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Efficacy of caspofungin combined with amphotericin B against azole-resistant *Candida albicans*

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The activity of caspofungin (CFG) combined with amphotericin B (AMB) against azole-resistant *Candida albicans* was evaluated *in vitro* (chequerboard) and *in vivo* (murine). CFG+AMB resulted in positive interactive effects *in vitro* (fractional inhibitory concentration index 0.75). Compared with untreated controls, CFG+AMB prolonged mouse survival ($P = 0.006$) and compared with AMB alone, CFG+AMB prolonged mouse survival ($P = 0.36$); however, the latter difference was not significant. CFG+AMB treatment significantly reduced kidney cfu compared with untreated controls and CFG-treated groups ($P \leq 0.05$ for both comparisons). In addition, this combination reduced brain cfu significantly compared with untreated controls and AMB-treated mice ($P = 0.005$ and 0.05, respectively).

Keywords: disseminated candidiasis, antifungal, therapy

Introduction

Candidal infections are associated with the highest crude mortality compared with other nosocomial bloodstream pathogens.[1] Therefore, there is a growing need for new approaches, such as combination therapy, to treat invasive candidiasis. The echinocandin caspofungin (CFG) is a new antifungal with activity against yeasts and moulds.[2] Unlike amphotericin B (AMB), which binds to membrane sterols, CFG inhibits the fungal (1,3)-β-D-glucan synthase enzyme complex that forms glucan polymers, a major fungal cell wall component.[3] Our hypothesis is that an effective way to inhibit *Candida albicans* is by combining a cell wall active agent (CFG) with a membrane active drug (AMB).

This study evaluates the activity of CFG+AMB against an azole-resistant *C. albicans* isolate using *in vitro* and *in vivo* methodologies. No antagonistic interactions were observed between the two agents, which showed a trend towards additivity.

Materials and methods

Caspofungin acetate (CFG, Merck Research Laboratories, Rahway, NJ, USA) and amphotericin B deoxycholate (AMB, Bristol-Myers Squibb, Princeton, NJ, USA) powders were dissolved in sterile distilled water. CFG was stored at –20°C and AMB at 4°C in the dark.

Candida albicans strain 12-99, a clinical isolate obtained from a patient for whom fluconazole therapy failed, was used. Antifungal susceptibility testing was performed according to M27-A methodology.[4] Before each experiment, *C. albicans* was grown overnight at 37°C in Sabouraud dextrose broth (SDB; Difco Laboratories, Detroit, MI, USA). Blastospores were washed twice and suspended in sterile normal saline (NS; 0.85%); their number was determined using a haemocytometer, and confirmed by quantitative culturing.

A chequerboard technique was used to evaluate drug–drug interactions. Different concentrations of CFG and AMB in RPMI 1640 medium were combined into wells of microtitre plates so that the concentration of each agent increased simultaneously. Two rows, consisting of serial dilutions of the individual drugs alone, were also included. The highest concentrations of CFG and AMB used were 128 and 64 mg/L, respectively. Wells were inoculated with *C. albicans* (between 0.5 and 2.5×10^3 cfu/mL) and plates incubated at 35°C for 48 h. Growth in each well was observed visually. The fractional inhibitory concentration index (FICI) was

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calculated. The activity was expressed as synergic when the FICI was < 0.5 and antagonistic when the FICI was > 4.0; FICI < 1 was regarded as a positive and FICI > 1 as a negative interaction.

The Institutional Animal Care and Use Committee approved the protocol for our murine model. Six- to eight-week-old male, BALB/c mice (Charles River, Wilmington, MA, USA) were housed in cages (three mice or fewer/cage) and acclimatized for 5 days before fungal challenge. Each mouse was inoculated with 0.1 mL of *C. albicans* 12-99 (prepared in sterile NS from an overnight, 37°C, SDB culture) via the lateral tail vein. To determine the optimal challenge inoculum, three groups of mice (five mice/group) were infected intravenously with different concentrations of *C. albicans* (5×10^5, 1×10^6 or 3×10^6 blastospores).

In subsequent experiments, the animals were infected with 5×10^5 cells in 100 μL of NS. They were divided into seven mice/group and received their therapies intraperitoneally 2 h post-challenge. In preliminary experiments, drug dosages that were below an effective dose when used singly were determined. The following dosages were evaluated: CFG 0.0005, 0.001 and 0.002 mg/kg; and AMB 0.008, 0.016 and 0.032 mg/kg daily for 14 days. Based on these experiments, the following dosages were selected for subsequent drug–drug interaction studies: CFG 0.002 mg/kg and AMB 0.016 mg/kg. Efficacy was evaluated by monitoring survival and tissue burden. Animals were monitored daily for evidence of infection and its severity, and deaths were noted. Each experiment was performed twice (totalling 14 animals/group) to determine the survival pattern. For determination of tissue fungal burden, seven mice per group were killed; their kidneys and brain were removed aseptically, weighed and homogenized. Diluted samples of homogenates were cultured on agar plates (at 37°C for 48 h), and the number of cfu counted and expressed as cfu/g of tissue.

Differences in survival were assessed by the Kaplan–Meier method, while the mean cfu were compared using the Mann–Whitney *U*-test (mean ± S.E.). A *P* value of <0.05 was considered statistically significant.

Results

The MICs of fluconazole (FLC), itraconazole (ITC), AMB and CFG for *C. albicans* 12–99 were 64.0, 2.0, 1.0 and 0.06 mg/L, respectively, indicating resistance to FLC and ITC. Combining AMB with CFG resulted in two- and four-fold reductions in MICs of AMB and CFG, respectively. Also, a combination of CFG+AMB revealed a positive interaction (FICI value 0.75).

Experiments directed at determining the optimal challenge inoculum, showed that mice infected with 1×10^6, or 3×10^6 cells of *C. albicans* exhibited clinical signs of infection (e.g. reduced activity, ruffled hair) on day 2, and all died within 2–3 days. Mice inoculated with 5×10^5 cells showed signs of infection on day 4, and survived until day 7. Thus, 5×10^5 cells/animal was selected as the optimal challenge inoculum.

Treating animals with a CFG+AMB combination significantly prolonged survival compared with infected, untreated controls (P = 0.006) (Figure 1). Treatment of mice with AMB+CFG, even at low dosage (0.016 and 0.002 mg/kg), also tended to prolong survival. Survival rates for untreated controls, CFG, AMB and CFG+AMB groups at day 21 were 0%, 22%, 50% and 72%, respectively. Thus, combining CFG with AMB increased the survival rate compared with the control group. Although animals treated with a CFG+AMB combination survived longer than those treated with AMB alone (72% versus 50%, respectively) this difference was not significant ($P = 0.36$).

Table 1 compares the kidney and brain tissue burden following treatment with CFG or AMB alone, or CFG combined with AMB. Compared with untreated controls, the only treatment regimen that resulted in a reduction of cfu in the kidneys was CFG+AMB (P = 0.05). A comparison of brain tissue candidal load showed that treatment with CFG alone or CFG+AMB reduced the invasion of the brain (P values 0.05 and 0.005, respectively). No statistically significant differences in brain cfu were noted between mice treated with CFG alone or as part of a combination ($P = 0.94$).

Discussion

Our data demonstrated that no antagonism was noted *in vitro* or *in vivo* between the test drugs CFG and AMB. Franzot &

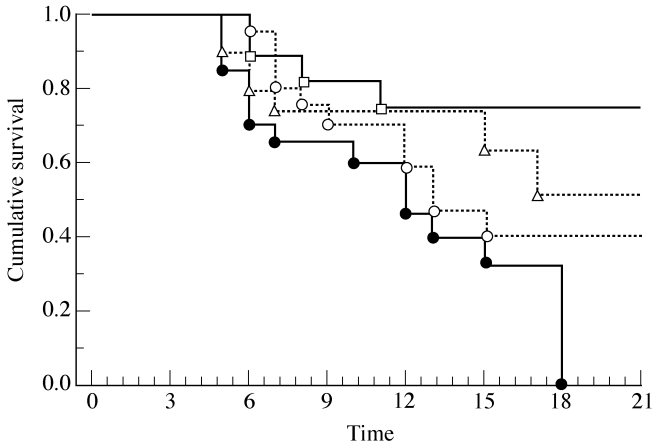

Figure 1. Effect of antifungal therapy on survival of mice infected with *C. albicans*: solid circles, untreated controls; open circles, mice treated with CFG (0.002 mg/kg); open triangles, mice treated with AMB (0.016 mg/kg); open squares, mice treated with CFG+AMB. Time indicates the number of days the animals survived. The survival curve was prepared using the Kaplan–Meier method based on Logrank (Mantel–Cox) analysis; the cumulative data were generated from two experiments.

Table 1. Effect of antifungal therapy on the tissue fungal burden of kidney and brain in mice infected with *C. albicans*

	Kidney (log_{10}cfu/g) mean ± S.E.	Brain (log_{10}cfu/g) mean ± S.E.
Untreated	6.54 ± 0.25	3.18 ± 0.39
CFG 0.002 mg/kg	6.73 ± 0.49	2.52 ± 0.28*
AMB 0.016 mg/kg	6.1 ± 0.33	2.66 ± 0.12
CFG+AMB	5.48 ± 0.33*	2.3 ± 0.08**

Data for the mycological study are the mean ± S.E. of the number of *C. albicans* in the kidney and brain (expressed as cfu) 24 h after the last day of treatment with different antifungal agents. Each group comprised seven mice. The Mann–Whitney *U*-test was performed to compare the mean cfu in tissues. Kidney cfu were significantly lower in CFG+AMB-treated mice than untreated controls and CFG-treated mice (*P value 0.05, for both comparisons). Similarly, brain cfu were significantly lower in CFG+AMB-treated mice than untreated controls and CFG-treated mice (**P value 0.005 and 0.05, respectively), and brain cfu were significantly lower in CFG-treated mice than untreated controls (*P value 0.05).

Casadevall tested the combination against *C. neoformans* and showed synergic effects.[5] Others demonstrated that the combination was synergic to additive against *Aspergillus* and *Fusarium* spp. isolates, whereas CFG alone was not effective against *Fusarium*. Importantly, similar to our data, no antagonism was seen with the CFG+AMB combination. Manavathu *et al*. reported a synergistic interaction by AMB with either micafungin (MFG) or CFG against *A. fumigatus*.[6] To our knowledge, this is the first study to evaluate the effect of combining CFG+AMB in the treatment of haematogenously disseminated candidiasis caused by an azole-resistant *C. albicans*. Our data show that combination therapy resulted in prolongation of survival and a reduction in the tissue fungal burden. In agreement with our findings, Flattery *et al*., using a disseminated candidiasis mouse model, demonstrated that the CFG+AMB combination was synergic against *C. albicans*.[7] A limitation of our study was that because the doses we selected were minimally effective, the difference in survival pattern and tissue fungal burden was marginal. This is often a problem when running *in vivo* combination studies. More strains, animals, doses and combinations would provide more biologically meaningful data.

The underlying mechanisms of synergic or additive effects for CFG+AMB are unknown. It is likely that inhibition of (1,3)-β-D-glucan formation by CFG leads to cell wall damage. This would allow AMB easier access to the fungal cell membrane, where it binds to membrane ergosterol, resulting in pore formation and cell lysis.[5] In this study, we focused on *in vivo* evaluation of the combined effects of CFG and AMB against an azole-resistant *C. albicans* strain. We did not include an azole-sensitive strain; such evaluation was beyond the scope of this study. Extending this work to cover more strains should be undertaken in the future.

In conclusion, our data demonstrate no evidence of antagonism between CFG and AMB when combined, and interactions *in vivo* and *in vitro* have tended to be favourable. Further work is needed to ascertain the clinical relevance of our findings.

Acknowledgements

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