Caspofungin in Combination with Amphotericin B against *Candida parapsilosis*

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*Candida parapsilosis* has emerged as an important nosocomial pathogen. In the present study, a checkerboard broth microdilution method was performed to investigate the in vitro activities of caspofungin (CAS) in combination with amphotericin B (AMB) against three clinical isolates of *C. parapsilosis*. Although there was a significant reduction of the MIC of one or both drugs used in combination, an indifferent interaction (fractional inhibitory concentration index greater than 0.50 and less than or equal to 4.0) was observed in 100% of cases. This finding was confirmed by killing curve studies. By a disk diffusion assay, the halo diameters produced by antifungal agents in combination were often significantly greater than those produced by each drug alone. Antagonism was never observed. In a murine model of systemic candidiasis, CAS at either 0.25 or 1 mg/kg/day combined with AMB at 1 mg/kg/day was significantly more effective than each single drug at reducing the colony counts in kidneys. Higher doses of the echinocandin (i.e., 5 and 10 mg/kg/day) combined with the polyeone did not show any advantage over CAS alone. Overall, our study showed a positive interaction of CAS and AMB against *C. parapsilosis*.

The frequency of invasive mycoses due to opportunistic fungal pathogens has increased dramatically over the past two decades, and now *Candida* spp. ranks as the fourth most common cause of nosocomial bloodstream infections (20). Although *Candida albicans* is the organism most often associated with serious fungal infections, other *Candida* spp. have emerged as clinically important pathogens associated with opportunistic infections (17, 20).

*Candida parapsilosis* is the second most frequent yeast species isolated from normally sterile body sites in North America, Europe, and Latin America (12, 20). Moreover, studies conducted in the United States showed that *C. parapsilosis* is the most common non-*C. albicans Candida* spp. in pediatric patients (17, 18, 23).

Caspofungin (CAS) is an echinocandin antifungal agent that has potent activity against many fungal species, including *Candida* spp. (13, 20, 22). Clinical studies have shown that CAS is at least as active as amphotericin B and fluconazole in the treatment of invasive candidiasis (13, 20).

Amphotericin B (AMB) targets fungal ergosterol, the main component of the fungal cell membrane, while CAS inhibits the synthesis of the fungal cell wall by blocking β-1,3-β-glucan (6, 7). Its innovative mechanism of action makes this drug a suitable candidate for antifungal combination therapy.

Although CAS MICs for *C. parapsilosis* can be higher than those seen for *C. albicans*, the echinocandin is generally effective in infections caused by this yeast species (1, 4, 6–8, 13, 20, 22). Similarly, amphotericin B is active in vitro and in vivo against *C. parapsilosis* (20).

In this study, we hypothesized that the combination of CAS and AMB could be advantageous over each monotherapy against *C. parapsilosis*. To investigate this interaction, we applied in vitro methods and an experimental mouse model of systemic infection.

**MATERIALS AND METHODS**

**Isolates.** Two clinical isolates of *C. parapsilosis* (no. 2 and no. 3) and *C. parapsilosis* ATCC 22019 were used in this study. Both clinical isolates were recovered from blood. Yeast isolates were identified at the species level by conventional morphological and biochemical methods and stored at −70°C in 10% glycerol. Before the initiation of the study, yeast isolates were subcultured on antifungal agent-free medium to ensure viability and purity.

**Drugs.** For both in vitro and in vivo studies, stock solutions of CAS (Merck Sharp & Dohme Ltd., Hoddesdon, United Kingdom) were prepared in distilled water. For in vitro studies, stock solutions of AMB (Sigma Chemical, Milan, Italy) were prepared in dimethyl sulfoxide (Sigma). Further dilutions were prepared in the test medium. For in vivo studies, stock solutions of AMB (Fungizone; Bristol-Myers Squibb S.p.A., Latina, Italy) were prepared in sterile distilled water.

**In vitro studies.** (i) **Microdilution method.** Drug activity was assessed by a checkerboard method derived from the standardized procedure established by the CLSI for broth microdilution antifungal susceptibility testing (14). Briefly, testing was performed in RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS; Gibco Laboratories, Milan, Italy) buffer. Volumes of 50 μl of each drug at a concentration of four times the targeted final concentration were dispensed in the wells of 96-well microtiter plates (Falcon 3072; Becton Dickinson). The final concentrations of the antifungal agents ranged from 0.03 to 2.0 μg/ml for AMB and from 0.03 to 64 μg/ml for CAS. Yeast inocula (100 μl), prepared spectrophotometrically and further diluted to obtain concentrations ranging from 1.0 × 103 to 5.0 × 103 CFU/ml (2× inoculum), were added to each well of the microdilution trays. The trays were incubated in air at 35°C and read at 24 and 48 h. Readings were performed spectrophotometrically at an optical density at 490 nm (OD490) with an automatic plate reader (ELx800; Biotek). Two MIC endpoints were considered: the first concentration of the antifungal agent tested alone or in combination at which the turbidity in the well was either 50% (50% inhibitory concentration...
Experiments were conducted in triplicate. Defined as a 100-fold decrease in killing compared with that achieved with the most active single agent, while antagonism was never observed. At 0, 2, 6, and 24 h following the introduction of the test isolate into the system, an aliquot from each dilution was streaked in triplicate onto Sabouraud dextrose agar plates for colony count determination. Following incubation at 35°C for 48 h, the number of CFU on each plate was determined. The limit of detection was 20 CFU/ml. Fungicidal activity was considered to have been achieved when the number of CFU per milliliter was less than 0.50, and less than or equal to 4.0, and antagonistic if the FIC index was greater than 0.50 and less than or equal to 0.25. The halo diameters produced by different concentrations of combination were never smaller than those produced by each drug alone.

Starting 24 h postchallenge with CAS at 0.25, 1, and 5 mg/kg/day (CAS 0.25, CAS 1, and CAS 5), with AMB at 1 mg/kg/day (AMB 1), or with their respective combinations (COMBO 0.25, COMBO 1, COMBO 5, and COMBO 10). Tissue burden studies were performed on day 5 postinfection. Drug efficacy was assessed by determining the number of CFU per kidney pair. Briefly, the mice were sacrificed, the kidneys were aseptically removed and homogenized, and diluted or undiluted aliquots, including the entire organ, were grown in cultures on Sabouraud dextrose agar for colony count determination. There were 8 animals in each group. Animal experiments were conducted with the approval of University of Ancona Ethics Committee.

Statistical analysis. The in vivo results were analyzed by either Mann-Whitney U test or Student’s t test considering a P value of <0.05 significant. The Mann-Whitney U test was performed to compare tissue burden counts. Due to multiple comparisons, a P value of <0.016 was considered statistically significant.

RESULTS

Susceptibility results of the three C. parapsilosis isolates are reported in Table 1. At 24 h, the CAS IC50 and IC90 ranged from 0.5 to 1.0 and from 0.5 to 4.0 μg/ml, respectively. At the same interval, the AMB IC50 and IC90 ranged from 0.125 to 0.25 and from 0.125 to 1.0 μg/ml, respectively. The combination therapy yielded a significant reduction in the IC50 and IC90 for both CAS and AMB. Similarly, the checkerboard assay evaluated at 48 h showed that the combination of CAS with AMB significantly decreased the IC50 and IC90 values with respect to each single drug. However, according to our definition, FIC indexes yielded 100% indifferent interactions either at 24 h or 48 h (FIC indexes ranging from 0.51 to 1.0). Antagonism was never observed.

To further characterize the effects of combination therapy against C. parapsilosis, we used a disk diffusion assay. The results are reported in Table 2. In general, at 24 h, the halo diameters produced by different concentrations of combination therapies never exceeded the largest inhibition zone obtained with either CAS or AMB alone. However, CAS at 10 μg combined with AMB at 1 μg yielded halo diameters superior to those obtained by each drug alone (P < 0.05). At 48 h, CAS at 1 or 10 μg combined with AMB at either 1 μg or 10 μg produced inhibition zones greater than those of each single drug (P < 0.05). Again, antagonism was never observed; in fact, the halo diameters of each drug combination were never smaller than those produced by each drug alone.

The results of killing experiments conducted with C. para-
**psilosis** strain no. 3 are reported in Fig. 1. Both CAS and AMB showed a dose-dependent activity. CAS used at 1× MIC did not exhibit any antifungal activity (growth at 24 h similar to that of the control), while the echinocandin showed a fungistatic effect at 8× the MIC. Similarly, AMB at 1× the MIC did not exert any antifungal activity, while the polyene yielded a reduction of 0.8 log₁₀ with respect to the initial inoculum at 8× the MIC. Although COMBO 1 was more effective than each drug alone, it yielded only 1.7 log₁₀ growth reduction with respect to AMB alone. COMBO 8 did not show any additional reduction compared to AMB alone. Therefore, combination therapy yielded indifferent interactions regardless of the drug concentrations. Finally, a fungicidal activity was never reached.

To investigate this interaction in vivo, neutropenic CD1 mice were infected intravenously with *C. parapsilosis* strain no. 3 and treated with several therapeutic regimens, including a scheme of combination therapy. The results are reported in Fig. 2. Amphotericin B was effective at reducing the fungal burden against the controls (P = 0.005). All CAS doses, with the exception of CAS 0.25, were effective at reducing the counts with respect to the controls (P = 0.001). Both COMBO 0.25 and COMBO 1 significantly reduced the tissue burden counts with respect to each single therapy (P ranging from 0.001 to 0.002). COMBO 5 and COMBO 10 were both significantly more effective than AMB alone (P = 0.001 and 0.004) but not more effective than the echinocandin given as a single drug.

**DISCUSSION**

In this study, we analyzed the in vitro and in vivo interactions of CAS and AMB against *C. parapsilosis*. To our knowledge, this is the first study addressing the relationship between these two drugs versus this important opportunistic pathogen. Our data are encouraging, since we did not observe any negative effect. The lack of antagonism was documented in vitro and confirmed in vivo.

In vitro interactions were explored by using different methods, including the classical checkerboard dilution methodology, a disk diffusion assay, and the killing curve assay. Although the first method did not support a synergistic interaction, we observed a significant MIC reduction of both drugs upon the combination. Killing curves confirmed these findings. In these experiments, the best interaction was noted when both drugs were combined at 1 rather than at 8 times the MIC. Similarly, the effects observed by the halo assay were influenced by the drug doses utilized in the combination. In particular, only AMB at 1 and 10 μg combined with similar doses of CAS was significantly more effective than each single drug. On the contrary, when the polyene was added to CAS at 100 μg, the combination approach was not superior to the echinocandin alone.

It is interesting that a similar phenomenon was also observed in vivo. Actually, CAS given at 0.25 and 1 mg/kg/day combined with AMB at 1 mg/kg/day was significantly more effective than each single drug at reducing the colony counts in the kidney, while higher doses of the compound (i.e., CAS at 5 and 10 mg/kg/day) added to the polyene did not show any advantage over CAS alone. This can be due to the fact that CAS given as a single drug did not show a significant improvement in the clearance of fungal burden with dose escalation above 1 mg/kg/day. These results are similar to those recently reported by others (1, 5, 9). Altogether, these data indicate that, to obtain a beneficial effect of this combination approach against *C. parapsilosis*, an adequate ratio of drug concentrations has to be achieved.

Literature data addressing the relationships between echinocandins and polyenes against *Candida* spp. are limited. Early
experience with cilofungin and AMB in mice with disseminated candidiasis due to *C. albicans* indicated improved survival and reduced tissue burden relative to the results seen with either agent alone (21). More recently, Hossain et al., evaluated in vitro and in vivo efficacies of CAS plus AMB against an azole-resistant strain of *C. albicans* (10). While they found an indifferent interaction in vitro by the checkerboard dilution method, the combination approach was the only treatment that resulted in significant reduction in kidney CFU counts. Recent data showed that either CAS or micafungin administered in combination with AMB were the only therapeutic approaches yielding organ sterilization in murine candidemia models due to *Candida glabrata* (2, 16). In the present study, we expanded the knowledge of this interaction in infections due to *C. parapsilosis*. Unlike previous reports on *C. glabrata*, we did not observe organ sterilization upon combination therapy. This finding can be due to the fact that, in the present study, AMB was utilized only at 1 mg/kg/day, while in previous studies with *C. glabrata*, kidney sterilization was reached when CAS was associated either with AMB at 3 mg/kg/day or with liposomal AMB at 7.5 mg/kg/day (2, 16).

The positive interaction between an echinocandin compound and a polyene can be explained by the fact that both drug families possess unique mechanisms of action. It can be postulated that the candins, which inhibit cell wall synthesis, may enhance the activity of AMB by increasing the rate or degree of their access to the cell membrane.

Although we found a positive interaction between CAS and AMB versus *C. parapsilosis*, an extrapolation of these results into the clinical practice should be done with caution. Actually, literature data showed that AMB, fluconazole, and CAS given as monotherapies are already effective in systemic infections due to this species of *Candida* (20). Thus, this combination approach should be reserved for special clinical settings, such as severe immunocompromised patients with systemic infections, patients with endocarditis, or patients with recurrent infections despite an appropriate antifungal therapy.

In conclusion, combinations of CAS and AMB appear to produce enhanced activity against *C. parapsilosis* both in vitro and in vivo. Additional studies involving several isolates and multiple dosing regimens are warranted to further elucidate the potential benefit of this combination approach in infections due to this common species of *Candida*. 

![Figure 2](http://aac.asm.org/)

**FIG. 2.** Kidney tissue burden of neutropenic CD1 mice. The mice were infected intravenously with $3.5 \times 10^5$ CFU/mice of *C. parapsilosis* strain no. 3. Animals were treated daily for four consecutive days with AMB at 1 mg/kg/day (AMB 1), with CAS at 0.25, 1, 5, or 10 mg/kg/day (CAS 0.25, CAS 1, CAS 5, CAS 10), or with their respective combinations (COMBO 0.25, COMBO 1, COMBO 5, and COMBO 10). Tissue burden experiments were performed on day 5 postinfection. The bars represent the medians. C, control; *, $P < 0.016$. 

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