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Calcineurin Inhibitor Agents Interact Synergistically with Antifungal Agents In Vitro against *Cryptococcus neoformans* Isolates: Correlation with Outcome in Solid Organ Transplant Recipients with Cryptococcosis▽

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Synergistic interactions were observed between CIs and antifungal agents against 53 (90%) of 59 *Cryptococcus neoformans* isolates from solid organ transplant recipients with cryptococcosis and may account for better outcomes in patients with cryptococcosis receiving these immunosuppressive agents.

Cryptococcosis occurs in 0.3 to 5% and an average of 3% of solid organ transplant (SOT) recipients (12, 13, 18, 23). Dissemination beyond the pulmonary focus and central nervous system (CNS) involvement have been documented in 52 to 61% of these patients (12, 13, 18, 23). Mortality rates in transplant recipients with cryptococcosis typically range from 15 to 20% and approach 40% in those with CNS infection (1, 12, 13, 18, 23).

Calcineurin inhibitor (CI)-based regimens are the mainstay of modern antirejection therapy in SOT recipients. Furthermore, CIs have been independently associated with improved outcomes in SOT recipients with cryptococcosis (19, 21). This beneficial effect of CI agents is in part considered to be attributable to their in vitro antifungal activity against *Cryptococcus neoformans* (8, 11, 17). The calcineurin pathway plays a vital role in cellular morphogenesis and virulence in *C. neoformans* (7). Inhibitors of this signaling pathway such as tacrolimus (FK506) and cyclosporine (CsA) target not only the mammalian but also the fungal homologs of calcineurin (11). *C. neoformans* also possesses TOR kinases, and their inhibitors, such as rapamycin, impair cell proliferation via the nutrient-sensing pathway of this yeast (5, 6). Additionally, in vitro data obtained with laboratory strains have shown synergistic interactions between the immunosuppressive and antifungal agents against pathogenic fungi, including *C. neoformans* (10, 14, 15). There are no clinical studies, however, that have determined the relevance of these in vitro findings. To this end, we examined the magnitude of in vitro interactions between CI agents or rapamycin and antifungals against *C. neoformans* clinical isolates and their correlation with the treatment outcomes of a recent cohort of SOT recipients with cryptococcosis.

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The study population was derived from a large cohort of SOT recipients with cryptococcosis who were studied prospectively between 1999 and 2006 (21). A total of 74 patients from whom *C. neoformans* isolates were available made up the patient population in the present study. *C. neoformans* infection

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TABLE 1. Interactions of AmB or fluconazole with immunosuppressive agents versus *C. neoformans* isolates

Drug combination	Median FIC index (range)	Synergistic interaction[a]	Additive interaction[a]
AmB + tacrolimus	0.25 (0.030–2)	67/74 (90)	7/74 (10)
AmB + CsA	0.12 (0.030–1)	65/73[b] (89)	9/73 (11)
AmB + rapamycin	0.12 (0.30–1)	67/73[b] (92)	7/73 (8)
Fluconazole + tacrolimus	0.25 (0.03–1)	61/74 (82)	13/74 (17)
Fluconazole + CsA	0.125 (0.031–1)	69/74 (93)	5/74 (7)
Fluconazole + rapamycin	0.125 (0.007–1)	71/74 (96)	3/74 (4)

[a] Data represent the number of patients whose isolates yielded the stated interaction/total number (percentage) of patients whose isolates were tested. None of the drugs demonstrated antagonism with any of the isolates.
[b] The isolate from one patient could not be tested.

TABLE 2. Outcomes (90-day survival) of patients stratified by the immunosuppressive and antifungal agents received

Drug combination (*n*)[a]	Survival[a] in CI recipients when drugs were:		Survival[a] of non-CI recipients
	Synergistic	Additive	
Tacrolimus + AmB (31)	25/30 (83)	1/1 (100)	
CsA + AmB (8)	7/7 (100)	1/1 (100)	
Non-CI + AmB (9)			5/9 (56)
Tacrolimus + fluconazole (14)	10/10 (100)	4/4 (100)	
CsA + fluconazole (3)	3/3 (100)		
Non-CI + fluconazole (4)			3/4 (75)
Any CI (56)	45/50 (90)	6/6 (100)	
Any non-CI (13)			8/13 (62)
Total (69)	45/50 (90)	6/6 (100)	8/13 (62)

[a] Each value in parentheses is the number of patients who received the drug combination.
[b] Data are presented for 69 of the 74 study patients; 2 patients who received a CI were not treated (diagnosed at autopsy), and 3 received an antifungal agent other than AmB or fluconazole as therapy for cryptococcosis.

was defined in accordance with the criteria set forth by the European Organization for Research and Treatment in Cancer and the Mycoses Study Group (3). Organ sites involved were classified as CNS, pulmonary, skin, soft tissue, osteoarticular, or other (12, 19). Disseminated infection was defined as CNS disease, fungemia, or involvement of two or more noncontiguous organs (12, 19).

In vitro drug interactions between CIs (tacrolimus or FK506 and CsA), rapamycin, and antifungal agents (amphotericin B [AmB] and fluconazole) against clinical isolates of *C. neoformans* were assessed (blinded to clinical data) by using the CLSI (formerly NCCLS) M38-A method modified for broth microdilution checkerboard testing as previously reported (19, 20, 22). In vitro MICs of the antifungal agents (AmB and fluconazole) and immunosuppressive agents (tacrolimus, CsA, and sirolimus) alone or in combination were determined by using concentrations of 0.125 to 64 μg/ml for fluconazole, 0.03 to 16 μg/ml for AmB, and 0.04 to 25 μg/ml for the three immunosuppressive agents (22). MICs were determined at 37°C by using RPMI 1640 medium and 2% glucose buffered to pH 7. Sterile normal saline was used as the solvent for the antifungal agents, and dimethyl sulfoxide (DMSO) was used as the solvent for the immunosuppressant drugs; the stock solution was prepared at 100 times the highest concentration tested and stored at −20°C. The final concentration was prepared from the antifungals and the immunosuppressant stock solution in RPMI plus 2% glucose. The final concentration of DMSO in each well was 0.1% or less. Before testing, all isolates were subcultured in liquid YPD medium to ensure optimal growth characteristics. Stock suspensions were prepared in sterile normal saline and adjusted to yield a final inoculum concentration of 1×10^6 to 5×10^6 cells/ml of stock solution. The stock solution was then diluted 1:50 in RPMI culture medium to obtain the final test inoculum (dilution of 1×10^4 to 5×10^4 cells/ml). Two well-characterized *Candida* isolates (*Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019) were tested in parallel with each checkerboard plate as quality control isolates (4).

Drug interactions were defined as synergistic, additive, or antagonistic on the basis of the fractional inhibitory concentration (FIC) index. The FIC index was considered to be the sum of the FICs of each of the drugs and defined as the MIC of the drug used in the combination divided by the MIC of the drug when used alone. Drug interactions were considered as synergistic if the lowest FIC index was ≤0.5, additive (i.e., no interaction) if the lowest FIC index was >0.5 and ≤4, and antagonistic if the highest FIC index was >4 (16).

Intercooled Stata 9.2 (StataCorp LP, College Station, TX) was used for statistical analysis. The Fisher exact test was used to compare categorical data. The end point was survival at 90 days. A logistic model was used to examine the effects of an immunosuppressant regimen on survival at 90 days. Factors added to the model were those known to contribute to a poor prognosis in this cohort of patients (renal failure and disseminated infection) as previously reported (19).

The MICs of AmB for the cryptococcal isolates ranged from 0.25 to 4 μg/ml (mean, 1 μg/ml), and those of fluconazole ranged from 4 to 64 μg/ml (mean, 16 μg/ml). The mean MICs of fluconazole were 17 μg/ml for isolates from the recipients of tacrolimus, 8 μg/ml for the recipients of CsA, and 15.3 μg/ml for isolates from non-CI recipients ($P = 0.96$). A synergistic interaction of AmB with tacrolimus was found for 67 (90%) of the 74 isolates, with CsA for 65/73 (89%), and with rapamycin for 67/73 (92%); one isolate was unavailable for CsA and rapamycin testing (Table 1). AmB interactions with these immunosuppressants were additive for the rest of the isolates and antagonistic for none (Table 1). The MICs for quality control strains were in agreement with the published ranges for each drug; i.e., those of AmB were 0.25 to 2 μg/ml, and those of fluconazole were 0.12 to 1 μg/ml (4).

Of 31 patients who received tacrolimus and AmB, 30 (97%) yielded *C. neoformans* isolates against which these two drugs showed synergy; 25 (83%) of these 30 patients survived (Table 2). CsA and AmB had synergistic interactions against the isolates from seven (87%) of the patients who received those drugs; the survival of these patients is shown in Table 2. The interaction of immunosuppressive agents and fluconazole against *C. neoformans* isolates is depicted in Table 1. Of 14

patients who received tacrolimus and fluconazole, 10 (71%) had isolates against which the drugs demonstrated synergy and 4 (29%) had isolates against which this drug combination showed additive interactions. The outcomes of these patients, as well as those of patients who received CsA and fluconazole, are shown in Table 2.

The survival at 90 days of the recipients of CIs treated with AmB or fluconazole was 91% (51/56), compared to 61.5% (8/13) for the recipients of non-CI agents who also received the same antifungal agents ($P = 0.020$). Of 10 patients who died, 9 received an AmB-based antifungal regimen as induction therapy for cryptococcosis for a median of 17 days (range, 5 to 34 days) and 1 received fluconazole. When adjusted for factors portending a poor outcome (renal failure and disseminated infection), the odds ratio for the survival of patients receiving a CI compared to those who received non-CIs was 6.19 (95% confidence interval, 1.3 to 34.9; $P = 0.018$). In this model, the odds ratio for the survival of patients with renal failure was 0.24 (95% confidence interval, 0.04 to 1.24; $P = 0.09$) and that for those with disseminated infection was 0.15 (95% confidence interval, 0.01 to 1.63; $P = 0.12$). Mortality in the recipients of CIs was too low to compare the outcomes of patients whose isolates yielded synergistic versus nonsynergistic interaction; 10% (5/50) of the patients with isolates against which the drugs demonstrated synergy and 0% (0/6) of those with isolates against which they did not show synergy died ($P = 0.99$).

Transplant recipients are unique hosts in that the immunosuppressants used in these patients have shown synergism with antifungal agents against several pathogenic yeasts and molds. For example, although fluconazole exhibits largely fungistatic activity against *Candida*, its combination with CIs is synergistic and potently fungicidal against *C. albicans*, including strains that are azole resistant (7, 15). The combination of tacrolimus and fluconazole is also synergistic in vitro against *C. neoformans* and resulted in an ~30-fold decrease in the MIC of tacrolimus and a 4-fold decrease in that of fluconazole for this yeast (10). The synergistic activity of tacrolimus with fluconazole is mediated via inhibition of multidrug-resistant pumps that export azoles from fungal cells and is independent of calcineurin inhibition (10). Despite the fact that the *FKS1* gene, which encodes β-1,3-glucan synthase, is essential in *C. neoformans*, the echinocandins have limited activity against this yeast. However, the combination of caspofungin with tacrolimus is synergistic against *C. neoformans* (10). Synergism has also been shown for CsA, tacrolimus, and rapamycin with AmB or an echinocandin against *Aspergillus fumigatus* (14).

To our knowledge, our study is the first attempt to determine in vitro drug interactions of immunosuppressants with antifungal agents in clinical isolates of *C. neoformans* and to correlate these with outcome in SOT patients with cryptococcosis. Synergy was observed between CIs and antifungal agents against 90% of the *C. neoformans* isolates, and patients receiving CIs had significantly higher survival than non-CI recipients, even when adjusted for renal failure and disseminated infection.

In the absence of prior exposure to the azoles, the MICs of fluconazole against cryptococcal isolates typically range from 1 to 4 μg/ml (2, 9). The median MIC of fluconazole against the isolates in our study was 16 μg/ml. Nevertheless, these strains demonstrated synergy with the CI agents. The relatively high MICs of fluconazole against the cryptococcal isolates, despite the fact that only 1 of the 74 patients in our study had previously received fluconazole, raises the possibility that immunosuppressive drugs could potentially influence susceptibility to the azole antifungal agents or the existence of yeast strains with altered susceptibility due to as-yet-unknown factors.

Our study has limitations that deserve to be acknowledged. Given a small number of patients receiving a non-CI agent-based regimen, comparison of these patients with the recipients of CIs must be done with caution. We note that a non-CI-based regimen was used as the standard of care and was not due to noncompliance with the CIs. Second, since none of the isolates showed antagonism, a meaningful comparison could not be conducted between patients with isolates against which the drugs showed synergistic versus antagonistic interactions. Finally, we were unable to correlate synergy with time to culture negativity, decline in antigen titers, or fungus-attributable mortality.

Improved outcomes in transplant recipients in the modern immunosuppressive era are due largely to a lower overall risk of rejection and greater graft survival. It is, however, plausible that in concert with conventional antifungal agents, the unique antifungal attributes of immunosuppressive agents potentially contribute to better outcomes in transplant recipients with opportunistic mycoses. Our findings raise the possibility that outcomes in transplant recipients with invasive mycoses may be further optimized by developing more potent inhibitors of fungal signaling pathways.

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