Calcineurin in fungal virulence and drug resistance: Prospects for harnessing targeted inhibition of calcineurin for an antifungal therapeutic approach

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ABSTRACT

Increases in the incidence and mortality due to the major invasive fungal infections such as aspergillosis, candidiasis and cryptococcosis caused by the species of Aspergillus, Candida, and Cryptococcus, are a growing threat to the immunosuppressed patient population. In addition to the limited armamentarium of the current classes of antifungal agents available (pyrimidine analogs, polyenes, azoles, and echinocandins), their toxicity, efficacy and the emergence of resistance are major bottlenecks limiting successful patient outcomes. Although these drugs target distinct fungal pathways, there is an urgent need to develop new antifungals that are more efficacious, fungal-specific, with reduced or no toxicity and simultaneously do not induce resistance. Here we review several lines of evidence which indicate that the calcineurin signaling pathway, a target of the immunosuppressive drugs FK506 and cyclosporine A, orchestrates growth, virulence and drug resistance in a variety of fungal pathogens and can be exploited for novel antifungal drug development.

KEYWORDS

antifungals; azoles; calcineurin; cyclosporine A; drug resistance; echinocandins; FK506; FKBP12; Hsp90; virulence

Introduction

With the ever increasing immunosuppressed patient population, infections due to opportunistic fungi are becoming more common and the most frequently isolated fungal pathogens include species of Candida, Cryptococcus and Aspergillus. Adding to this complexity are the insufficient therapeutic options available for these invasive fungal infections due to the limited number of antifungal drugs, their toxicity and the emergence of drug resistance. There is a critical need for new antifungal agents with broad spectrum antifungal activity, little or no drug resistance, and reduced adverse effects compared to currently available drugs. Therefore, approaches toward identifying novel fungal-specific targets and designing customized inhibitors are required.

In recent years, signal transduction mechanisms involving the key secondary messenger calcium have gained significance due to its requirement for adaptation and survival of multiple fungi and in varied environments. Calcineurin has been identified as one of the important regulators of intracellular calcium homeostasis in several fungi. Furthermore, antifungal resistance has also been linked to calcium and calcineurin signaling cascade. For example, combinations of calcium and calcineurin inhibitors with known antifungal compounds have been shown to inhibit the growth of drug resistant fungal strains. Therefore, inhibition of calcineurin signaling is a novel antifungal strategy that both attenuates fungal virulence and increases the efficacy of the existing antifungals with concomitant suppression of antifungal resistance.

Calcineurin is a conserved Ca\textsuperscript{2+}-calmodulin (CaM) activated protein phosphatase 2B belonging to the phospho-protein phosphatase family of enzymes and is involved in calcium-dependent signaling and regulation of several important cellular processes in both yeasts and filamentous fungi (Fig. 1). It is a heterodimer comprised of a catalytic subunit (calcineurin A; CnA) and a regulatory subunit (calcineurin B; CnB). The catalytic subunit contains an N-terminal phosphatase domain, the regulatory subunit binding helix (CnBBH) along with the CaM-binding domain (CaMBD). An autoinhibitory domain (AID) that blocks the catalytic activity is also present at the C-terminal end of the protein.
Association of CaM causes a conformational change that removes the AID and activates the phosphatase complex. Ca$^{2+}$ signals are then transmitted via the active trimeric complex (CnA-CnB-CaM) to elicit downstream responses by regulating its key transcription factor, Crz1, a fungal homolog of mammalian NFAT, through dephosphorylation and its nuclear translocation (Fig. 1). This results in the induction of several calcineurin-dependent target genes which function in a wide array of cellular functions.

Following the discovery of calcineurin as a CaM-binding protein of the nervous system, it has since been characterized in several organisms ranging from the prokaryotes to the higher eukaryotes. Calcineurin has links to neuronal metabolism, T-lymphocyte proliferation, immune suppression, regulation of intracellular Ca$^{2+}$ homeostasis, and various human diseases that have been well recognized.

In fungi, a requirement of calcineurin for regulating stress responses and growth was demonstrated, revealing its diverse and multifunctional roles across various species. Calcineurin biology has gained significance over the years due to its target role of the immunosuppressive drugs, cyclosporine A (CsA) and FK506 (tacrolimus), which inhibit the cellular activity of calcineurin via their interaction with the respective immunophilins, cyclophilin A and FKBP12.

Efforts to understand the mechanism of inhibition of calcineurin by these compounds have led to a detailed characterization of the residues involved in drug-protein interactions. Owing to the seminal importance of calcineurin for growth and pathogenesis in plant and human fungal pathogens, recent studies are focused on capitalizing upon calcineurin as a potential antifungal target. While the currently available anti-calcineurin drugs are immunosuppressive in nature, it would be greatly beneficial to design fungal-specific and alternative strategies.
Calcineurin is required for virulence and drug resistance in a diverse group of fungi

The first evidence for the requirement of calcineurin for virulence came from work on *Cryptococcus neoformans*. It followed by other human fungal pathogens, including *Candida albicans*, along with other species of *Candida*, *Cryptococcus* and the filamentous fungal pathogen *Aspergillus fumigatus*. Furthermore, a role for calcineurin in the pathogenesis of prominent plant fungal pathogens such as *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Magnaporthe oryzae*, *Ustilago maydis* and *U. hordei* has also been described. Interestingly, despite the spectrum of host niches and varied modes of infection of these diverse and morphologically distinct pathogens, calcineurin maintains a conserved role in virulence or pathogenic traits. In a majority of these fungi, calcineurin is required for growth, transition between morphological states, cation homeostasis and stress responses. These characteristics are expected to be the most plausible attributes for the observed defects in virulence of these pathogens in different host settings in the absence of calcineurin function. The established role for calcineurin in the cell wall integrity pathway of different fungi is another important feature contributing to survival in the host environment and for virulence. The fungal cell wall, consisting largely of glucan and chitin moieties, is thought to be a unique feature that imparts protection against environmental stressors and also host immunity. While azole antifungals inhibit the ergosterol biosynthesis pathway and lead to membrane stress by compromising the integrity of the cell membrane, the echinocandins target cell wall β-glucan synthesis. Therefore, antifungal agents targeting the cell membrane and cell wall are being used for antifungal therapy. The calcineurin mediated signal transduction pathway has been shown to impact both the cell membrane and cell wall integrity through the regulation of other downstream effectors that influence the biosynthesis of ergosterol, chitin and β-glucan.

**Candida, Cryptococcus and emerging molds**

In *C. albicans*, calcineurin is required for morphogenesis, azole tolerance, membrane stress, survival in serum and virulence. *C. albicans* is the major causative agent of invasive candidiasis in immunocompromised patients, yet several other species of *Candida* (*C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis*) are also associated with disease. While the requirement of calcineurin for hyphal growth in *C. albicans* remains controversial, it varies according to the developmental stage (hyphal or pseudohyphal) in other *Candida* species. In *C. dubliniensis* and *C. tropicalis*, calcineurin is required for hyphal growth, but in *C. lusitaniae* it is only necessary for regulating pseudohyphal growth. In addition, temperature sensitivity was noted only in the *C. glabrata* calcineurin mutants but not the other *Candida* species examined.

Calcineurin also regulates antifungal susceptibility. Characterization of calcineurin and crz1 mutants across different *Candida* species indicated a divergence in their functions with respect to antifungal activity. For instance, fungicidal action of azoles was noted in combination with calcineurin inhibitors against *C. albicans*, *C. glabrata* and *C. krusei*. Both *C. albicans* and *C. dubliniensis* calcineurin and crz1 mutants exhibited reduced tolerance to azole and echinocandin antifungal drugs. On the contrary, only calcineurin mutants showed reduced tolerance to azoles in *C. lusitaniae*, while crz1 mutants actually showed increased drug tolerance, indicative of calcineurin-independent functions for Crz1 in *C. lusitaniae*. Although isolates of *C. dubliniensis* are susceptible to azole antifungal agents, emergence of resistance during therapy has been observed. Similarly, in *C. lusitaniae* a unique resistance to amphotericin B and dimorphic switching between the yeast and filamentous forms was noted, but calcineurin or Crz1 did not seem to have any role in amphotericin B tolerance.

Calcineurin inhibition along with fluconazole treatment also has been shown to have a synergistic effect against azole-resistant *C. albicans*. FK506 also had a synergistic effect with caspofungin on echinocandin resistant strains of *C. dubliniensis* and *C. lusitaniae* revealing their potential use in combination therapy for these emerging drug-resistant isolates. CsA also showed a synergistic effect with other antifungals against *C. parapsilosis* and related species. Furthermore, non-azole drugs such as terbinaine and fenpropimorph that target other enzymes in the ergosterol biosynthetic pathway also exhibit synergism with FK506 and CsA against *C. albicans*. Overall these variations observed across different *Candida* species and the unexplored drug resistance mechanisms prompt the need for an in depth understanding of the calcineurin network for effective targeting.

Cryptococcosis caused by *C. neoformans* and *C. gattii* is one of the major opportunistic infections in immunocompromised patients worldwide and initial induction treatment includes amphotericin B combined with flucytosine, followed by azole therapy for maintenance therapy. *C. gattii* also infects immunocompetent healthy individuals and causes both pulmonary...
infections and life-threatening meningoencephalitis.

The ability of *C. neoformans* and *C. gattii* to grow at host body temperature (37°C) is one of the virulence attributes that is controlled by the calcineurin signaling pathway. In addition, calcineurin is also required for growth at alkaline pH, hyphal elongation during mating and monokaryotic fruiting. During thermal stress, *C. neoformans* calcineurin was shown to localize as endoplasmic reticulum (ER)-associated puncta co-localizing with P-bodies and stress granules, possibly reflective of a role in post-transcriptional control under temperature stress. Although Cryptococcal species are susceptible to amphotericin B, fluconazole and triazoles (fluconazole, itraconazole, posaconazole and voriconazole), they are resistant to echinocandins (anidulafungin, caspofungin and micafungin) and also develop resistance to fluconazole. Surprisingly, it was shown that in contrast to *C. neoformans* in *C. gattii* calcineurin is required for fluconazole tolerance, revealing its divergent role in this closely related species. While the requirement of calcineurin for azole resistance in the majority of *Candida* species is known, the same is not yet clear in *C. neoformans* or *C. gattii*. Although FK506 exhibits a synergistic antifungal effect with fluconazole against *C. neoformans*, this phenomenon seems to be FKBP12 and calcineurin-independent, indicating the possibility for additional targets of FK506 in *C. neoformans*. For instance, synergistic activity of FK506 with bafilomycin A1, an inhibitor of H(+) ATPase, has been demonstrated in *C. neoformans* strains lacking calcineurin.

The development and use of well-designed non-immunosuppressive FK506 and CsA analogs that specifically target fungal calcineurin without host cross-reactivity certainly seems promising. One such FK506 analog is L-685,818 (12-ethyl-FK506), which has been previously tested on the growth of *C. neoformans* and other fluconazole-resistant clinical isolates and found to exhibit susceptibilities similar to that of FK506. Two other non-immunosuppressive CsA derivatives have also been shown to inhibit *C. neoformans* calcineurin. Therefore, further studies on designing and testing of non-immunosuppressive analogs are warranted to exploit this approach.

Recent work on the emerging pathogenic molds belonging to Mucorales, including *Rhizopus* and *Mucor* species that cause invasive mucormycosis, have also revealed that the use of calcineurin inhibitors in combination with other antifungals has beneficial outcomes. The genome of *M. circinelloides* revealed an unusually high number of genes encoding for calmodulin (9 genes) and calcineurin catalytic subunits (3 genes), but with a single calcineurin regulatory subunit. Pharmacologic and genetic studies showed that calcineurin is required for the dimorphic transition (yeast to hyphal form) and the calcineurin regulatory subunit is required for virulence.

**Aspergillus fumigatus**

*A. fumigatus* is a filamentous fungal pathogen known to cause allergic, chronic and invasive aspergillosis. Invasive aspergillosis is a leading cause of death in leukemic patients and haematopoietic stem cell or solid-organ transplant recipients. Although *A. fumigatus* infections can be treated with polyene, triazole, or echinocandin antifungals, their efficacy is limited, azole resistance is increasing, and mortality due to invasive aspergillosis remains high.

Calcineurin is involved in regulating hyphal growth, septation and virulence of this opportunistic pathogen. While the deletion of *A. fumigatus crzA* (*crz1* homolog) also conferred defects in growth, conidiation and virulence, it was suggested to play a minor role in the regulation of β-1,3-glucan biosynthesis. Also, deletion of another closely related gene encoding the calcineurin binding protein (*cbpA*), belonging to the calcipressin family, resulted in only a minor hyphal growth defect and limited attenuation of virulence.

Calcineurin inhibitors are active in vitro against *A. fumigatus* as monotherapy and potentiate the effect of the echinocandin, caspofungin. However, following treatment with high concentrations of caspofungin, the “paradoxical growth effect,” or reversal of growth inhibition, is observed. While deletion of calcineurin abolishes the paradoxical growth response, it also causes reduction in β-1,3-glucan content, leading to a compensatory increase in chitin. It appears that the glucan-chitin interaction is controlled by calcineurin signaling through the transcriptional regulation of chitin synthases. This apparent cell wall stress response is ideal for potential future combination therapy in which calcineurin, β-1,3-glucan, and chitin targets can all be blocked by drugs, supporting a multi-faceted attack on cell wall targets and hyphal growth in *A. fumigatus*. The antifungal activity of calcineurin inhibitors was also demonstrated against various azole- and echinocandin-resistant *A. fumigatus* clinical isolates.

The formation and extension of *Aspergillus* hyphae confers the ability to actively penetrate host tissue and spread disease. Recent studies have indicated that the calcineurin complex (the catalytic and regulatory subunits) localizes at the actively growing hyphal tips and septa to direct hyphal elongation and proper septation. Although mutations in functional domains of
calcineurin have revealed important residues required for localization and function of calcineurin at the septum, the exact mechanism of how calcineurin regulates septation is still unclear. A filamentous fungal-specific serine-proline rich (SPRR) linker domain between the CnBBH and CaMBD was also identified in the *A. fumigatus* calcineurin catalytic subunit. This SPRR was phosphorylated at all 4 clustered serine residues and mutations in this region caused hyphal growth and virulence defects, implicating the importance of calcineurin phosphorylation for its function. Identification of key regions for calcineurin function that are unique to fungi and absent in humans is a promising step toward the development of novel antifungal therapies.

### Plant fungal pathogens

In the filamentous plant pathogenic fungi, calcineurin is important for sclerotal development in *S. sclerotiorum* and formation of the infectious structure appressorium in *M. oryzae*. In the necrotrophic fungus *B. cinerea*, deletion of *crz1* caused defects in cell wall and membrane integrity and defects in hyphal penetration into the plant tissue. In the plant pathogenic basidiomycete *U. maydis* deletion of the calcineurin catalytic subunit resulted in multiple budding and reduced mating, and in a related species *U. hordet* calcineurin is required for adaptation to a variety of environmental stresses, cell-wall integrity, mating and virulence. The costs of treating fungal diseases in agriculture are equally high as for human health and exploiting conserved signaling pathways could lead to beneficial outcomes in both sectors. One such example is a recent effort to identify novel targets in the MAP kinase signaling pathway by the ARIDANE consortium focusing on plant pathogenic fungi (http://cordis.europa.eu/result/rcn/161258_en.html) that yielded important results and may have potential impact on treatment of fungal infections. Along similar lines, an explicit understanding of the calcineurin signaling pathway and its substrates in various fungal pathogens will prove useful in the identification of new targets for novel antifungal therapies.

### Primary targets of the calcineurin signaling pathway

With the plethora of cellular functions that calcineurin regulates, several studies have focused on determining its key downstream effectors and substrates through genetic and biochemical approaches. To date, one of the well-characterized substrates of calcineurin is the mammalian NFAT ortholog, Crz1, which mediates transcriptional response triggered by calcineurin activation in response to stress conditions in *Candida species*, *C. neoformans* and *A. fumigatus*. As mentioned earlier, another important binding partner of calcineurin is the calcineurin binding protein, Cbp1, an element involved in fine tuning of calcineurin signaling that was also characterized from *C. neoformans* and *A. fumigatus*. The respective mutant strains exhibited only moderate growth defects under stress conditions, indicating yet unexplored downstream substrates of calcineurin.

Calcineurin exerts its influence on other key pathways regulating cell wall integrity and response to antifungal drugs and this is evident from study of *C. neoformans* MAP kinase regulation. Loss of calcineurin function results in transcriptional activation of the *FKSI* gene encoding the β-1,3-glucan synthase that is essential for the synthesis of β-glucan and dependent on MAP kinase. Recently, whole proteomic analyses to identify calcineurin interacting proteins in the model budding yeast *Saccharomyces cerevisiae* identified 18 calcineurin interactors from among 70 candidate calcineurin substrates. These were involved in polarized growth, glucose sensing, membrane structure, cell wall integrity and other important processes, indicative of diverse roles for calcineurin in cellular processes. Another study also analyzed the calcineurin associated proteome in *C. neoformans*, and identified 139 potential interactors. Multiple proteins involved in membrane trafficking, protein folding, sphingolipid biosynthesis, trehalose synthesis and stress were identified. Importantly, an association of calcineurin with COPI (Sec28) and COPII (Sec13) complexes was demonstrated implicating calcineurin’s role in membrane trafficking and stress response mechanisms.

### Heat shock protein 90 and calcineurin inhibitors: Another promising combination

The heat shock protein 90 (Hsp90) is an essential, abundant and highly conserved molecular chaperone that facilitates proper folding, assembly and maturation of proteins in eukaryotes. Studies on Hsp90 in *C. albicans* have clearly demonstrated its role in biofilm formation and the evolution of drug resistance. Although Hsp90 inhibitors have potent synergistic antifungal activity in combination with azoles and echinocandins, we are faced with the challenge of overcoming toxic effects of current Hsp90 inhibitors in use. Hsp90 is known to regulateazole resistance through its key downstream effectors, calcineurin and the Mkc1 kinase in *C. albicans*. Hsp90 stabilizes calcineurin by direct interactions so the inhibition of Hsp90 is expected to result in depletion or inactivity of the client protein calcineurin.
Recent work in *A. fumigatus* has shown that Hsp90 is required for the caspofungin mediated paradoxical growth response, and that disrupting Hsp90 circuitry potentiates the antifungal activity of caspofungin. Furthermore, targeting the Hsp90–calcineurin pathway through the combination of respective inhibitors not only resulted in fungicidal activity againstazole-resistant *A. fumigatus* strains but also showed distinct patterns of susceptibility among different fungal species revealing this to be a promising alternative strategy.17,91

**Calcineurin: An attractive antifungal target**

Due to limited drug efficacy and emerging resistance to drugs, current antifungal classes would benefit from adjunctive agents focused on separate cellular pathways that can be used in combination therapy. Research on calcineurin biology in various fungal pathogens clearly demonstrates its potential for such exploitation. Although currently available calcineurin inhibitors are unlikely to gain approval for clinical application as antifungal agents, new and more potent fungal-specific calcineurin inhibitors are required that do not cross-react with human calcineurin and do not induce immune suppression.

Advances in drug development have enabled the design and synthesis of non-immunosuppressive calcineurin inhibitors that hold promise in the treatment of human diseases. For example, SDZ PSC-833, a non-immunosuppressive analog of CsA, known to reverse the resistance to chemotherapy of cancer cells, was shown to increase survival time and to some extent cure leukemic mice. Other non-immunosuppressive analogs of CsA, alisporivir (Debio-025) and SCY-635, have been shown to be beneficial for treatment of chronic hepatitis. In addition, the non-immunosuppressive FK506 analog, L-685,818, enhanced the function and morphological recovery of crushed sciatic nerves in rats, showing promise for use in the treatment of neurodegenerative diseases. As mentioned earlier, L-685,818, was also effective on the growth of *C. neoformans* and other fluconazole-resistant isolates with similar susceptibilities as that of FK506.4

The inhibitory activity of FK506 and CsA on calcineurin is mediated by binding of the immunosuppressants to their respective immunophilins, FKBP12 prolyl isomerase and cyclophilin. Characterization of FKBP12 homologs in *C. neoformans* and *A. fumigatus* revealed that the immunophilin is not required for growth and virulence but mediates the interaction between FK506 and calcineurin. The FKBP12 deletion strains were resistant to FK506 and rapamycin, the inhibitor of the TOR signaling pathway. Because of the role of FKBP12 in the inhibition of calcineurin, and with the homology between fungal FKBP12s and human FKBP12 between 40–50%, it is an attractive target for novel antifungal drug development. Recent structural elucidation of FKBP12 proteins from *A. fumigatus*, *C. albicans* and *C. glabrata* provided novel insights into the self-catalyzing function of *A. fumigatus* and *C. albicans* FKBP12s indicating that they may function as their own substrates. This self-dimerization occurs at the 80s loop region where a proline residue is present, and previous studies have shown that the 80s loop serves as a key region for FKBP12 interactions with other proteins.

These unique findings lend support to the idea of exploiting the differences between fungal FKBP12s and human FKBP12 for generation of fungal-specific FK506 analogs. Certainly the development of novel non-immunosuppressive analogs of the calcineurin inhibitors CsA and FK506 that would retain antifungal activity hold promise as better antifungal drugs. On the other hand, identification of fungal-specific domains in calcineurin and an in-depth understanding of the calcineurin pathway and its specific interactors in each of these pathogenic fungi will also help in effective designing of better drugs and also identifying new targets for combating fungal diseases. Elucidation of the complete crystal structure of the calcineurin complexes in these different fungal pathogens would also be very useful in future development of novel drugs that can specifically target the pathogen.

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No potential conflicts of interest were disclosed.

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