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


M. D. Anderson Cancer Center, University of Texas, Houston, Texas; Duke University Medical Center, Durham, North Carolina; Institut Pasteur and Faculté de Médecine Paris Descartes, Hôpital Necker-Enfants malades, Paris, France; Institut Pasteur, Paris, France; Postgraduate Institute of Medical Education and Research, Chandigarh, India; Christian Medical College Hospital, Vellore, India; Henry Ford Hospital, Detroit, Michigan; Baylor University Medical Center, Dallas, Texas; Emory University, Atlanta, Georgia; University of Chicago, Chicago, Illinois; Northwestern University, Chicago, Illinois; Hospital General Universitario Gregorio Marañón, Madrid, Spain; University of Washington, Seattle, Washington; University of Nebraska, Omaha, Nebraska; University of Virginia, Charlottesville, Virginia; Ochsner Clinic, New Orleans, Louisiana; University Health Network, Toronto General Hospital, Toronto, Ontario, Canada; University of South Florida, Tampa, Florida; University of Western Ontario, London, Ontario, Canada; Medical University of South Carolina, Charleston, South Carolina; Oregon Health Sciences University, Portland, Oregon; University of Miami, Miami, Florida; Virginia Commonwealth University, Richmond, Virginia; and VA Medical Center and University of Pittsburgh, Pittsburgh, Pennsylvania.

Received 30 July 2007/Returned for modification 23 September 2007/Accepted 27 October 2007

Synergistic interactions were observed among CI agents 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 has 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.

(The data presented here were previously presented in part at the 47th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, 17 to 20 September 2007.)

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...
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 \( \mu \)g/ml for fluconazole, 0.03 to 16 \( \mu \)g/ml for AmB, and 0.04 to 25 \( \mu \)g/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 \(-20^\circ\)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 the antifungals and the immunosuppressant stock solution in RPMI plus 2% glucose. The final concentration of DMSO in the antifungals and the immunosuppressant stock solution in RPMI plus 2% glucose.

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.

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

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

* 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. 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**

<table>
<thead>
<tr>
<th>Drug combination (in)</th>
<th>Survival* in CI recipients when drugs were:</th>
<th>Survival* of non-CI recipients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Synergistic</td>
<td>Additive</td>
</tr>
<tr>
<td>Tacrolimus + AmB (31)</td>
<td>25/30 (83)</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>CsA + AmB (8)</td>
<td>7/7 (100)</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>Non-CI + AmB (9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tacrolimus + fluconazole (14)</td>
<td>10/10 (100)</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>CsA + fluconazole (3)</td>
<td>3/3 (100)</td>
<td></td>
</tr>
<tr>
<td>Non-CI + fluconazole (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any CI (56)</td>
<td>45/50 (90)</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td>Any non-CI (13)</td>
<td>8/13 (62)</td>
<td></td>
</tr>
<tr>
<td>Total (69)</td>
<td>45/50 (90)</td>
<td>6/6 (100)</td>
</tr>
</tbody>
</table>

* Each value in parentheses is the number of patients who received the drug combination.

**Notes:** Antimicrob. Agents Chemother.
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 CsA 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.

This study was supported by research grants from NIH/NIAID (R01 AI 054719-01) and Astellas to N.S. and by The University of Texas M. D. Anderson Faculty E. N. Cobb Scholar Award Research Endowment to D.P.K.

Astellas played no role in study design, data collection, laboratory assays, interpretation of results, or manuscript preparation. Dimitrios Kontoyiannis and Russell Lewis have received research support from Merck, Astellas, and Enzon. Lorraine A. Dowdy has received research support from Enzon and Astellas. K. J. Pursell serves on the speaker’s bureau for Merck. N. Singh has received research support from Scheering and Enzon. There are no conflict-of-interest disclosures for the other authors.

The following members of the French Cryptococcosis Study Group contributed data and isolates for this study: Corinne Antoine (Saint-Louis Hospital, Paris, France), Benoit Barrou (Pitié-Salpêtrière Hospital, Paris, France), Anne-Elisabeth Heng (Gabriel Montpied Hospital, Clermont-Ferrand, France), Christophe Legendre (Necker-Enfants malades Hospital, Paris, France), Christian Michelet (Pontchaillou Hospital, Rennes, France), Bénédicte Ponceau (Croix-Rousse Hospital, Lyon, France), Nacéra Ouali (Tenon Hospital, Paris, France), and Marc Stern (Foch Hospital, Suresnes, France).

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