Comparison of the efficacy of combination and monotherapy with caspofungin and liposomal amphotericin B against invasive candidiasis

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ABSTRACT

الأهداف: التحقق ما إذا كان العلاج التركيبي بعقار ليبوسومال أمفوتيريسين ب (LAmB) وعقار كاسبوفونجين (CAS) هو فوق المعالجات الأحادية في نموذج تجريبي لداء المبيضات المقاومة.

الطريقة: أجريت هذه الدراسة في الفترة ما بين أكتوبر 2006م وأغسطس 2007م، بجامعة سيلال بيار – تركيا. شملت الدراسة إجمالي عدد 144 فأراً وتم تقسيمهم إلى أربع مجموعات، مجموعة التحكم (عدد=36)، المجموعة التي تلقت المعالجة CAS (عدد=36)، والمجموعة التي تلقت المعالجة LAmB (عدد= 36)، ومجموعة المعالجة المركبة (عدد=36). تم تقييم فعالية المعالجة بواسطة تحديد النجاة كذلك انخفاض الكثافات في قطر النسيج.

النتائج: انخفضت كثافات القطر في الأنسجة بشكل ملحوظ وطالة معدلات النجاة مع المعالجة بـ CAS فقط أو بـLAmB فقط، أو مع المعالجة المركبة مقارنةٍ مع مجموعة التحكم $p<0.05$. لم يكن هنالك فرقاً ملحوظاً بين مجموعات المعالجة الأحادية. كانت الإنخفاضات في كثافات قطر النسيج ملحوظة في CAS و 1mg/kg – LAmB، ومجموعة المعالجة المركبة مقارنتاً بمجموعة 1mg/kg – CAS و 1mg/kg – LAmB لمجموعة CAS $p=0.004$، نسبة $p=0.009$ لمجموعة LAmB. كانت معدلات النجاة متشابهة في كلتا مجموعتي المعالجة.

خاتمة: كانت المعالجة المركبة أعلى بمقدار 1mg/kg من جرعات عقار LAmB وعقار CAS في جوانب إنقاص كثافة قطر النسيج. على الرغم مع المعالجة المركبة طالة من معدلات النجاة في جميع المجموعات الفرعية. لم يكن هنالك فرقاً ملحوظاً بين المعالجة المركبة والأحادية يمكن إظهاره. يجب إجراء المزيد من الدراسات الإضافية مع أعداد كبيرة من الحالات للتحقق من علو العلاج المركب.

Objective: To investigate if combination therapy with liposomal amphotericin B (LAmB), and caspofungin (CAS) is superior to monotherapies in an experimental model with azole-resistant *Candida albicans*

Methods: This study was carried out between October 2006 and August 2007 in Celal Bayar University, Manisa, Turkey. A total of 144 mice were included in the study, and divided into 4 groups as: control (n=36), CAS treatment group (n=36), LAmB treatment group (n=36), and combination therapy group (n=36). Treatment efficacy was assessed by determining survival, as well as, the decrease in tissue fungal densities.

Results: The fungal densities in tissues were significantly reduced, and the survival rates were prolonged with either CAS only, or LAmB only, or with combination therapy compared to those of controls ($p<0.05$). There was no significant difference between monotherapy groups. Decrease in tissue fungal densities were significant in CAS and LAmB (1mg/kg) combination group, compared to CAS (1mg/kg) and LAmB (1mg/kg) groups ($p=0.004$ for CAS, $p=0.009$ for LAmB). Survival rates were similar in both treatment groups.

Conclusion: The combination treatment was superior with 1mg/kg of doses of LAmB and CAS in terms of reducing the tissue fungal burden. Although with combination therapy the survival rates prolonged in all subgroups, no significant difference between the combination and monotherapies could be shown. Additional studies with a large number of cases are warranted to investigate the superiority of combination therapy.

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The incidence of invasive candidiasis has increased dramatically over the last 3 decades, and it has become a significant cause of mortality and morbidity, especially in immune-compromised patients.[1,2] Over the past decade, there was a great progression in antifungal drug researches, and the clinical development of several new compounds, however, the need for more effective drugs is increasing as the improvement in the therapy success could not be reached according to expected level.[3-5] On the other hand, resistance to antifungal drugs among pathogenic yeasts is increasing, particularly among the azole group of drugs. Recently, *Candida* species' resistance to fluconazole (FLU) has been highlighted as a worldwide problem, with treatment failures leading to fatal outcomes.[4,6] The emergence of resistance to azole antifungals and of systemic toxicity to polyene agents, (namely, Amphotericin B [AmB]) has raised the issue of using such antifungals in combination to optimize therapeutic outcome. Antifungal combinations may increase the magnitude and rate of microbial killing in vivo, shorten the total duration of therapy, prevent the emergence of drug resistance, expand the spectrum of activity, and decrease drug-related toxicities by allowing the use of lower doses of antifungals.[7,8] The polyene compound AmB has been the mainstay of antifungal therapy for invasive candida infections.[9,10] However, due to its severe and dose-limiting adverse effects, alternatives to AmB, and lipid based AmB are being used, either alone, or as part of a combination therapy.[11] Echinocandins are amongst the recently-developed alternative antifungal groups which act by blocking 1-3-ß-glucan synthetase, thus inhibiting cell wall synthesis. The echinocandin compound caspofungin (CAS) has a potential fungicidal activity against *Candida* spp. and has been shown to be better tolerated than AmB.[12-14] In a double-blind randomized study comparing CAS and AmB deoxycholate in the treatment of invasive candidiasis, CAS was found to be as effective as AmB, while fewer drug-related adverse reactions were determined in the CAS group.[15] In this study, we hypothesized that a combination of a membrane active drug (liposomal amphotericin B [LAmB]), and a cell wall active agent CAS could be more effective against invasive candida infections than monotherapy. To investigate this activity, we used an experimental murine model of azole-resistant *Candida albicans (C. albicans)* infection.

Methods. This study was performed between October 2006 and August 2007 in the experimental laboratory of Celal Bayar University, Manisa, Turkey. Liposomal amphotericin B (Ambisome) was obtained commercially from Gilead Sciences Inc., (Dublin, Ireland). A fresh LAmB solution (1 mg/ml) in sterile distilled water, was prepared prior to intervention. The commercial form of CAS (Cancidas, Merck Sharp & Dohme, Rahway, NJ, USA) was also used, and dissolved in sterile distilled (1 mg/ml) water. Both drugs were administered at a volume of 0.1 ml per dose. Male BALB/c mice, 8 to 10 weeks old, and weighing between 22 and 25 grams were purchased from Bornova Veterinary Research Laboratory. The mice were allowed free access to food and water. The mice were randomized into 12 groups, each comprising 12 animals. Animals were housed in plastic boxes, with 6 animals per container. All procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals, and were approved by the Animal Research Ethics Committee of Celal Bayar University. The *C. albican* strain, utilized to establish disseminated candidiasis in this murine model, was obtained from a patient with candidemia in the intensive care unit. The isolate was stored at -70°C in 10% glycerol. Before each experiment, *C. albicans* was grown overnight at 37°C, in Sabouraud dextrose broth (Difco Laboratories, Detroit, MI, USA). This *C. albicans* strain was tested for susceptibility to FLU, CAS, and AmB by the broth micro dilution method as described by Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS), utilizing the recommended buffered RPMI-1640 medium, an inoculum of $0.5x10^3$-$2.5x10^3$ colony forming unit (CFU)/ml, and an incubation temperature of 35°C for 48 hours.[16] Minimal inhibitory concentration values for FLU were 64 mg/L, CAS was 0.06 mg/L, and AmB was 0.125 mg/L. Prior to each intervention, yeast cells were prepared in fresh Sabouraud dextrose broth (Difco Laboratories, Detroit, MI, USA) from frozen stock cultures, washed twice with sterile normal saline (NS), counted with a hemocytometer, and diluted to the desired concentrations with NS. Hemocytometer counts were routinely verified by quantitative cultures on agar. To determine the optimal challenge inoculum (in other words, induce disseminated candidiasis in >95% of the mice [95% infective dose] without significant mortality), mice were infected intravenously with 6 different concentrations of *C. albicans* ($1x10^5$, $5x10^5$, $1x10^6$, $5x10^6$, $1x10^7$, $5x10^7$ CFU/ml). All mice infected with an inoculum greater than $1x10^6$ CFU/ml died within 3 days of infection, while mice infected with $1x10^6$ CFU/ml showed a 20%

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survival rate over a 7-day period. All animals infected with an inoculum greater than $5x10^6$ died hours after infection. Therefore, an inoculum of $1x10^6$ CFU/ml was considered optimal, and intravenously (0.1 ml) administered via the lateral tail vein. A total of 144 mice were included in the study. Initially, mice were randomly divided into 4 groups as: controls (n=36), CAS group (n=36), LAmB group (n=36), and CAS with LAmB combination therapy group (n=36). Infected mice were treated with antifungals administered intraperitoneally for 7 consecutive days, starting with a 24-hour post infection. The control groups were sham-treated with sterile distilled water, on the same therapy schedule as the treatment groups. The doses of LAmB and CAS were given in 3 different doses to each treatment group, as 0.5-1-2 mg/kg/day for monotherapy and combination therapy. For this reason, each treatment group was then divided into 3 subgroups, and each group consisted of 12 mice. Efficacy was measured either by reduction in colony counts, or by prolongation of survival observed over a period of 21 days. For this reason, each subgroup was divided into 2 groups comprising 6 animals each: one for examination of fungal tissue burden, and one for survival ratio. For evaluation of fungal tissue burden, 6 mice per group were sacrificed a day after the last treatment (post infection day 8) by cervical dislocation. The spleen, liver, and kidneys were removed aseptically, weighed, and each organ was homogenized in 5 ml NS. The homogenates were serially diluted, and plated onto Sabouraud dextrose agar (Difco, Laboratories, Detroit, MI, USA). The colonies were counted after 2 days of incubation at 35°C. Organ fungal densities were quantified as $\log_{10}$ CFU/gram of tissue. To determine the survival rate, the remaining 6 mice were monitored daily for any evidence of infection and its severity, and deaths were noted during an observation period of up to 21 days.

Statistical Package for Social Sciences (SPSS Windows version 11.0) program was used. Tissue burden counts were analyzed by the Mann-Whitney U test. Differences in cumulative survival rates were assessed by the Kaplan-Meier survival analysis, and compared among groups by using log rank test. A *p*-value of <0.05 was considered statistically significant.

Results. After treating the animals with different doses of antifungal agents, 6 animals in each group were sacrificed on day 8 of infection, and examined for the tissue fungal burden. In each dose group (0.5 mg/kg/day, 1 mg/kg/day, 2 mg/kg/day), LAmB monotherapy, CAS monotherapy, or LAmB combined with CAS, yielded significant reductions in tissue fungal burden compared to the untreated controls ($p<0.05$). Fungal densities are presented in **Figure 1**. The difference between the CAS only and LAmB only groups was not statistically significant. Comparison of monotherapies with the combined administration of CAS+LAmB showed a substantial reduction in fungal tissue burden with combination therapy. However, reduction in fungal densities was significant only in combination treatment at 1 mg/kg/day dose. The remaining 6 animals in each treatment group were evaluated daily for 21 days, for the progression of infection, and death. The survival rates of control mice, and those treated with CAS and LAmB mono therapies, and CAS+LAmB combination for all dose groups (0.5, 1, and 2 mg/kg/day) are presented in **Figure 2**. All infected, however, untreated mice died of infection between days 2 and 18. Survival rates for CAS (0.5 mg/kg/day) were 33.3%, CAS (1 mg/kg/day) were 66.7%, LAmB (0.5 mg/kg/day) were 50%, and LAmB (1 mg/kg/day) were 83.3% at day 21. All other mice survived. Compared to the untreated controls, treatment with only LAmB, only CAS, or the combination of LAmB with CAS prolonged the survival of animals. The prolongations in survivals were also statistically significant ($p<0.05$), except the 0.5 mg/kg/day CAS-only group ($p=0.09$). Even though survival rates in groups that received combination therapy at 0.5 mg/kg and 1 mg/kg doses were higher than the monotherapy groups, differences in survival rates between groups that received combination therapy and monotherapies, or between monotherapy groups were not statistically significant. No mortality was detected on day 21 among animals that had either combination or monotherapy at 2 mg/kg dosing.

Discussion. *Candida* species are the most common cause of fungal infections, and produce infections that range from mucocutaneous illnesses, to life-threatening invasive diseases. *Candida albicans* is the pathogen in approximately one half to two thirds of candidemia episodes, and is generally susceptible to FLU. However, FLU resistance in *C. albicans* among these patients is increasing, now estimated at 5%, particularly among severely immune-suppressed patients who received repeated fluconazole treatment and prophylaxis.[17,18] Amphotericin B has been tried in many patients resistant to FLU, however, the toxicity of this drug is problematic, and all patients do not respond to the therapy. Consequently, there has been an increasing effort to develop new antifungals with high efficacy, and less toxicity. The echinocandins are amongst these antifungals.[19]

Caspofungin causes inhibition of ß-1, 3-glucan synthesis, leading 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 lyses.[13,20] Our aim in carrying

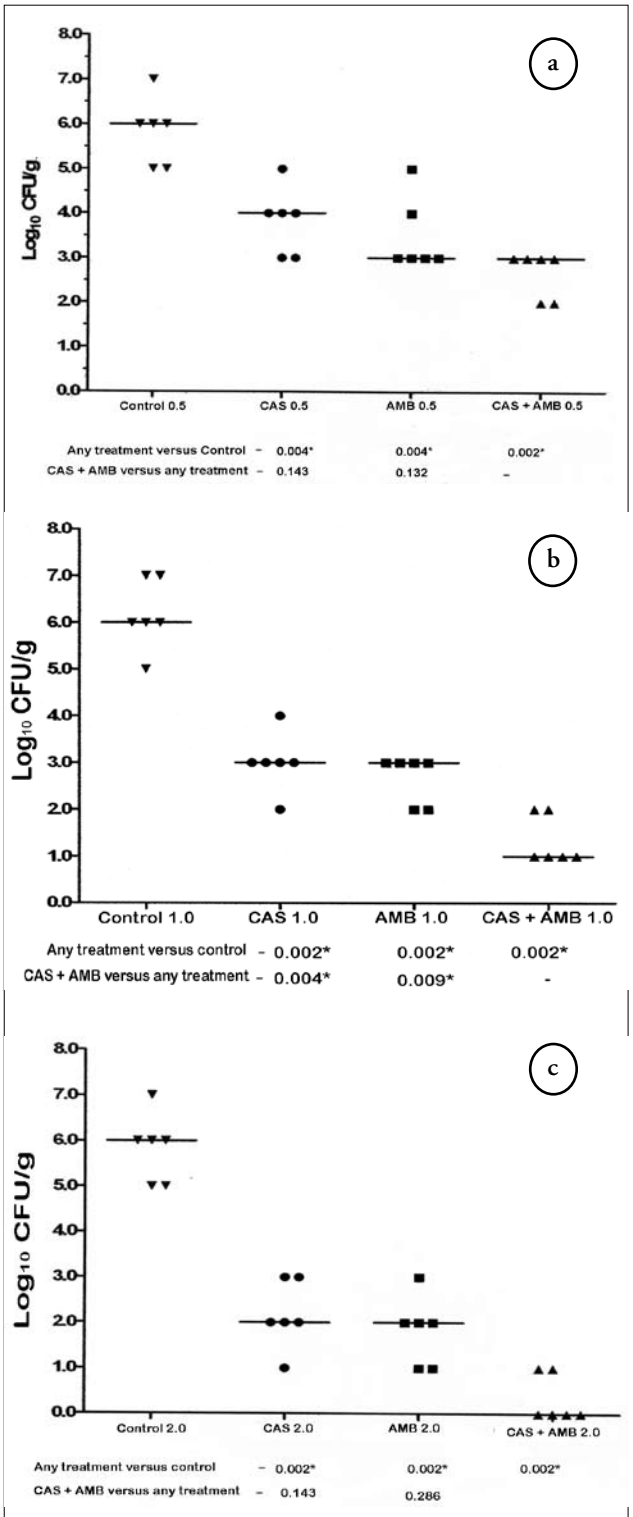

Figure 1 - Effect of antifungal therapy on the tissue fungal burden in mice infected with azole-resistant *Candida albicans*. Results of treatment with Amphotericin B (AmB), caspofungin (CAS), or AmB + CAS at **a)** 0.5, **b)**1, or **c)** 2 mg/kg/day doses and untreated controls were schematized at graphics. Horizontal bars represent the median values. The asterisks at the bottom of graphics indicate *p* values of <0.05.

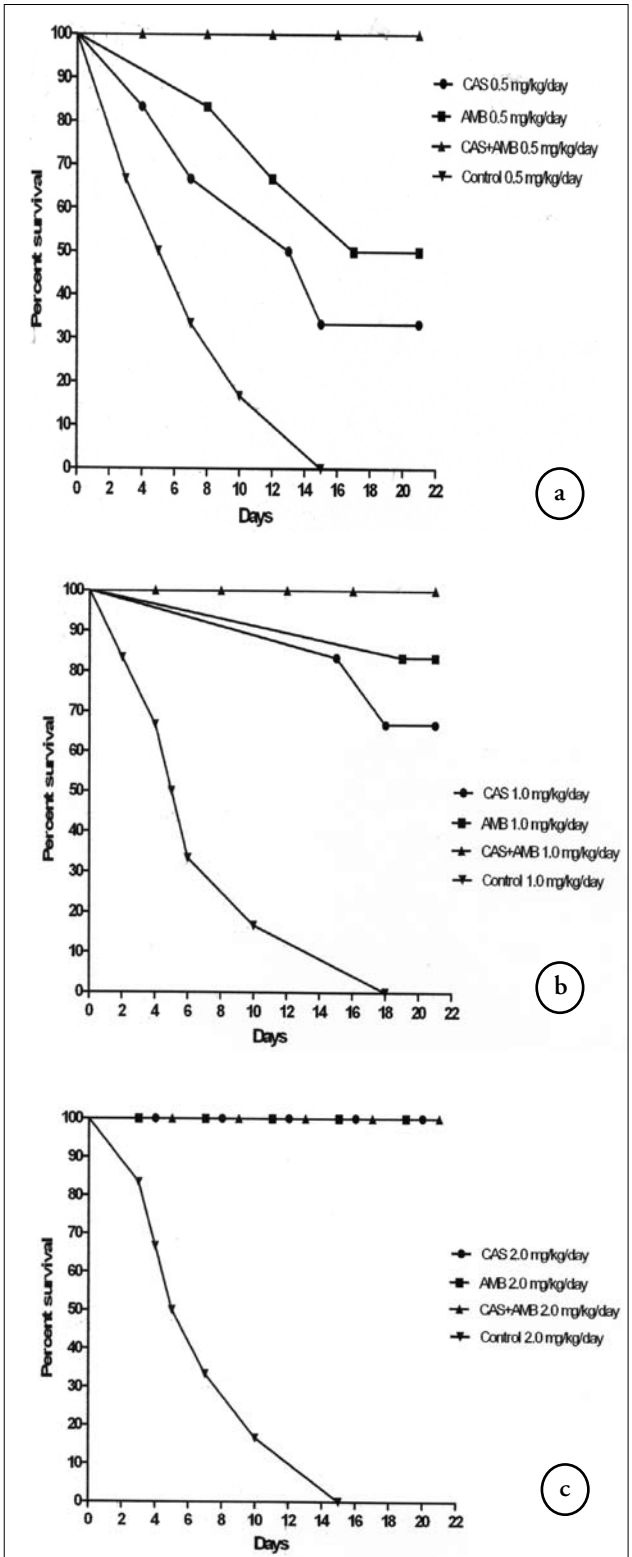

Figure 2 - Plot of cumulative mortality against time in the murine model. Time indicates the number of days the animals survived. The survival curve was prepared using the Kaplan-Meier method based on Log-rank analysis. **a)** Percentage of survival rate with 0.5 mg/kg/day dose, **b)** 1.0 mg/kg/day dose, and **c)** 2.0 mg/kg/day dose. CAS - caspofungin, AmB- amphotericin B.

out the present study was to explore if combined use of these 2 drugs with different modes of action delivered better results, in the management of problematic azole-resistant *C.albicans* infections, than monotherapies with either drug. Furthermore, Johnson et al[21] showed that efficacy, and safety of combination antifungal treatment with low doses in invasive candida infections, were better than monotherapy.

In the present study, the indicators of the experiment's success were the decrease in fungal tissue burden, and the prolongation of survival. Indeed, we found that monotherapy with either drug was effective in reducing the fungal burden, and prolonging the survival compared to untreated control animals (p<0.05). However, statistical comparison of monotherapies revealed no significant difference. Barchiesi et al's[22] study also showed that, similar to AmB, CAS had a potential fungicidal effect in candida infections. In our study, the efficacies of the monotherapies with regard to decrease in fungal tissue burden, and prolongation of survival rate significantly increased with higher dosages of CAS and LAmB (**Figures 1 & 2**). This dose-dependent effect was also emphasized in Barchiesi et al's,[22,23] and Hossain et al's[24] experimental studies.

Numerous studies evaluated the effects of combination therapies with AmB formulations, and echinocandins in different murine models.[22-27] Hossain et al[24] reported that concomitant use of AmB and CAS, significantly prolonged the survival of mice infected with azole resistant *C. albicans* compared to untreated controls. In their study, combination therapy significantly reduced kidney CFU compared with untreated controls, and CAS alone treated groups (p<0.05). In addition, this combination reduced brain CFU significantly, compared with untreated controls and AmB alone treated groups. Their study is the only one evaluating the effect of CAS and AmB combination, in the treatment of invasive candidiasis caused by an azole-resistant *C. albicans*. Although we performed a similar study to Hossain et al,[24] we obtained different results. We suggest that this might be related to differences in the groups, and the forms of AmB in the 2 studies. The sample size was less than our study group, they only had one combination subgroup, and used the more toxic deoxycholate form of AmB. The results of the previous studies indicate that the combination of CAS and AmB therapy has a synergistic, or additive efficacy in vivo.[21-27] There was also no antagonism between the 2 antifungals. In our study, with the combination therapy the tissue fungal burden decreased, and the survival rate prolongation was evident compared to monotherapies, however, a statistically significant decrease in tissue fungal burden was only determined at a 1 mg/kg/day dose. In other dose groups, significance could not be shown between the combination and mono therapies.

The important limitation of the study is the small sample group, and this might be the cause of this insignificance. However, lack of statistical significance between combination treatment and monotherapy at higher doses, may be attributed to the dose dependent efficiency. In other words, monotherapies with high doses of either drug produced similar effect as the combination treatment.

In conclusion, our data obtained in a murine model support the results of previous studies by demonstrating that combined LAmB and CAS treatment, improved the therapeutic outcome in azole-resistant *C. albicans* infection. Additional experimental and clinical studies with a large number of cases are warranted to investigate the superiority of combination therapy.

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