical reversal of growth inhibition at high doses of caspofungin remains unknown, the data available thus far indicate that the lack of paradoxical growth seen in the calcineurin pathway mutants is an outcome directly related to cell wall stress induced via caspofungin treatment and calcineurin pathway mutation. Recent works indicated that calcineurin may regulate key proteins involved in caspofungin-mediated paradoxical growth through localization at the hyphal tip and the hyphal septum and via its activation by phosphorylation (Figure 1) [51]. Mutations in key domains of CnaA that caused mislocalization of calcineurin from the hyphal septum [52] also showed defects in caspofungin-mediated paradoxical growth [53]. For the first time, mass spectrometry revealed the phosphorylation of CnaA at a filamentous fungal-specific domain (designated as the serine-proline rich region [SPRR]) that is completely absent in human calcineurin, and mutation of the phosphorylated residues within the SPRR completely abolished paradoxical growth due to caspofungin [53].

Although the regulation of chitin synthases by Ca^{2+} and the calcineurin pathway has only been explored in a few fungal species, calmodulin, a key modulator of calcineurin, has been implicated in the proper activation of chitin synthase activity in *Neurospora crassa* [54]. In addition, in the dimorphic fungus *Benjaminiella poitrasii*, upregulation of chitin synthase activity can be attenuated by blocking Ca^{2+} channels [55]. Based on in vitro data, calcineurin activity and calmodulin are important for wild-type levels of chitin synthase activity in *A. fumigatus* [48]. This role likely involves the calmodulin-mediated activation of CnaA in response to cell wall stress following caspofungin treatment. Activated CnaA that is phosphorylated can subsequently dephosphorylate its transcription factor, CrzA, which, in turn, induces transcription of key cell wall–related genes encoding for the chitin synthases and β-1,3-glucan synthase. These chitin synthases also may be responsible for the chitin biosynthetic response to β-1,3-glucan synthase inhibition. Although our data support this model, we cannot rule out the possibility that calcineurin may posttranslationally control other proteins important for chitin biosynthesis, including the chitin synthases themselves, via dephosphorylation. In fact, the increased chitin content observed in the *A. fumigatus* Δ*crzA* mutant at high caspofungin concentrations supports the possibility of an alternative mechanism for CnaA function in chitin biosynthesis. A deeper understanding of these mechanisms could lead to even greater therapeutic benefits from echinocandin treatment.

Undoubtedly, the use of calcineurin inhibitors in combination with cell wall inhibitors as treatment regimen is therefore potentially attractive. Recently caspofungin-mediated paradoxical growth was also evident in 60% of *Candida* species isolated from the bloodstream, which was eliminated by calcineurin pathway inhibitors (tacrolimus and cyclosporine A), and the

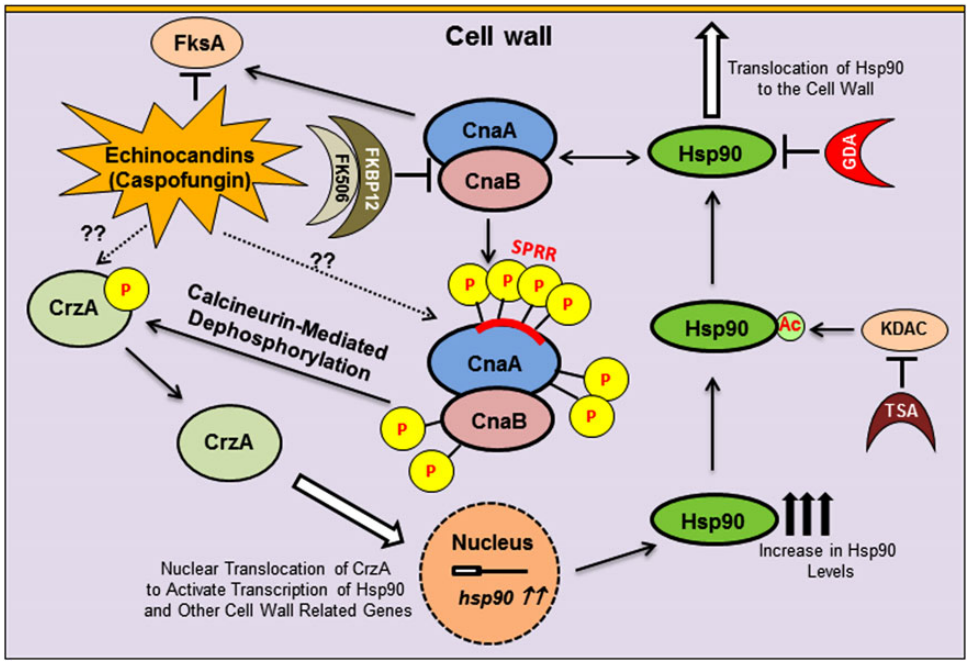

Figure 1. Schematic model of calcineurin and heat shock protein 90 (Hsp90) network in response to caspofungin treatment. Caspofungin inhibits β-1,3-glucan synthase (FksA). This cell wall stress is believed to trigger an increase in the expression of calcineurin. Calcineurin, a heterodimer comprising of the catalytic subunit (CnaA) and the regulatory subunit (CnaB), is then activated in response to cell wall stress. Calcineurin function is inhibited by the binding of the immunophilin-immunosuppressant complex (FK506-FKBP12). CnaA is activated through phosphorylation at 4 serine residues in the serine-proline rich region (SPRR) and also at 2 serine residues in the C-terminus, as well as 2 serine residues in the N-terminus of CnaB. The phosphorylated [P] calcineurin complex may bind to the phosphorylated transcription factor, CrzA, and dephosphorylate it, resulting in its nuclear translocation to mediate the transcription of other genes. It is not known if caspofungin directly influences the phosphorylation status of calcineurin or CrzA. Calcineurin also orchestrates its function through interaction with Hsp90, which is a target of geldanamycin (GDA). The activated calcineurin-Hsp90 complex is involved in the regulation of stress response, cell wall integrity, growth, and drug resistance. Hsp90 responds to cell wall stress through an increase in its expression and by localizing to the sites of cell wall damage. Lysine deacetylases (KDAC) control the acetylation status (Ac) and function of Hsp90. FK506, GDA, and trichostatin A (TSA) represent different ways to interfere with this compensatory response and to potentiate the action of caspofungin. Known and putative interactions are represented by solid and dotted single arrows, respectively. White arrows represent a change of localization. Black and bold arrows indicate increased expression of *hsp90*.

chitin synthase inhibitor, nikkomycin Z, with these inhibitors also being synergistic with caspofungin [10].

HSP90 PATHWAY: EMERGING AS A KEY REGULATOR OF PARADOXICAL GROWTH

As a molecular chaperone, heat shock protein 90 (Hsp90) controls the maturation, folding, and activation of a major part of the eukaryotic proteome, and assumes the role of guardian of intracellular homeostasis by governing multiple networks of stress adaptation [56]. Hsp90 has emerged as an essential trigger of antifungal resistance to both azoles and echinocandins in *C. albicans*, and this effect appears to result from the interaction with its client protein calcineurin [57–59]. The crucial role of Hsp90 in basal echinocandin resistance was also demonstrated in *A. fumigatus* and *A. terreus* [57, 60, 61]. Its role in the caspofungin paradoxical effect has been mainly described in *A. fumigatus* [61, 62].

While Hsp90 represents one of the most abundant cytosolic protein under basal growth conditions, its expression increases drastically under various stress conditions, such as heat shock [63]. The cell wall stress induced by caspofungin results in an approximately 2-fold increase of *hsp90* transcript levels in *A. fumigatus* [62] and is associated with a shift of Hsp90 from the cytosol to the cell wall, septa, and sites of hyphal regeneration (Figure 1) [61], which supports its crucial role in this response. This significant increase of *hsp90* expression seems to be required for proper adaptive mechanisms in response to caspofungin stress. Various genetic modifications of the *A. fumigatus hsp90* promoter resulting in decreased *hsp90* expression were associated with significant hypersensitivity to caspofungin and loss of the paradoxical effect at higher caspofungin concentrations (4 μg/mL) [61, 62]. Pharmacologic inhibition of Hsp90 by geldanamycin (GDA) resulted in a similar effect [61].

We have also shown that the 100-bp proximal region of the *A. fumigatus hsp90* promoter (p100) was crucial for basal resistance to caspofungin including the paradoxical response [62]. Interestingly, the *hsp90* promoter of *C. albicans* does not seem to contain a sequence similar to *A. fumigatus* p100 and was not able to restore the paradoxical response in *A. fumigatus* lacking the p100 promoter region. These observations support the existence of divergences in compensatory responses to caspofungin-induced cell wall stress among fungi, as illustrated by the species-specific characteristics of the paradoxical effect.

Hsp90 activation at the posttranslational level is also crucial for triggering the cascade of Hsp90-dependent pathways of echinocandin resistance in *A. fumigatus*. Although phosphorylation is an important process in governing Hsp90 function in eukaryotes [64], mechanisms of acetylation and deacetylation appear to be predominant in this case. Two acetylation sites that are crucial for Hsp90 function have been identified in fungi: K27 and K270 (*Saccharomyces cerevisiae*)/K271 (*A. fumigatus*) [65, 66]. A deletion or acetylation-mimetic mutation of K27 in *A. fumigatus* resulted in decreased basal resistance to caspofungin and abolition of the paradoxical effect, which could be restored by a deacetylation-mimetic mutation [66]. Although the single mutation of K271 had no phenotypic impact, the acetylation-mimetic mutation of both K27 and K271 resulted in further loss of thermal adaptation, as well as a growth defect and decreased virulence similar to Hsp90 repression. A comparable effect was achieved with trichostatin A, a broad-spectrum inhibitor of both class I and II lysine deacetylases (KDAC), which was shown to induce Hsp90 acetylation in eukaryotes [66, 67]. These results support the role of KDAC in governing Hsp90 function in echinocandin resistance pathways.

Hsp90, working in concert with calcineurin, is another essential trigger of caspofungin basal resistance in *A. fumigatus*, and its function in this pathway can be compromised by various means. Impaired *hsp90* expression at the transcriptional level, direct inhibition of Hsp90 by competitive binding of GDA to the ATPase pocket, and induction of Hsp90 acetylation by the KDAC inhibitor trichostatin A (TSA) all result in increased susceptibility to caspofungin and abolition of the paradoxical effect. Both GDA and TSA demonstrated in vitro synergism with caspofungin against *A. fumigatus*, and positive interactions have also been observed in other *Aspergillus* species [66, 68].

PARADOXICAL EFFECT SUMMARY

From all previous published studies, the paradoxical effect appears to not be a true resistance mechanism, but rather a phenomenon of dose-dependent tolerance in response to cell wall stress and damage. This microbiological effect occurs much more commonly with caspofungin than with the other 2 available echinocandins, and the effect has been documented in vitro at concentrations that are achievable in human sera with conventional dosing strategies, and potentially more relevant in higher dosing schemes. While it is very clear that the paradoxical effect is a cell stress response, the exact molecular mechanisms of this have not been completely deciphered. This serves as an effective tool to study, in great detail, the complex cellular actions and responses following antifungal drug exposure in an effort to optimize our therapies and develop new strategies for treatment.

What is unclear is the potential clinical relevance of this effect. In vitro studies are clear that it reliably exists in a certain percentage of fungal strains, and in vivo studies are somewhat contradictory in that some show no effect and others have reasonable suggestions of decreased efficacy using higher echinocandin dosing. The few clinical studies that have even tangentially addressed this issue are also not definitive. As larger daily or once-weekly doses of echinocandins will lead to super-therapeutic concentrations in the serum and infected tissues, this issue needs to continue to be explored to both define specific mechanisms of action as well as optimally deliver life-saving antifungal agents.

Notes

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References

1. Pappas PG, Kauffman CA, Andes D, et al. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis **2009**; 48:503–35.
2. Walsh TJ, Anaissie EJ, Denning DW, et al. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis **2008**; 46:327–60.
3. Eagle H, Musselman AD. The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. J Exp Med **1948**; 88:99–131.
4. Hall GS, Myles C, Pratt KJ, Washington JA. Cilofungin (LY121019), an antifungal agent with specific activity against *Candida albicans* and *Candida tropicalis*. Antimicrob Agents Chemother **1988**; 32:1331–5.
5. Pfaller MA, Wey S, Gerarden T, Houston A, Wenzel RP. Susceptibility of nosocomial isolates of *Candida* species to LY121019 and other antifungal agents. Diagn Microbiol Infect Dis **1989**; 12:1–4.
6. Bartizal C, Odds FC. Influences of methodological variables on susceptibility testing of caspofungin against *Candida* species and *Aspergillus fumigatus*. Antimicrob Agents Chemother **2003**; 47:2100–7.
7. Ramage G, VandeWalle K, Bachmann SP, Wickes BL, Lopez-Ribot JL. In vitro pharmacodynamic properties of three antifungal agents against preformed *Candida albicans* biofilms determined by time-kill studies. Antimicrob Agents Chemother **2002**; 46:3634–6.
8. Stevens DA, Espiritu M, Parmar R. Paradoxical effect of caspofungin: reduced activity against *Candida albicans* at high drug concentrations. Antimicrob Agents Chemother **2004**; 48:3407–11.

9. Stevens DA, White TC, Perlin DS, Selitrennikoff CP. Studies of the paradoxical effect of caspofungin at high drug concentrations. Diagn Microbiol Infect Dis **2005**; 51:173–8.
10. Shields RK, Nguyen MH, Press EG, Clancy CJ. Five-minute exposure to caspofungin results in prolonged postantifungal effects and eliminates the paradoxical growth of *Candida albicans*. Antimicrob Agents Chemother **2011**; 55:3598–602.
11. Melo AS, Colombo AL, Arthington-Skaggs BA. Paradoxical growth effect of caspofungin observed on biofilms and planktonic cells of five different *Candida species*. Antimicrob Agents Chemother **2007**; 51: 3081–8.
12. Walraven CJ, Bernardo SM, Wiederhold NP, Lee SA. Paradoxical antifungal activity and structural observations in biofilms formed by echinocandin-resistant *Candida albicans* clinical isolates. Med Mycol **2014**; 52:131–9.
13. Antachopoulos C, Meletiadis J, Sein T, Roilides E, Walsh TJ. Comparative in vitro pharmacodynamics of caspofungin, micafungin, and anidulafungin against germinated and nongerminated *Aspergillus conidia*. Antimicrob Agents Chemother **2008**; 52:321–8.
14. Stevens DA, Ichinomiya M, Koshi Y, Horiuchi H. Escape of *Candida* from caspofungin inhibition at concentrations above the MIC (paradoxical effect) accomplished by increased cell wall chitin; evidence for beta-1,6-glucan synthesis inhibition by caspofungin. Antimicrob Agents Chemother **2006**; 50:3160–1.
15. Bizerra FC, Melo AS, Katchburian E, et al. Changes in cell wall synthesis and ultrastructure during paradoxical growth effect of caspofungin on four different *Candida* species. Antimicrob Agents Chemother **2011**; 55:302–10.
16. Rueda C, Cuenca-Estrella M, Zaragoza O. Paradoxical growth of *Candida albicans* in the presence of caspofungin is associated with multiple cell wall rearrangements and decreased virulence. Antimicrob Agents Chemother **2014**; 58:1071–83.
17. Shields RK, Nguyen MH, Du C, Press E, Cheng S, Clancy CJ. Paradoxical effect of caspofungin against *Candida* bloodstream isolates is mediated by multiple pathways but eliminated in human serum. Antimicrob Agents Chemother **2011**; 55:2641–7.
18. Lee KK, Maccallum DM, Jacobsen MD, et al. Elevated cell wall chitin in *Candida albicans* confers echinocandin resistance in vivo. Antimicrob Agents Chemother **2012**; 56:208–17.
19. Walker LA, Munro CA, de Bruijn I, Lenardon MD, McKinnon A, Gow NA. Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. PLoS Pathog **2008**; 4:e1000040.
20. Szilagyi J, Foldi R, Bayegan S, Kardos G, Majoros L. Effect of nikkomycin Z and 50% human serum on the killing activity of high-concentration caspofungin against *Candida* species using time-kill methodology. J Chemother **2012**; 24:18–25.
21. Chamilos G, Lewis RE, Albert N, Kontoyiannis DP. Paradoxical effect of echinocandins across *Candida* species in vitro: evidence for echinocandin-specific and *Candida* species-related differences. Antimicrob Agents Chemother **2007**; 51:2257–9.
22. Fleischhacker M, Radecke C, Schulz B, Ruhnke M. Paradoxical growth effects of the echinocandins caspofungin and micafungin, but not of anidulafungin, on clinical isolates of *Candida albicans* and *C. dubliniensis*. Eur J Clin Microbiol Infect Dis **2008**; 27:127–31.
23. Khlif M, Bogreau H, Michel-Nguyen A, Ayadi A, Ranque S. Trailing or paradoxical growth of *Candida albicans* when exposed to caspofungin is not associated with microsatellite genotypes. Antimicrob Agents Chemother **2010**; 54:1365–8.
24. Hadrich I, Neji S, Makni F, Ayadi A, Elloumi M, Ranque S. Trailing or paradoxical growth of *Aspergillus flavus* exposed to caspofungin is independent of genotype. J Med Microbiol **2014**; 63:1584–9.
25. Clemons KV, Espiritu M, Parmar R, Stevens DA. Assessment of the paradoxical effect of caspofungin in therapy of candidiasis. Antimicrob Agents Chemother **2006**; 50:1293–7.
26. Abruzzo GK, Flattery AM, Gill CJ, et al. Evaluation of the echinocandin antifungal MK-0991 (L-743,872): efficacies in mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. Antimicrob Agents Chemother **1997**; 41:2333–8.
27. Graybill JR, Bocanegra R, Luther M, Fothergill A, Rinaldi MJ. Treatment of murine *Candida krusei* or *Candida glabrata* infection with L-743,872. Antimicrob Agents Chemother **1997**; 41:1937–9.
28. Zanette RA, Kontoyiannis DP. Paradoxical effect to caspofungin in *Candida* species does not confer survival advantage in a *Drosophila* model of candidiasis. Virulence **2013**; 4:497–8.
29. Petraitiene R, Petraitis V, Groll AH, et al. Antifungal efficacy of caspofungin (MK-0991) in experimental pulmonary aspegillosis in persistently neutropenic rabbits: pharmacokinetics, drug deposition, and relationship to galactomannan antigenemia. Antimicrob Agents Chemother **2002**; 46:12–23.
30. Lewis RE, Albert ND, Kontoyiannis DP. Comparison of the dose-dependent activity and paradoxical effect of caspofungin and micafungin in a neutropenic murine model of invasive pulmonary aspergillosis. J Antimicrob Chemother **2008**; 61:1140–4.
31. Moretti S, Bozza S, D'Angelo C, et al. Role of innate immune receptors in paradoxical caspofungin activity in vivo in preclinical aspergillosis. Antimicrob Agents Chemother **2012**; 56:4268–76.
32. Wiederhold NP, Kontoyiannis DP, Chi J, Prince RA, Tam VH, Lewis RE. Pharmacodynamics of caspofungin in a murine model of invasive pulmonary aspergillosis: evidence of concentration-dependent activity. J Infect Dis **2004**; 190:1464–71.
33. Gumbo T, Drusano GL, Liu W, et al. Once-weekly micafungin therapy is as effective as daily therapy for disseminated candidiasis in mice with persistent neutropenia. Antimicrob Agents Chemother **2007**; 51:968–74.
34. Ibrahim AS, Bowman JC, Avanessian V, et al. Caspofungin inhibits *Rhizopus oryzae* 1,3-beta-D-glucan synthase, lowers burden in brain measured by quantitative PCR, and improves survival at a low but not a high dose during murine disseminated zygomycosis. Antimicrob Agents Chemother **2005**; 49:721–7.
35. Betts RF, Nucci M, Talwar D, et al. A multicenter, double-blind trial of a high-dose caspofungin treatment regimen versus a standard caspofungin treatment regimen for adult patients with invasive candidiasis. Clin Infect Dis **2009**; 48:1676–84.
36. Pappas PG, Rotstein CM, Betts RF, et al. Micafungin versus caspofungin for treatment of candidemia and other forms of invasive candidiasis. Clin Infect Dis **2007**; 45:883–93.
37. Safdar A, Rodriguez G, Rolston KV, et al. High-dose caspofungin combination antifungal therapy in patients with hematologic malignancies and hematopoietic stem cell transplantation. Bone Marrow Transplant **2007**; 39:157–64.
38. Safdar A, Rodriguez G, Zuniga J, Akhrass FA, Pande A. High-dose caspofungin as a component of combination antifungal therapy in 91 patients with neoplastic diseases and hematopoietic stem cell transplantation: a critical review of short-term and long-term adverse events. J Pharm Pract **2015**; 28:175–82.
39. Yamazaki S, Nakamura F, Yoshimi A, Ichikawa M, Nannya Y, Kurokawa M. Safety of high-dose micafungin for patients with hematological diseases. Leuk Lymphoma **2014**; 55:2572–6.
40. Wiederhold NP, Kontoyiannis DP, Prince RA, Lewis RE. Attenuation of the activity of caspofungin at high concentrations against *Candida albicans*: possible role of cell wall integrity and calcineurin pathways. Antimicrob Agents Chemother **2005**; 49:5146–8.
41. Steinbach WJ, Schell WA, Blankenship JR, Onyewu C, Heitman J, Perfect JR. In vitro interactions between antifungals and immunosuppressants against *Aspergillus fumigatus*. Antimicrob Agents Chemother **2004**; 48:1664–9.
42. Steinbach WJ, Singh N, Miller JL, et al. In vitro interactions between antifungals and immunosuppressants against *Aspergillus fumigatus* isolates from transplant and nontransplant patients. Antimicrob Agents Chemother **2004**; 48:4922–5.
43. Steinbach WJ, Cramer RA Jr, Perfect BZ, et al. Calcineurin inhibition or mutation enhances cell wall inhibitors against *Aspergillus fumigatus*. Antimicrobial Agents Chemother **2007**; 51:2979–81.

44. Steinbach WJ, Cramer RA Jr, Perfect BZ, et al. Calcineurin controls growth, morphology, and pathogenicity in *Aspergillus fumigatus*. Eukaryotic Cell **2006**; 5:1091–103.
45. Steinbach WJ, Reedy JL, Cramer RA Jr, Perfect JR, Heitman J. Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. Nat Rev Microbiol **2007**; 5:418–30.
46. Cramer RA Jr, Perfect BZ, Pinchai N, et al. Calcineurin target CrzA regulates conidial germination, hyphal growth, and pathogenesis of *Aspergillus fumigatus*. Eukaryotic Cell **2008**; 7:1085–97.
47. Fortwendel JR, Juvvadi PR, Pinchai N, et al. Differential effects of inhibiting chitin and 1,3-beta-D-glucan synthesis in Ras and calcineurin mutants of *Aspergillus fumigatus*. Antimicrob Agents Chemother **2009**; 53:476–82.
48. Fortwendel JR, Juvvadi PR, Perfect BZ, Rogg LE, Perfect JR, Steinbach WJ. Transcriptional regulation of chitin synthases by calcineurin controls paradoxical growth of *Aspergillus fumigatus* in response to caspofungin. Antimicrobial Agents Chemother **2010**; 54:1555–63.
49. Rogg LE, Fortwendel JR, Juvvadi PR, Lilley A, Steinbach WJ. The chitin synthase genes chsA and chsC are not required for cell wall stress responses in the human pathogen *Aspergillus fumigatus*. Biochem Biophys Res Commun **2011**; 411:549–54.
50. Muszkieta L, Aimanianda V, Mellado E, et al. Deciphering the role of the chitin synthase families 1 and 2 in the in vivo and in vitro growth of *Aspergillus fumigatus* by multiple gene targeting deletion. Cell Microbiol **2014**; 16:1784–805.
51. Juvvadi PR, Lamoth F, Steinbach WJ. Calcineurin as a multifunctional regulator: unraveling novel functions in fungal stress responses, hyphal growth, drug resistance, and pathogenesis. Fungal Biol Rev **2014**; 28:56–69.
52. Juvvadi PR, Fortwendel JR, Rogg LE, Burns KA, Randell SH, Steinbach WJ. Localization and activity of the calcineurin catalytic and regulatory subunit complex at the septum is essential for hyphal elongation and proper septation in *Aspergillus fumigatus*. Mol Microbiol **2011**; 82:1235–59.
53. Juvvadi PR, Gehrke C, Fortwendel JR, et al. Phosphorylation of calcineurin at a novel serine-proline rich region orchestrates hyphal growth and virulence in *Aspergillus fumigatus*. PLoS Pathog **2013**; 9:e1003564.
54. Suresh K, Subramanyam C. A putative role for calmodulin in the activation of *Neurospora crassa* chitin synthase. FEMS Microbiol Lett **1997**; 150:95–100.
55. Deshpande MV, O'Donnell R, Gooday GW. Regulation of chitin synthase activity in the dimorphic fungus *Benjaminiella poitrasi*i by external osmotic pressure. FEMS Microbiol Lett **1997**; 152:327–32.
56. Leach MD, Klipp E, Cowen LE, Brown AJ. Fungal Hsp90: a biological transistor that tunes cellular outputs to thermal inputs. Nat Rev Microbiol **2012**; 10:693–704.
57. Cowen LE, Lindquist S. Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. Science **2005**; 309:2185–9.
58. Cowen LE, Carpenter AE, Matangkasombut O, Fink GR, Lindquist S. Genetic architecture of Hsp90-dependent drug resistance. Eukaryot Cell **2006**; 5:2184–8.
59. Singh SD, Robbins N, Zaas AK, Schell WA, Perfect JR, Cowen LE. Hsp90 governs echinocandin resistance in the pathogenic yeast *Candida albicans* via calcineurin. PLoS Pathog **2009**; 5:e1000532.
60. Cowen LE, Singh SD, Köhler JR, et al. Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. Proc Natl Acad Sci U S A **2009**; 106:2818–23.
61. Lamoth F, Juvvadi PR, Fortwendel JR, Steinbach WJ. Heat shock protein 90 is required for conidiation and cell wall integrity in *Aspergillus fumigatus*. Eukaryot Cell **2012**; 11:1324–32.
62. Lamoth F, Juvvadi PR, Gehrke C, Asfaw YG, Steinbach WJ. Transcriptional activation of heat shock protein 90 mediated via a proximal promoter region as trigger of caspofungin resistance in *Aspergillus fumigatus*. J Infect Dis **2014**; 209:473–81.
63. Lamoth F, Juvvadi PR, Steinbach WJ. Heat shock protein 90 (Hsp90): a novel antifungal target against *Aspergillus fumigatus*. Crit Rev Microbiol **2014**; 22:1–12.
64. Mollapour M, Neckers L. Post-translational modifications of Hsp90 and their contributions to chaperone regulation. Biochim Biophys Acta **2012**; 1823:648–55.
65. Robbins N, Leach MD, Cowen LE. Lysine deacetylases Hda1 and Rpd3 regulate Hsp90 function thereby governing fungal drug resistance. Cell Rep **2012**; 2:878–88.
66. Lamoth F, Juvvadi PR, Soderblom EJ, Moseley MA, Asfaw YG, Steinbach WJ. Identification of a key lysine residue in heat shock protein 90 required for azole and echinocandin resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother **2014**; 58:1889–96.
67. Scroggins BT, Robzyk K, Wang D, et al. An acetylation site in the middle domain of Hsp90 regulates chaperone function. Mol Cell **2007**; 25:151–9.
68. Lamoth F, Juvvadi PR, Gehrke C, Steinbach WJ. In vitro activity of calcineurin and heat shock protein 90 inhibitors against *Aspergillus fumigatus* azole- and echinocandin-resistant strains. Antimicrob Agents Chemother **2013**; 57:1035–9.