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Combination of caspofungin with inhibitors of the calcineurin pathway attenuates growth *in vitro* in *Aspergillus* species

Dimitrios P. Kontoyiannis[1]*, Russell E. Lewis[1,2], Nir Osherov[3], Nathaniel D. Albert[3] and Gregory S. May[3]

[1]Department of Infectious Diseases, Infection Control & Employee Health, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd, Box 402, Houston, TX 77030; [2]Department of Clinical Sciences and Administration, University of Houston College of Pharmacy, Houston, TX; [3]Department of Laboratory Medicine, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

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Work in *Saccharomyces cerevisiae* and *Cryptococcus neoformans* suggests that caspofungin could interact with the calcineurin pathway. We examined the *in vitro* interaction of caspofungin with calcineurin inhibitors (FK506, cyclosporin, FK520 and L685,818) and the TOR inhibitor rapamycin in 13 isolates of *Aspergillus* species. Caspofungin activity was enhanced by calcineurin/TOR inhibitors for all *Aspergillus* isolates studied. Further investigation of this pathway is warranted.

Keywords: caspofungin, calcineurin inhibitors

Introduction

Caspofungin (CAS), a novel echinocandin, inhibits 1,3-β-D-glucan synthase, a fungus-specific enzyme that is critical for cell-wall biosynthesis.[1,2] Against *Aspergillus* species, CAS displays a complex pattern of growth inhibition that results in death of actively growing hyphal tips.[3] Little is known, however, about the mechanisms regulating CAS action in *Aspergillus* spp.[4] Genetic studies in *Saccharomyces cerevisiae* have shown that glucan synthase is composed of two subunits: a putative catalytic subunit encoded by the genes *FKS1* and *FKS2* and a regulatory subunit composed of a GTP-bound protein encoded by *RHO1*.[2]

Interaction between CAS and the evolutionarily conserved calcineurin pathway is suggested by the observation that mutations in *FKS1*, the putative target of CAS activity, result in hypersensitivity of *S. cerevisiae* to the immunosuppressive agent FK506,[2] whose action is mediated through inhibition of the phosphatase calcineurin.[5] In addition, the second *FKS* homologue, *FKS2*, is regulated by calcineurin.[2] Furthermore, FK506 and L685,818, a non-immunosuppressive analogue of FK506,[6,7] have demonstrated synergic interactions with CAS against *Cryptococcus neoformans*, a fungus with inherent resistance to CAS.[8]

Materials and methods

We evaluated the *in vitro* interaction between CAS and several structurally unrelated inhibitors of the calcineurin pathway (FK506, cyclosporin, FK520 and L685,818) and rapamycin, a potent immunosuppressive inhibitor of TOR kinase, against *Aspergillus* spp., for which CAS demonstrates a lack of complete growth inhibition *in vitro*. Both FK506 and rapamycin form complexes with the immunophilin protein FKBP12.[9] Ten clinical isolates of *Aspergillus fumigatus* and three isolates each of *Aspergillus flavus* and *Aspergillus terreus* from the Mycology Research Laboratory at The University of Texas M.D. Anderson Cancer Center were used. CAS powder (Merck & Co., Rathway, NJ, USA) was dissolved in sterile water at 10 mg/mL. Stock concentrations of FK520 (Merck & Co.; 12 μg/mL in DMSO), L685,818 (Merck & Co.; 16 μg/mL in DMSO), cyclosporin (Sigma Chemicals, St Louis, MO, USA; 5 μg/mL in DMSO), FK506 (Fujisawa Healthcare, Inc., Deerfield, IL, USA;

*Corresponding author. Tel: +1-713-792-6237; Fax: +1-713-745-6839; E-mail: dkontoyi@mdanderson.org

5 mg/mL in DMSO) and rapamycin (Sigma Chemicals; 2 μg/mL in DMSO) were prepared and stored at –70°C until use. Disc diffusion testing was carried out on YAG plates (0.5% yeast extract, 1% dextrose, 1.5% agar) by plating a standardized suspension (1×10^7 conidia/mL) of *Aspergillus* isolates. After plates were allowed to dry, three sterile 1/4 inch paper discs (Schleicher and Schuell, Keene, NH, USA) were placed on the agar surface and inoculated (10 μL) with 1 μg of CAS, 1 μg of one of the calcineurin inhibitors (FK506, cyclosporin, FK520 and L685,818), rapamycin or the combination of 1 μg of CAS with 1 μg of one of the aforementioned inhibitors placed on the same disc. Because of the lack of significant activity of the combination of L685,818 with CAS when 1 μg of L685,818 was put on the disc, 2 μg of L685,818 was used either alone or in combination with 1 μg of CAS. Plates were then incubated for 48 h at 37°C. The radius of the zone of growth inhibition was measured using a micrometer at 24 and 48 h at 37°C. Measurements were compared by Kruskal–Wallis One Way Analysis of Variance on Ranks. Differences between CAS and CAS/inhibitor combinations were further analysed *post-hoc* by Tukey's test for multiple comparisons. DMSO (1 μg of 100% solution) served as the vehicle control. The experiments were repeated three times.

Results

A positive interaction between CAS in combination with any of the calcineurin inhibitors or rapamycin was observed in all *A. fumigatus* isolates tested (Figure 1). The *A. fumigatus* isolates tested showed strain-dependent differences; however, consistent enhancement of the activity of CAS followed by exposure to the calcineurin inhibitors or rapamycin was seen in all isolates. There was a time-dependent effect of the combination as the observed attenuation of growth was best appreciated after 48 h of incubation (Table 1). The combination of CAS with the non-immunosuppressive analogue L685,818 had the least antifungal activity. Growth attenuation was barely perceptible when 1 μg of L685,818 and 1 μg of CAS were put on the disc (Table 1). All calcineurin inhibitors demonstrated little or no intrinsic antifungal activity when used alone (Figure 1). The combinations resulted in attenuation of growth but did not produce a clear zone of inhibition. The combination of CAS with either FK506 or rapamycin exerted the most significant effect (Table 1). The combination of CAS with L685,818 chequerboard method did not result in a fungicidal effect at several concentrations of CAS or L685,818 used (data not shown). The combination of CAS with the calcineurin inhibitors or rapamycin resulted in a similar attenuation of growth in all isolates of *A. terreus* and *A. flavus* tested, especially after 48 h of growth (Table 2).

Finally, the inhibition of growth seen between the calcineurin inhibitors and CAS was not specific to echinocandins, since attenuation of growth was also observed with the combination of calcineurin inhibitors (FK506 and L685,818) with other antifungals (1 μg of itraconazole/disc) or cellular growth inhibitors (1 μg of cyclohexamide/disc, 10 μL of 3% peroxide disc) (Figure 2).

Discussion

Our findings suggest that a functional link might exist between the calcineurin pathway and the tolerance to antifungals in *Aspergillus* spp. This interaction does not appear to be *Aspergillus* species specific. Recent evidence in *Candida albicans*[10] suggests that the calcineurin and TOR pathways play a conserved role in the regulation of the stress response of fungi against a variety of cellular growth inhibitors. Inhibition or down-regulation of the calcineurin or TOR pathways could decrease the threshold of deleterious effects of antifungals in moulds such as *Aspergillus* spp.

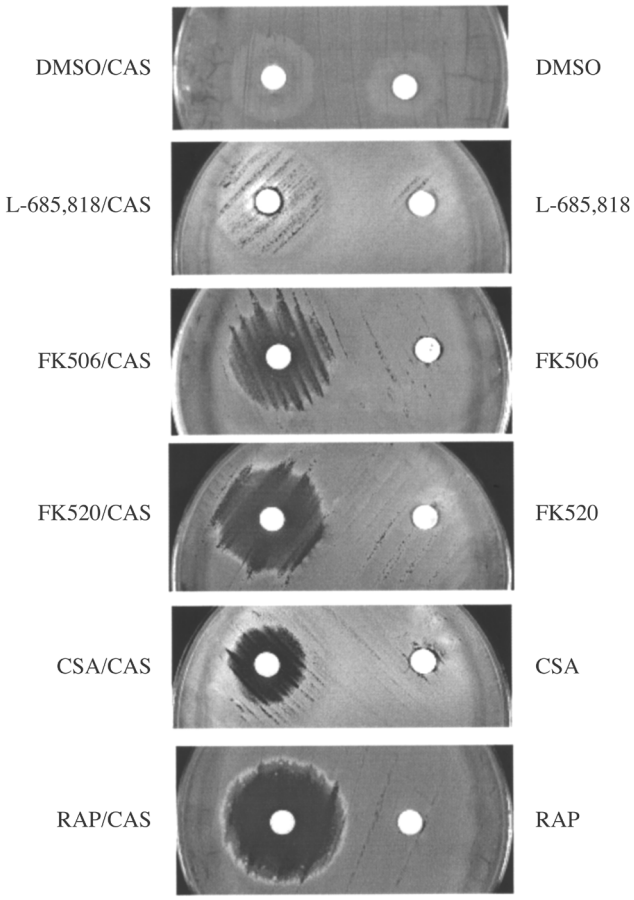

Figure 1. Combination of calcineurin inhibitors or rapamycin with CAS results in attenuation of growth in *A. fumigatus*. Disc diffusion assay in a representative isolate of *A. fumigatus* (isolate 10). The discs contained 1 μg of DMSO, caspofungin (CAS), FK520, cyclosporin (CSA), rapamycin (RAP), FK506 or 2 μg of L685,818 alone (right-hand side) or in combination with 1 μg of CAS (left-hand side). The radius of the zone of inhibition was measured after 48 h of growth at 37°C.

Table 1. Summary of the growth of inhibition responses of 10 clinical isolates of *A. fumigatus* by disc diffusion assay

Drug[a]	Zone of inhibition (mm)[c] 24 h	48 h
Caspofungin	5.44 (±0.51)	NGI
DMSO	NGI	NGI
L685,818	NGI	NGI
L685,818[b]	NGI	NGI
FK506	NGI	NGI
FK520	NGI	NGI
Cyclosporin	NGI	NGI
Rapamycin	11.9 (±1.17)*	NGI
DMSO/caspofungin	5.44 (±1.34)	0.11 (±0.36)
L685,818/caspofungin	5.44 (±0.19)	NGI
L685,818[b]/caspofungin	8.00 (±0.54)	7.95 (±0.82)**
FK506/caspofungin	6.61 (±0.19)	5.28 (±0.62)**
FK520/caspofungin	5.50 (±0.50)	4.73 (±0.72)
Cyclosporin/caspofungin	3.83 (±0.60)	2.33 (±0.46)
Rapamycin/caspofungin	9.00 (±1.59)	7.57 (±0.65)**

NGI, no growth inhibition (radius of zone of inhibition ≤1 mm).
[a]1 μg of each drug.
[b]2 μg of L685,818.
[c]Mean ± S.D., measurement of zone of inhibition was performed at two time points, 24 and 48 h of incubation at 37°C.
*$P < 0.05$ combination versus DMSO/CAS alone at 24 h.
**$P < 0.05$ combination versus DMSO/CAS alone at 48 h.

Alternatively, other indirect mechanisms might be operative. Hence, it is possible that calcineurin/TOR pathway inhibitors affect separate, unrelated metabolic processes in *Aspergillus* spp., and their combination with CAS-induced inhibition of cell wall integrity results in growth arrest. For example, cyclosporin and FK506 have been reported to be toxic against *A. fumigatus*[6] and rapamycin has been reported to be toxic against *C. albicans* and *C. neoformans*.[11]

Nevertheless, specific interactions are possible. Thus, an inhibition of the expression of the *A. fumigatus FKS1* homologue[4] could occur by the combination of CAS and the calcineurin or TOR inhibitors. Since pneumocandins can be naturally detected in *Aspergillus* spp. themselves,[1,2] it will be interesting to examine the specific role of the calcineurin pathway in the orchestrated regulation of cell wall sensing pathways and stress response.

Although the non-immunosuppressive compound L685,818 was the least potent in its interaction with CAS, itraconazole or other cellular inhibitors, the mechanisms and the clinical utility of the observed synergy between calcineurin inhibitors and glucan synthase inhibitors needs further investigation. Preliminary clinical experience suggests that the combination of the echinocandins with some calcineurin inhibitors (tacrolimus) is well tolerated. Finally, in view of the regulation of virulence of *C. neoformans* by the calcineurin pathway,[5] it will be of interest to examine the virulence of *A. fumigatus* when exposed to combinations of novel agents targeting this and other signalling pathways. Of note, components of the

Table 2. Summary of the growth of inhibition responses of three clinical isolates of *A. flavus* and *A. terreus* by disc diffusion assay

Drug[a]	Zone of inhibition (mm)[c] *A. flavus* (24 h)	*A. terreus* (24 h)	*A. flavus* (48 h)	*A. terreus* (48 h)
DMSO	NGI	NGI	NGI	NGI
L685,818[b]	1.00 (±1.73)	3.22 (±0.19)	NGI	NGI
FK506	12.9 (±6.20)	6.66 (±0.66)	12.6 (±0.84)	NGI
FK520	16.2 (±3.53)*	7.55 (±0.38)	14.0 (±3.40)*	NGI
Cyclosporin	8.11 (±2.36)	8.50 (±0.92)	2.66 (±1.80)	NGI
Rapamycin	16.8 (±4.37)	9.33 (±0.66)	NGI	NGI
DMSO/caspofungin	8.11 (±2.83)	6.33 (±1.55)	1.78 (±2.52)	0.44 (±0.77)
L685,818[b]/caspofungin	7.22 (±2.41)	10.7 (±0.10)	6.00 (±0.58)	5.67 (±0.8)**
FK506/caspofungin	15.0 (±3.17)*	10.8 (±1.35)	13.4 (±1.07)*	6.50 (±0.76)**
FK520/caspofungin	16.6 (±3.28)*	10.9 (±0.70)	14.0 (±3.40)*	6.78 (±0.70)**
Cyclosporin/caspofungin	11.3 (±0.25)*	11.3 (±0.67)	4.22 (±1.68)	1.50 (±0.86)
Rapamycin/caspofungin	11.0 (±0.88)*	10.5 (±0.35)	8.44 (±0.70)	4.88 (±1.20)**

NGI, no growth inhibition (radius of zone of inhibition ≤1 mm).
[a]1 μg of each drug.
[b]2 μg of L685,818.
[c]Mean ± S.D., measurement of zone of inhibition was performed at two time points, 24 and 48 h of incubation at 37°C.
*$P < 0.05$ combination versus DMSO/CAS alone at 24 h.
**$P < 0.05$ combination versus DMSO/CAS alone at 48 h.

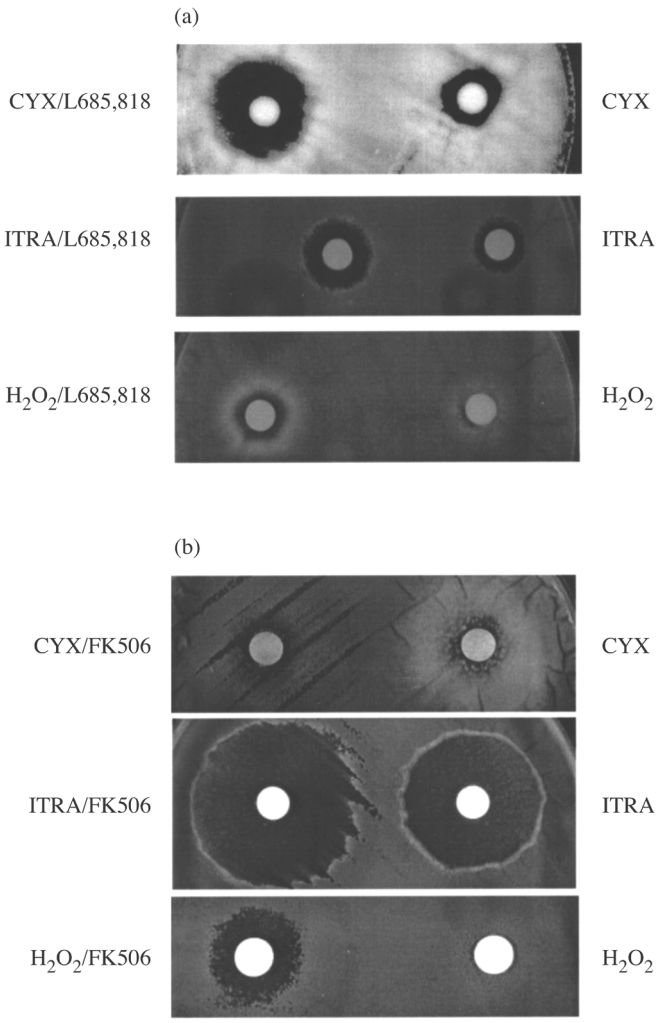

Figure 2. Combination of calcineurin inhibitors and other cellular inhibitors results in attenuation of growth in *A. fumigatus*. (a) Disc diffusion assay in *A. fumigatus* (isolate 9). The discs contained 1 μg of itraconazole (ITRA), 1 μg of cyclohexamide (CYX) and 10 μg of 3% hydrogen peroxide (H_2O_2) alone (right-hand side) or in combination with 2 μg of L685,818 (left-hand side). The radius of the zone of inhibition was measured after 48 h of growth at 37°C. (b) Disc diffusion assay in *A. fumigatus* (isolate 9). The discs contained 1 μg of itraconazole (ITRA), 1 μg of cyclohexamide (CYX) and 10 μg of 3% hydrogen peroxide (H_2O_2) alone (right-hand side) or in combination with 1 μg of FK506 (left-hand side). The radius of the zone of inhibition was measured after 48 h of growth at 37°C.

calcineurin pathway have been reported to regulate hyphal growth in *Aspergillus nidulans*.[12]

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References

1. Georgopapadakou, N. H. (2001). Update on antifungals targeted to the cell wall: focus on beta-1,3-glucan synthase inhibitors. *Expert Opinion on Investigational Drugs* **10**, 269–80.

2. Kurtz, M. B. & Douglas, C. M. (1997). Lipopeptide inhibitors of fungal glucan synthase. *Journal of Medical and Veterinary Mycology* **35**, 79–86.

3. Bowman, J. C., Hicks, P. S., Kurtz, M. B., Rosen, H., Schmatz, D. M., Liberator, P. A. *et al.* (2002). The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus in vitro*. *Antimicrobial Agents and Chemotherapy* **46**, 3001–12.

4. Beauvais, A. & Latge, J. P. (2001). Membrane and cell wall targets in *Aspergillus fumigatus*. *Drug Resistance Update* **4**, 38–49.

5. Hemenway, C. S. & Heitman, J. (1999). Calcineurin. Structure, function, and inhibition. *Cell Biochemistry and Biophysics* **30**, 115–51.

6. Hatanaka, H., Kino, T., Miyata, S., Inamura, N., Kuroda, A., Goto, T. *et al.* (1988). FR-900520 and FR-900523, novel immunosuppressants isolated from a *Streptomyces*. II. Fermentation, isolation and physico-chemical and biological characteristics. *Journal of Antibiotics* **41**, 1592–601.

7. Odom, A., Del Poeta, M., Perfect, J. & Heitman, J. (1997). The immunosuppressant FK506 and its nonimmunosuppressive analog L-685,818 are toxic to *Cryptococcus neoformans* by inhibition of a common target protein. *Antimicrobial Agents and Chemotherapy* **41**, 156–61.

8. Del Poeta, M., Cruz, M. C., Cardenas, M. E., Perfect, J. R. & Heitman, J. (2000). Synergistic antifungal activities of bafilomycin A, fluconazole, and the pneumocandin MK-0991/caspofungin acetate (L-743,873) with calcineurin inhibitors FK506 and L-685,818 against *Cryptococcus neoformans*. *Antimicrobial Agents and Chemotherapy* **44**, 739–46.

9. Xu, X., Su, B., Barndt, R. J., Chen, H., Xin, H., Yan, G. *et al.* (2002). FKBP12 is the only FK506 binding protein mediating T-cell inhibition by the immunosuppressant FK5061. *Transplantation* **73**, 1835–8.

10. Cruz, M. C., Goldstein, A. L., Blankenship, J., Del Poeta, M., Davis, D., Cardenas, M. E. *et al.* (2002). Calcineurin is essential for survival during membrane stress in *Candida albicans*. *EMBO Journal* **21**, 1–14.

11. Cruz, M. C., Goldstein, A. L., Blankenship, J., Del Poeta, M., Perfect, J. R., McCusker, J. H. *et al.* (2001). Rapamycin and less immunosuppressive analogs are toxic to *Candida albicans* and *Cryptococcus neoformans* via FKB12-dependent inhibition of TOR. *Antimicrobial Agents and Chemotherapy* **45**, 3162–70.

12. Lu, K. P., Rasmussen, C. D., May, G. S. & Means, A. R. (1992). Cooperative regulation of cell proliferation by calcium and calmodulin in *Aspergillus nidulans*. *Molecular Endocrinology* **6**, 365–74.