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In Vivo Studies with a *Candida tropicalis* Isolate Exhibiting Paradoxical Growth *In Vitro* in the Presence of High Concentration of Caspofungin

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We investigated the activity of caspofungin against a *Candida tropicalis* clinical isolate showing paradoxical growth *in vitro*. BALB/c mice immunosuppressed by cyclophosphamide were infected intraperitoneally using 10^7 CFU/mouse. Caspofungin was administered intraperitoneally once daily for 5 days or as a single dose using the following doses: 0.12, 0.25, 1, 2, 3, 5, and 15 mg/kg. The single dose of caspofungin was effective only at 5 and 15 mg/kg concentrations (100% survival). Five-day caspofungin treatment led to 100% survival at doses of 1 mg/kg or higher. Caspofungin treatment significantly decreased the number of viable yeasts in the peritoneal lavage samples as well as in the infected abscesses at doses 1, 3, 5, and 15 mg/kg caspofungin as compared to the untreated control ($P<0.001$ in all cases), and even to the group treated with 0.12 mg/kg caspofungin ($P<0.05$ in all cases). At 2 mg/kg caspofungin dose, sterilization of the internal organs was reproducibly incomplete, suggesting that the role of paradoxical growth in the late clinical failure cannot be excluded.

***Keywords*:** caspofungin, paradoxical growth, murine, *C. tropicalis*

Caspofungin is a member of the new class of echinocandin antifungals with potent activity against *Candida* and *Aspergillus* species. Caspofungin, similarly to other echinocandins, is fungicidal against *Candida* species and the actively growing tip of *Aspergillus* hyphae (Deresinski and Stevens, 2003). Caspofungin proved to be highly effective both in animal models and in clinical situations (Wiederhold *et al.*, 2004).

In a persistently neutropenic rabbit model of invasive pulmonary aspergillosis, however, Petraitiene *et al.* (2002) found higher fungal burden in the lungs after high (3 and 6 mg/kg/day) doses of caspofungin treatment when compared to the lower doses. A similar result was obtained by Wiederhold *et al.* (2004) in their murine model. Additionally, they observed an increase in fungal viability at 16-32 mg/L caspofungin concentrations *in vitro* besides the expected decline in viability at lower caspofungin concentrations in the case of both *A. fumigatus* and *C. albicans*.

Paradoxical growth of *C. albicans* strains in the presence of high concentration of caspofungin was throughly investigated *in vitro* by Stevens and colleagues (Stevens *et al.*, 2004, 2006). The frequency of this phenomenon was found to be 21% and 18% in the case of *C. albicans* and non-*albicans Candida* species, respectively.

In contrast, in our previous work using MIC, MFC, and killing curve determinations, we have noted growth at high (≥6.25 mg/L) but not at low caspofungin concentrations in the case of 14 out of 15 (93%) *C. tropicalis* clinical isolates (Soczo *et al.*, 2007). We have demonstrated that caspofungin acts as a fungistatic drug at 6.25-12.5 mg/L concentrations, but is fungicidal at lower concentrations against *C. tropicalis*. However, the clinical relevance of this phenomenon has not yet been investigated in *C. tropicalis* infections. Therefore, the aim of our present study was to investigate the importance of paradoxical growth in an *in vivo* murine model.

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Materials and Methods

Clinical isolate

C. tropicalis, an isolate obtained from a human peritoneal abscess, was used, which grew at both 6.25 and 12.5 mg/L caspofungin concentrations in the time-kill experiment, but was killed at concentrations from 0.048 to 3.12 mg/L of caspofungin within 24 h (MIC=0.024 mg/L) (Soczo *et al.*, 2007).

Animals

Mice were given food and water ad libitum. The animals were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals; the experiments were approved by the Animal Care Committee of the University of Debrecen, Debrecen, Hungary (No. 56/2005).

Protocols

We used the intraperitoneal abscess model described by Ninomiya *et al.* (2005) using immunosuppressed (with two 200 mg/kg cyclophosphamide doses four days prior to and one day after infection) female BALB/c mice weighing 18-20 g. Briefly, autoclaved caecal content from mice was mixed with equal amount of fungal suspension (0.25-0.25 ml) and was inoculated into mice intraperitoneally (final inoculum 10^7 CFU/mice). Inoculum density was confirmed by plating

serial dilutions on Sabouraud agar plates.

In the first set of our *in vivo* experiments, we used caspofungin in a single dose (Cancidas, commercial preparation). Animals were randomised into eight groups each containing five mice as follows: no treatment (control), 0.12, 0.25, 1, 2, 3, 5, and 15 mg/kg of body weight caspofungin. Intraperitoneal caspofungin treatment was administered 1 h after the infection. Mice were followed up for seven days and the survival rate was determined.

In the second set of experiments, caspofungin was administered as an intraperitoneal injection once daily for 5 days, at the same doses as described above. We used five to eight mice in each group. This experiment was repeated with all caspofungin doses.

Mice were monitored two times daily. Mice that became immobile and showed signs of severe illness were euthanized and recorded as having died on the following day. Surviving mice were sacrificed and colony numbers of viable fungi were determined from an abdominal lavage sample taken by washing the peritoneal cavity with 1 ml sterile saline. The number of abdominal abscesses formed and the fungal colony numbers in each abscess were also determined (Ninomiya *et al.*, 2005).

Statistical analysis

The relationship between treatment and survival of mice was analysed using a chi-square test. Numbers of fungal CFUs (both those obtained by peritoneal lavage and those yielded from peritoneal abscesses) were compared by Kruskal-Wallis test with Dunn's post-testing. Values of $P<0.05$ were considered to be significant. For statistical analysis, GraphPad Prism version 4.03 for Windows was used.

Results

All untreated control mice succumbed to infection within five days. Single doses of 0.12, 0.25, 1, 2 or 3 mg/kg caspofungin did not decrease mortality (100% mortality within five days), however, 100% survival rate was observed at 5 and 15 mg/kg doses (data not shown).

Five days of caspofungin treatment significantly decreased the mortality rate when compared to the control group (Fig. 1) irrespective of the dose ($P<0.0001$); differences among the caspofungin treated groups were statistically not significant ($P>0.05$). Moreover, caspofungin treatment significantly decreased the number of viable yeasts in the peritoneal lavage samples (Fig. 2, panel A) in the case of all groups as compared to the control ($P<0.001$ in each case), with the exception of the 0.12 mg/kg dose group ($P>0.05$).

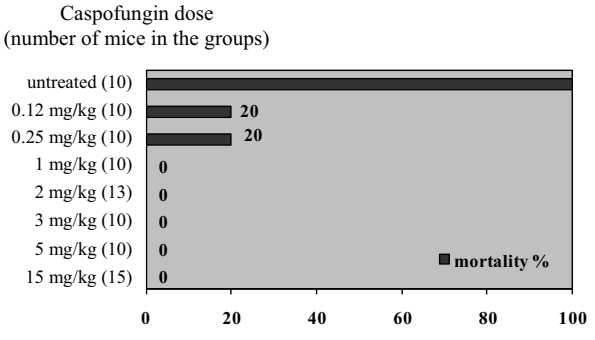

Fig. 1. Cumulative mortality of mice after infection with *C. tropicalis* (10^7/CFU/mouse) in the untreated controls and the caspofungin treated groups.

The vast majority of abscesses were found in the liver (Fig. 2, panel B). The number of abscesses containing viable yeasts as well as yeast CFU numbers in the abscesses decreased significantly in groups treated with 1, 2, 3, 5, and 15 mg/kg caspofungin ($P<0.001$ in case of 1, 3, 5, and 15 mg/kg and $P<0.01$ in case of 2 mg/kg), in comparison with the control group. There were no differences between the groups treated with the two lowest doses of caspofungin (0.12 and 0.25 mg/kg) and the control group ($P\geq0.05$ in all cases) (Fig. 2, panels B and C). Viable yeast CFU numbers significantly decreased in groups treated with 1, 3, 5, and 15 mg/kg caspofungin doses ($P<0.05$) as compared to the 0.12 mg/kg caspofungin treated group. These data were reproducible in the second independent experiments (Fig. 2, panel C).

In the two experiments using 2 mg/kg caspofungin, one and two out of five and eight mice, respectively, yielded viable yeasts in the abscesses after treatment, i.e., sterilization of the abscesses was not achieved (20,000 CFU/ml in the first and 180 and 560 in the second experiment in the single liver abscesses, respectively, Fig. 2: panel C). These yeast CFU numbers in the case of the 0.12 and 2 mg/kg caspofungin-treated groups did not differ significantly from each other. To test the reproducibility of these results found in the 2 mg/kg caspofungin group, we repeated the experiment with this dose a third time. This third experiment yielded similar results (one of eight mice remained infected, carrying 6,300 CFU/ml in a single abscess). The results of this third experiment were statistically comparable to the former ones.

Discussion

Dose escalation is one of the possible strategies for the echinocandin treatment of severe invasive candidiasis. Although the maximum tolerated dose of caspofungin is unknown, caspofungin at two or three times the standard (50 mg/day) dosing regimen was well tolerated in adult non-candidemic and candidemic patients (Cornely *et al.*, 2007; Betts *et al.*, 2009). High daily doses of caspofungin and micafungin produced a numerically higher favourable overall response rate in patients infected with *C. albicans* and *C. parapsilosis* as compared to the standard dosing regimen (Pappas *et al*., 2007; Betts *et al.*, 2009). In contrast, high daily doses of caspofungin and micafungin produced numerically lower favourable overall response rates in patients infected with *C. tropicalis* when compared to the standard daily dose group. However, these differences were statistically not significant and were not considered to be associated with the phenomenon of paradoxical growth (Pappas *et al.*, 2007; Betts *et al.*, 2009).

In our present work, caspofungin proved to be highly effective in an immunocompromised murine model against a *C. tropicalis* strain showing paradoxical growth *in vitro*. As expected, the subtherapeutic daily doses of 0.12 and 0.25 mg/kg did not improve the survival significantly, and could not eradicate the infection, but the daily dose of 1 mg/kg, which is considered to be standard dose, led to 100% survival and effectively cleared the infection from the peritoneal cavity. Supratherapeutic daily caspofungin doses (2, 3, 5, and 15 mg/kg/day) were also effective in decreasing lethality and in

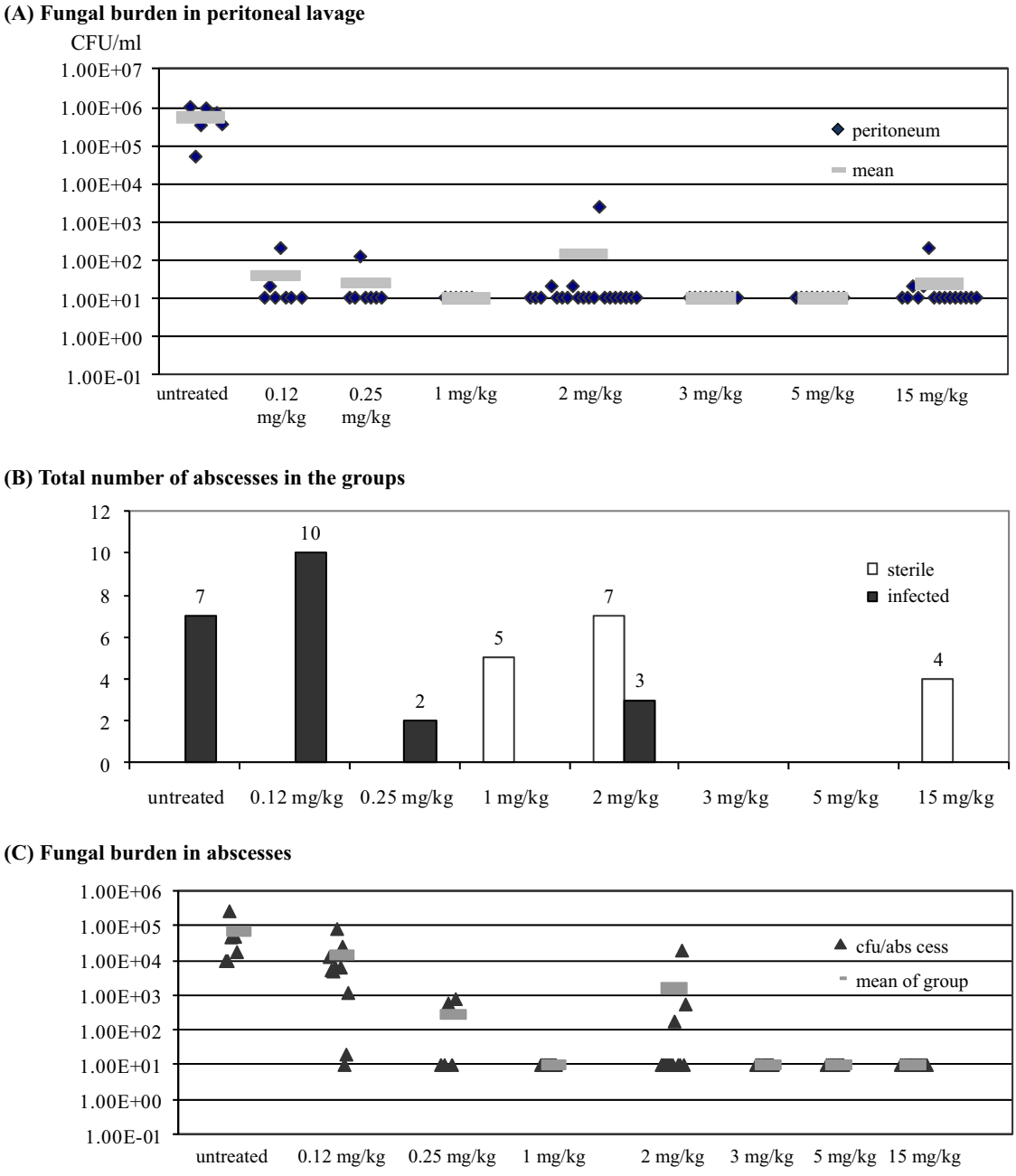

Fig. 2. Therapeutic efficacy of caspofungin against *C. tropicalis* in neutropenic mice determined in an intraperitoneal abscess model. Caspofungin was administered intraperitoneally at doses of 0 (control), 0.12, 0.25, 1, 2, 3, 5, and 15 mg/kg/day. Treatment once daily was started 1 h after fungal inoculation, and continued for 5 days. (A) the number of viable yeast number obtained from peritoneal lavage; (B) the number of sterile and infected abscesses formed; (C) viable yeast number of the infected abscesses.

preventing abscess development. In contrast to total sterilization of abscesses by 1 mg/kg caspofungin, three of 13 mice receiving a 2 mg/kg dose remained infected; this was confirmed in a third repeated experiment. However, the CFU numbers found in the group treated 2 mg/kg caspofungin were not statistically different from the zero CFU numbers found in the group treated with 1 mg/kg. Similar results were observed by Clemons *et al*. (2006) in case of *C. albicans* strains showing paradoxical growth *in vivo* at 20 mg/kg caspofungin dose upon examination of kidney of the mice. However, they could not reproduce their findings and concluded that paradoxical growth had limited *in vivo* significance.

Present results suggest a trend that supratherapeutic doses may, at least in some mice, lead to incomplete sterilization of the mice and may be a source for relapse after the discontinuation of therapy. This challenges the earlier conclusions of Pappas *et al.* (2007) and Betts *et al.* (2009), who discarded the *in vivo* role of paradoxical growth on the basis of the lack of

statistical differences between groups treated with normal and with supratherapeutic caspofungin doses, in spite of the lower number of therapeutic successes in the supratherapeutic dose groups in case of *C. tropicalis*. Therefore, we believe that a certain role of paradoxical growth in the late clinical failure cannot be excluded unequivocally.

Caspofungin was shown to reach concentrations between ca. 6 and 9 mg/L in the murine liver when the standard 1 mg/kg dose was given, however, the drug levels produced by slightly supratherapeutic doses are unknown (Hajdu *et al.*, 1997). Viewing the available earlier results in the light of the present findings draws the attention to the necessity of further investigations, including determination of serum drug levels produced by supratherapeutic echinocandin doses.

Pharmacokinetic and pharmacodynamic studies suggest that echinocandins efficacy is strongly associated with the AUC/MIC (the area under the concentration-time curve per MIC) or with the C_{max}/MIC (the maximum concentration of the drug in serum per MIC). Either result means that intermittent dosing regimens with echinocandins will be effective. Louie *et al.* (2005) in their disseminated candidiasis model found that the early outcome in caspofungin therapy (up to day 3) appears to be most closely linked to the C_{max}/MIC ratio; however, the outcome in the later treatment period (day 7) is better predicted by the AUC/MIC ratio, presumably due to the prolonged tissue distribution of caspofungin. They concluded that both pharmacodynamic relationships support the administration of large doses of echinocandin infrequently. In our study, the 5 and 15 mg/kg caspofungin doses (corresponding to 1 and 3 mg/kg daily doses, respectively) were able to improve the survival rate when given in a single dose corresponding well to the suggestion of Louie *et al*. (2005).

Our study has some limitations. First, we only examined one *C. tropicalis* clinical isolate, and other isolates may behave differently. Second, single dose experiments were conducted only with evaluation of lethality; the efficacies in eradication of the infection by single caspofungin doses remain to be examined. Third, longer follow up of surviving mice would be needed to evaluate the risk of clinically significant persistence or relapse of infection posed by different dosing regimens.

In summary, our results suggest that higher than recommended *in vivo* daily dosing regimen of caspofungin is neither superior nor inferior to the normal dose for the treatment of invasive candidiasis caused by *C. tropicalis*, even in the case of an isolate showing paradoxical growth *in vitro*. However, the role of paradoxical growth in the late clinical failure cannot be excluded when using supratherapeutic caspofungin doses. In addition, the excellent survival rate (100%) elicited by a single dose (5 and 10 mg/kg) of caspofungin indicates that this dosing regimen is worth further evaluation not only for prophylaxis but also for treatment of infections caused by *C. tropicalis*.

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