Correlation between Antifungal Susceptibilities of *Coccidioides immitis* In Vitro and Antifungal Treatment with Caspofungin in a Mouse Model

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Caspofungin (Merck Pharmaceuticals) was tested in vitro against 25 clinical isolates of *Coccidioides immitis*. In vitro susceptibility testing was performed in accordance with the National Committee for Clinical Laboratory Standards document M38-P guidelines. Two *C. immitis* isolates for which the caspofungin MICs were different were selected for determination of the minimum effective concentration (MEC), and these same strains were used for animal studies. Survival and tissue burdens of the spleens, livers, and lungs were used as antifungal response markers. Mice infected with strain 98-449 (48-h MIC, 8 μg/ml; 48-h MEC, 0.125 μg/ml) showed 100% survival to day 50 when treated with caspofungin at ≥1 mg/kg. Mice infected with strain 98-571 (48-h MIC, 64 μg/ml; 48-h MEC, 0.125 μg/ml) displayed ≥80% survival when the treatment was caspofungin at ≥5 mg/kg. Treatment with caspofungin at 0.5, 1, 5, or 10 mg/kg was effective in reducing the tissue fungal burdens of mice infected with either isolate. When tissue fungal burden study results were compared between strains, caspofungin showed no statistically significant difference in efficacy in the organs of the mice treated with both strains. A better in vitro-in vivo correlation was noted when we used the MEC instead of the MIC as the endpoint for antifungal susceptibility testing. Caspofungin may have a role in the treatment of coccidioidomycosis.

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* Coccidioides immitis is the etiologic agent of coccidioidomycosis. The fungus lives in the soil of arid regions of the United States, Mexico, and Central and South America. There are an estimated 100,000 infections annually in the United States (17). Coccidioidomycosis is a systemic fungal infection and is frequently refractory to treatment. Unfortunately, conventional antifungal therapy is associated with therapeutic failures, relapses, and toxicity (5, 7, 8, 18). However, there is some hope that major improvements in therapy for coccidioidomycosis will come from drugs with novel mechanisms of action.

The fungal cell wall is a vital and complex structure containing mannoproteins, chitin, and glucans. Any disruption in its integrity should affect growth. The cell wall provides a unique therapeutic opportunity for antifungal agents by targeting a structure not found in mammalian cells.

In a prior study, we found that the organism concentration at 95% of the isolates were vortexed and adjusted to the proposed standard slants prepared in-house (16). Isolates were evaluated by using National Committee for Clinical Laboratory Standards broth macrodilution proposed standard reference method M38-P for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi (15). Briefly, the mycelium was overlaid with sterile distilled water and suspensions were made by gently scraping the colonies with wooden applicators. Heavy fragments were allowed to settle, and the upper, homogenous supernatant was transferred to sterile tubes. The conidial-small mycelial fragment suspensions were vortexed and adjusted to the 95% transmittance at 530 nm setting with a spectrophotometer (Spectronic 21; Milton Roy Company). In a prior study, we found that the organism concentration at 95% transmittance corresponds to 1 x 10^6 to 5 x 10^6 CFU/ml (G. González González, R. Tijerina, D. A. Sutton, and M. G. Rinaldi, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999; abstr. C-172, 1999). CAS and FLU were tested in RPMI 1640 medium with L-glutamine and morpholinepropanesulfonic acid (PAA; Lonza, Basel, Switzerland). AMB was reconstituted with sterile distilled water and injected intraperitoneally in a 0.2-ml volume. CAS was given at 0.01, 0.1, 0.5, 1.0, 5.0, and 10.0 mg/kg per day on days 2 through 22 postinfection. The control group received sterile distilled water intraperitoneally. Deaths were recorded through 50 days postinfection. Moribund mice were terminated, and their deaths were recorded as occurring on the next day. At the end of the study, survivors were sacrificed by inhalation of metofane, followed by cervical dislocation. Spleens and livers were removed aseptically. The organs were homogenized in 2 ml of sterile saline, and the entire organs were plated onto potato dextrose agar and incubated at 35°C for a week.

For tissue burden studies, the treatment was the same but mice were sacrificed on day 24. Livers, spleens, and lungs were removed, weighed, and homogenized in 2 ml of sterile saline, and serial 10-fold dilutions were plated onto potato dextrose agar and incubated at 35°C for a week to determine the number of viable CFU in each organ.

Statistics. For survival studies, the log rank and Wilcoxon tests were used. The P values for determining significance varied because of correction for multiple comparisons. For tissue burden studies, Dunnett’s one-tailed t test or the rank sum test (Wilcoxon scores) was used. A P value of ≤0.05 determined the significance of differences compared only with controls.

RESULTS

Antifungal susceptibility. The MIC and MLC ranges, geometric mean MICs and MLCs, and MICs and MLCs of CAS, FLU, and AMB necessary to inhibit and kill 50% and 90% of the C. immitis isolates tested are summarized in Table 1. MICs were reported at 48 h. The 48-h MIC ranges of CAS, FLU, and AMB for the P. variotii control strain were as follows: CAS, 0.125 to 0.5 μg/ml; FLU, 4 to 8 μg/ml; AMB, 0.5 to 1 μg/ml. The results for this organism were within the expected ranges.

The result of the microscopic measurement of the 48-h MEC was 0.125 μg/ml for the pair of strains. We observed inflated and divided hyphae in the tubes with different concentrations of CAS (Fig. 1) in both strains. The results obtained when we used the MEC as the endpoint for in vitro susceptibility testing were dramatically different from those obtained when we employed the MIC defined as ≥80% inhibition of growth.

Survival study with strain 98-449. The results of the survival study with strain 98-449 are displayed in Fig. 2. All control mice died between days 12 and 22. Every mouse treated with CAS at 1, 5, or 10 mg/kg survived to day 50. CAS doses of ≥0.5 mg/kg significantly prolonged survival (P ≤ 0.0085).

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**TABLE 1. In vitro activities of CAS, FLU, and AMB against 25 clinical strains of C. immitis**

<table>
<thead>
<tr>
<th>Compound</th>
<th>48-h MIC (μg/ml)</th>
<th>48-h MLC (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Range</td>
<td>GM 50% 90%</td>
</tr>
<tr>
<td>CAS</td>
<td>8–64</td>
<td>17.4 32</td>
</tr>
<tr>
<td>FLU</td>
<td>16–64</td>
<td>28.6 64</td>
</tr>
<tr>
<td>AMB</td>
<td>0.25–0.5</td>
<td>0.34 0.5</td>
</tr>
</tbody>
</table>

* GM, geometric mean.
+ MIC at which 50% of the isolates were inhibited.
§ MLC at which 50% of the isolates were killed.
© MLC at which 90% of the isolates were killed.
ND, not done.

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FIG. 1. Photomicrographs (original magnification, ×400) after 48 h of incubation showing morphology changes in C. immitis strain 98-571. (A) RPMI 1640 medium control tube; (B) 1 μg of CAS per ml.
Survival study with strain 98-571. Figure 3 shows that all of the control mice died between days 14 and 22. All mice treated with CAS at 10 mg/kg survived to day 50. Sixty and twenty percent of the mice treated with CAS at 1 and 5 mg/kg died between days 27 and 45 and days 35 and 45, respectively. CAS was slightly less effective in survival studies with strain 98-571 than with strain 98-449 at 1 mg/kg. However, significant prolongation of survival was noted at CAS doses of ≥0.5 mg/kg ($P \leq 0.0085$).

Tissue burden with strain 98-449. The tissue fungal burden of mice was determined when they died or on day 24 postchallenge for those who survived. Treatment of mice with CAS at
TABLE 2. Fungal burdens in spleens, livers, and lungs of mice infected with 200 arthroconidia of C. immitis strains 98-449 and 98-571

<table>
<thead>
<tr>
<th>Amt of CAS (mg/kg) administered</th>
<th>Mean log_{10} no. of CFU/organ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>None (control)</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>6.979</td>
</tr>
<tr>
<td>0.1</td>
<td>6.661</td>
</tr>
<tr>
<td>0.5</td>
<td>6.489</td>
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<tr>
<td>1</td>
<td>5.385a</td>
</tr>
<tr>
<td>5</td>
<td>4.570a</td>
</tr>
<tr>
<td>10</td>
<td>4.077a</td>
</tr>
<tr>
<td></td>
<td>2.450c</td>
</tr>
</tbody>
</table>

a MIC, 8 µg/ml.
b MIC, 64 µg/ml.
c P ≤ 0.05 in comparison with control group.

Tissue burden with strain 98-571. Treatment of mice with CAS at 0.01 mg/kg daily was ineffective in reducing the fungal burden and there was no significant difference from the control value (P ≤ 0.05) (Table 2). CAS at ≥0.5 mg/kg significantly reduced spleen, liver, and lung fungal counts in a clearly dose-dependent manner.

The results of this study demonstrated poor in vitro antifungal activity against C. immitis, with 48-h MICs ranging from 8 to 64 µg/ml. Nevertheless, CAS was highly efficacious in the treatment of experimental coccidioidomycosis, despite MICs that differed between the strains. MIC endpoints were determined according to the conventional criteria outlined in NCCLS document M38-P. The NCCLS has not evaluated the echinocandins. Therefore, use of the same method to determine an endpoint may not be valid. In order to develop a clinically useful antifungal susceptibility method, perhaps entirely new criteria should be established for this class of compounds.

Studies of Aspergillus spp. have shown that endpoint readings with pneumocandins have been modified to obtain correlation with other parameters of antifungal activity. Kurtz et al. (13) used an endpoint MIC definition of complete absence of growth in ordinary broth microdilution to assess in vitro antifungal activities of pneumocandins A₀ and B₀. They found those compounds to be active in vitro against Candida spp. and inactivative against Aspergillus spp. However, when they determined the MIC by microscopic examination of microdilution wells of Aspergillus spp., their observations showed that in the presence of drugs, the hyphae were extremely divided tips with inflated germ tubes. Macroscopically, these changes corresponded to production of very condensed clumps in dilution wells. They proposed the use of the MEC as the new endpoint for determination of susceptibility to lipopeptides.

For the two isolates of C. immitis we used in animal studies, there were discrepancies between the MIC and MEC evaluations. For these isolates, we found very different MICs but the same MEC of 0.125 µg/ml, which we considered an indication of sensitivity. The MEC results established that CAS is active in vitro against both isolates of C. immitis. This MEC could explain the similar results of survival and tissue burden studies done with both strains.

Visualization of the MEC is slightly more onerous because it demands microscopic inspection, but this inspection could be easily eliminated because the microscopic changes observed in the hyphae are analogous to the alterations seen on macroscopic assessment of the dilution tubes.

CAS is an antifungal compound that challenges the present methodology used for in vitro testing. Endpoint criteria for antifungal susceptibility testing with lipopeptide agents seem to occupy an important position among the numerous variables that may influence the results of in vitro tests. Reading of the MEC seems to hold great promise for the determination of in vitro susceptibility to this kind of compound. However, more studies are necessary to generalize this determination as a definitive endpoint.

One of the goals of this study was to permit comparison of the MICs of CAS for C. immitis in vitro with different doses of CAS against experimental systemic coccidioidomycosis. For this, we used six doses of CAS. CAS-treated mice survived longer than controls when subjected to the 1-, 5-, and 10-mg/kg treatment regimens. CAS was slightly less effective in survival than controls when subjected to the 1-, 5-, and 10-mg/kg treatment regimens. When tissue burden results were compared between strains, CAS showed no statistically significant difference in efficacy in the organs of mice infected with strains 98-449 and 98-571.

In this study, different in vitro CAS activities were detected, depending on the endpoints applied. A limited association was displayed between in vitro testing with the MIC as the endpoint and antifungal treatment in this animal model. A better
in vitro-in vivo correlation was noted when we used the MEC as the endpoint in antifungal susceptibility testing.

CAS may have a role in the treatment of coccidioidomycosis and should be evaluated in more seriously ill patients.

REFERENCES