THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Efficacy of higher doses of caspofungin for treatment of invasive candidiasis caused by *Candida albicans* and *Candida tropicalis* in neutropenic murine models.

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Contents

List of Abbreviations

AmB: Amphotericin B

AUC: Area Under the Curve

BMD: Broth Microdilution

CBP: Clinical Break Point

CFU: Colony-forming Unit

CLSI: Clinical and Laboratory Standards Institute

CNS: Central Nervous System

CT scan: Computer Tomography Scan

FDA: Food and Drug Administration

IA: Invasive Aspergillosis

IV: Intravenous

MEC: Minimum Effective Concentration

MIC: Minimum Inhibitory Concentration

PAFE: Post-antifungal Effect

1. Introduction

Candida species are yeasts which are normally present as individual cells and which predominantly replicate asexually by budding or fission [1, 2]. The term *"yeast fungus"* is most commonly applied to yeasts fall under the kingdom of the fungi. *Candida* species are comprised of around 200 species, although few are important for man [1]. The most important of these are *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *C. krusei*, *C. kefyr*, *C. dubliniensis*, and *C. lusitaniae*. One reason that this list is short is that most *Candida* species are incapable of growing at 37 ºC [1, 3].

Candida species are the leading cause of invasive fungal infections in humans, producing infections that range from non–life-threatening mucocutaneous disorders to invasive disease that can involve any organ. Invasive candidiasis is largely a disorder of medical progress, reflecting the great advances in health care technology over the past decades [4].

Invasive candidiasis has a significant impact on patient outcomes, based on findings of Gudlaugsson et al. [5] patients who develop candidemia are still very likely to die during hospitalization [5]. In a study with a group of patients, the attributable mortality rate has been reported ranging from 5% to 71%, and crude mortality rates have been reported to be as high as 81% [6].

Nowadays, the population at risk for fungal infections has increased because of changing demographic patterns, in particular an aging population with a higher incidence of chronic illness and debilitation, and expanding population of immunosuppressed patients [7, 8]. Normally, the immune system can efficiently control the colonization of *Candida* species by both specific and non-specific immune system (intestinal flora, peristalsis, intestinal enzymes, defensins, and others) [9]. *Candida* yeasts are classified as opportunistic pathogens, meaning that they are pathogens only under certain conditions [1].

One of the major causes for the continuing high mortality rates despite the availability of active antifungal agents is the inability to recognize and diagnose early invasive fungal infection, resulting in inappropriate or delayed initiation of therapy, with increased costs of care and excess length of hospitalization [10]. During recent years numerous studies and efforts have been directed toward a better understanding of the pathogenesis of invasive

candidiasis and might aid the development of new treatment strategies that could reduce the high mortality associated with nosocomial candidiasis [9, 11].

Despite recent advances in antifungal pharmacotherapy, the morbidity and mortality caused by invasive fungal infections remains unacceptably high [10]. This thesis will describe therapeutic implementation of caspofungin doses in two different species of *Candida* in two different studies.

2. Literature review

2.1. *Candida species*

Among clinically important *Candida* species, *C. albicans* is the leading agent and following *non-albicans* most frequent species distribution are varied in different geographical area and it is distributed through all ages [12]. *C. tropicalis* is one of the more common *Candida* causing human disease in tropical areas [13]. *C. tropicalis* is taxonomically close to *C. albicans* and it shares many pathogenic characteristics with *C. albicans* [13].

The epidemiology of species responsible for invasive candidiasis, both worldwide and on the local levels, has been changing, a shift to increased prevalence of infections caused by non-albicans *Candida* species, which can be resistant to fluconazole (*C. krusei* and *C. glabrata*) or difficult to eradicate because of biofilm production by *C. parapsilosis* isolates [6]. These findings emphasize the importance of defining the epidemiology of candidemia in every setting.

A review of discharge data on approximately 750 million hospitalizations in the United States has identified 10,319,418 cases of sepsis over the 22-year period. The study found that the annual number of cases caused by fungal organisms in the United States increased by 207% between 1979 and 2000. Among the organisms reported to have caused sepsis in 2000, fungi for 4.6 percent was the fourth most common cause of blood stream infection [14].

The distribution of causative *Candida* species shows considerable geographical variation, particularly in the relative proportion of episodes caused by *C. glabrata* (higher in the United States) or *C. parapsilosis* (higher in some European centers and south America) [15]. Due to this increase in proportion of *non-albicans* species, especially *C. glabrata*, has aroused

concern due to its tendency toward decreased susceptibility to the first-line azole fluconazole [16].

A very dramatic increase in the relative proportion of ICU-associated candidemia episodes caused by *non-albicans Candida* species was observed in the United States through the 1990s. But other recent multicenter studies still suggest that *C. albicans* remains the predominant invasive *Candida* species among ICU patient cohorts, accounting for 40–60% of candidemia episodes [15].

A recent study has highlighted variation in *Candida* species causing bloodstream infection. The following were the most commonly isolated of 1239 *Candida* BSI isolates from 79 medical centers in 2008 to 2009: 50.0%, 17.4%, 17.4%, 9.8%, and 1.8% were *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, respectively [17].

2.2. *Aspergillus species*

Aspergillus species have emerged as being an important initiate of life-threatening infections in immunocompromised and critically ill patients [18, 19]. Given the public health importance of invasive aspergillosis, emphasis is placed on invasive pulmonary aspergillosis, disseminated aspergillosis, sinus aspergillosis, and several types of single-organ invasive aspergillosis [18]. The usual route of transmission of the etiologic agents of this infection is thought to be inhalation of airborne spores [20]. Clinical diagnosis of invasive aspergillosis is based on pulmonary CT scan findings and non-culture tests such as galactomannan or DNA detection in blood or bronchoalveolar lavage samples [20, 21].

In recent years novel antifungal agents have been released, significantly increasing options for the treatment of most serious fungal infections [22]. The most recent approved antifungal drugs include those in the echinocandin class (caspofungin, micafungin, and anidulafungin), as well as the newer generation triazoles like voriconazole and posaconazole [22]. Due to the release of new antifungal agents with improved efficacy such as voriconazole or caspofungin, mortality rates have declined among patients with IA during the past decade, but still more than 50% of cases are fatal [21].

Among all records were reviewed for the presence of *Aspergillus* isolates, *A. fumigatus* is by far the most common species (60-70%), followed by *A. flavus*, *A. niger* and *A. terreus* (4–15% each), whereas *A. nidulans*, *A. ustus* and other rare *Aspergillus* species typically each represent <2% of isolates [23, 24].

2.3. Antifungal agents

Systemic antifungal drugs have shown to be effective for the treatment of candidiasis consist of 4 major categories: the polyenes (AmB deoxycholate, liposomal AmB, AmB-lipid-complex, and AmB colloidal dispersion), the triazoles (fluconazole, itraconazole, voriconazole, and posaconazole), the echinocandins (caspofungin, anidulafungin, and micafungin), and flucytosine. To attain the maximum effect from these agents, clinicians should become familiar with strategies to optimize efficacy through an understanding of relevant pharmacokinetic properties [4, 25].

Table 1. Characteristics of selected systemic antifungal agents [25-38]

Parameters		Azoles			Echinocandins		
	AmB-D	FLU	VOR	POS	CAS	MICA	ANI
Available formulation	IV	IV/PO	IV/PO	PO	IV	IV	IV
Bioavailability%	100	95	96	Not available	Not available	Not available	Not available
Effect of food	Not available	No Effect	Decreases	Increases-optimal with high fat-meal	Not available	Not available	Not available
Protein binding%	>95	10	58	99	97	84	99
Volume of distribution (L/kg)	3-5	40	322	1774	Not available	0.24	0.7-0.9
Elimination half-life	15 days	31h	6h	25h	9-11h	11-17h	24-26h
Elimination	<5% in urine and bile	Urine	Renal	Feces	35% in feces, 41% in urine	40% in feces, <15% in urine	Primarily in feces
Metabolism	Renal	Minor hepatic	Hepatic	Hepatic	Via chemical degradation to inactive metabolites	Via peptide hydrolysis and N-acetylation	Via arylsulfatase and catechol-O-methyltransferase

AmB-D, Amphotericin B deoxycholate; FLU, fluconazole; VOR, voriconazole; POS, posaconazole; CAS, caspofungin; MICA, micafungin; ANI, anidulafungin. IV, intravenous; PO, per os.

2.3.1. Amphotericin B

Amphotericin B has been discovered in the late 1950s, and it was approved for human use as an antimycotic in 1960. For decades, amphotericin B has been the sole drug available for the prevention and treatment of invasive fungal infections, which is known to cause significant nephrotoxicity [39, 40]. AmB is the drug of choice in treating complicated *Candida* infections including infective endocarditis, central nervous system candidiasis and other cases of refractory candidiasis [41].

Amphotericin B is too toxic to be used as an ideal therapeutic agent [25]. In fact, its great affinity for cholesterol in the mammalian cell membrane likely plays a role in its toxicity [25]. Administration of AmB in association with deoxycholate is complicated by immediate

reactions such as fever and chills in 40–70% of cases, and renal insufficiency may develop in 30-50% of cases. The latter can occur during the first days of therapy and may become irreversible after a cumulative dose beyond 4 or 5 g of amphotericin B [42].

Recently three lipid-based preparations of amphotericin B have been developed. Despite their significant cost, many institutions preferentially utilize the lipid formulations owing to reduced adverse reactions. Lipid-based formulations have significantly improved AmB tolerability while maintaining the efficacy of AmB deoxycholate [43]. But unfortunately, lipid associated AmB formulations have no influence on AmB resistant clinical isolates [44].

Amphotericin B is active against most of the common molds, yeasts, and dimorphic fungi causing human infection. In a few instances some organisms are naturally resistant to amphotericin B [22]. *C. lusitaniae*, *C. guilliermondii*, *Scedosporium apiospermum*, *Scopulariopsis* species, and *Fusarium* species generally are considered intrinsically resistant to amphotericin B [45, 46]. The E-test represents one of the more reliable ways to detect resistant isolates and it has been proved to be useful for determination of amphotericin B MICs [45].

2.3.2. Azoles

The first systemic azole antifungal agent, ketoconazole, was introduced in 1979 [25]. Azole agents exert their antifungal activity by blocking the demethylation of lanosterol, thereby inhibiting the biosynthesis of ergosterol [25]. Ketoconazole was followed chronologically by fluconazole, itraconazole, and voriconazole [25]. The azole antifungal agents in clinical use of fungal treatment contain either two or three nitrogens in the azole ring and are thereby classified as imidazoles (e.g., ketoconazole and miconazole, clotrimazole) or triazoles (e.g., fluconazole, voriconazole, and posaconazole) [47]. Each of these agents offers a specific antifungal spectrum [25].

Additionally, one main difference associated with the triazoles is the volume of protein binding with lower values for fluconazole, intermediate values for voriconazole, and high binding with all the remaining compounds [48].

2.3.2.1. Fluconazole

Fluconazole is water soluble and is available in both oral and intravenous forms. Renal excretion is the major pathway for fluconazole elimination and approximately 80% of fluconazole is recovered in urine in unchanged form. Only 11% of the dose is excreted as metabolites [49, 50].

Fluconazole is unique among current agents in that food has no effect on its absorption [25, 47]. An increased population with weakened or impaired immune system demonstrates the increasing importance of *non-albicans Candida* species, particularly *C. tropicalis* and *C. glabrata* concerning pathogenic potential, ability to cause systemic life-threatening infections, acquired fluconazole resistance and resulting mortality in such patients [51].

Fluconazole possesses the narrowest *in vitro* spectrum in that it exhibits relatively poor activity against the filamentous organisms or common *molds* with only moderate activity against dimorphic fungi [25]. Many strains of *C. glabrata* and *C. krusei* are also resistant *in vitro* to fluconazole in clinically achievable concentrations. The most remarkable example of intrinsic resistance involving azoles is the universal resistance to fluconazole among isolates of *C. krusei* [25].

2.3.2.2. Voriconazole

Voriconazole is a second-generation triazole, and it was developed via systematic chemical manipulation of fluconazole to produce a compound with enhanced potency and spectrum of activity [52]. Voriconazole was approved by the FDA for clinical use in 2002 for the treatment of invasive aspergillosis and refractory infections due to *S. apiospermum* and *Fusarium* species [52].

Voriconazole is more active than fluconazole and itraconazole against *Candida* species [53]. The activity of voriconazole against filamentous fungi, particularly *Aspergillus*, was found to be superior to that of amphotericin B [54]. In addition, the vast majority of fluconazole-resistant *C. krusei* strains remain susceptible to voriconazole [55].

Voriconazole has been approved in both oral (tablet and powder) and intravenous (IV) formulations. The bioavailability of voriconazole is >90% in healthy volunteers and is optimal in the fasted state, approximately 1 h before or after a meal [56]. Voriconazole absorption is not influenced by gastric pH [52, 57]. Voriconazole is 58% protein bound in the serum with a volume of distribution of 4.6 L/kg [56]. Tissue distribution of voriconazole is extensive, while metabolism takes place via the hepatic cytochrome P-450 enzymes and eliminates in urine or bile as inactive metabolite [58].

Studies from patients receiving voriconazole for treatment of invasive aspergillosis have reported favorable results associated with trough concentrations greater than 1–2 mg/mL [59]. Uncommon cases of severe, and sometimes lethal, hepatic failure and encephalopathy linked to voriconazole indicate practitioners must be more vigilant in monitoring hepatic function and neurological adverse effects such as encephalopathy in patients receiving this agent [60].

As a consequence, voriconazole concentrations in serum can vary around 100-fold from one patient to another or within a particular affected person based on age, concurrent illness, drug dose, drug-drug interactions, and underlying liver function [61]. Lack of extra sources and rapid and complete neurological recovery just after therapy discontinuation highly suggest the potential connection between these encephalopathies and voriconazole overdosing [59]. The logistic regression analysis proved a significant link between voriconazole trough concentrations and neurotoxicity [59].

2.3.2.3. Posaconazole

Posaconazole (Noxafil®; Schering-Plough), a hydroxylated analogue of itraconazole, has been developed by the Schering-Plough Research Institute and approved by FDA (2006) [62-64] for use in adolescents aged ≥13 years who are at risk for invasive fungal infection due to severe immunocompromise [62, 63].

Posaconazole is already available in the market solely for an oral suspension formulated with polysorbate 80 as an emulsifying agent. The suspension is cherry-flavored and possesses 40 mg of posaconazole in each milliliter [65]. Protein binding for posaconazole is very similar to itraconazole (>98%) [66].

Posaconazole shows a high activity against a large number of *filamentous* fungi. It is also active against *Zygomycetes*, which are refractory to some other azoles [67]. Posaconazole exhibits *in vitro* activity against *Candida* species and *Cryptococcus neoformans* [67]. Due to lack of intravenous formulation of posaconazole, it has a limited role against invasive candidiasis [68].

The recommended dose of posaconazole is 800 mg per day to treat fungal infection and 600 mg daily basis for prophylaxis of fungal infection and it is required to be divided into 2 to 4 daily doses [64, 69]. Because of the saturable absorption, loading doses are commonly not recommended for this antifungal [70]. Unlike azoles, the use of food, particularly a high-fat meal, greatly increases absorption of posaconazole [66, 71]. However, absorption of posaconazole is decreased by co-administration of drugs that increase the gastric pH [71].

Posaconazole has been discussed to be superior to fluconazole, itraconazole, and amphotericin B against most frequent fungal pathogens at *in vitro* and animal studies [72, 73]. It is licensed for prophylaxis of invasive fungal infections (candidiasis and aspergillosis) in immunocompromised patients as well as the management of oropharyngeal candidiasis [74].

All the triazole class members demonstrates *in vitro* activity against *C. neoformans* [56]. A small number of *C. glabrata* isolates that exhibit resistance to fluconazole are still susceptible to second generation triazoles [56]. Voriconazole and posaconazole, the newest triazoles, are also active *in vitro* against dimorphic fungi [75, 76].

When it comes to stem cell transplant populations, antifungal prophylaxis, particularly using fluconazole [77] and posaconazole [65], can show good results by reducing systemic fungal infections.

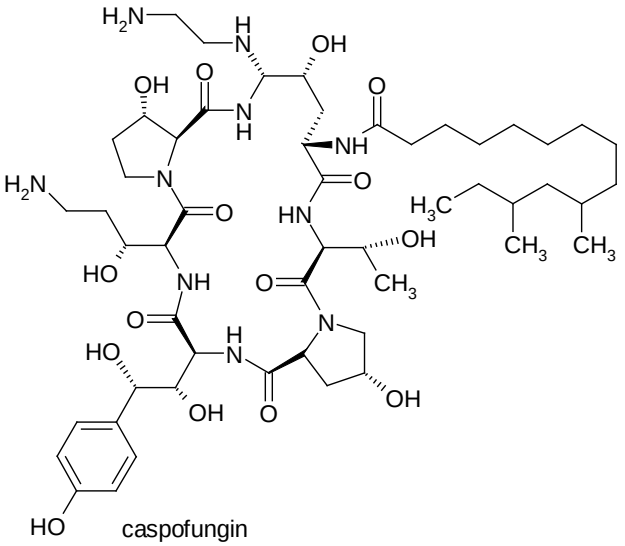
2.3.3. Echinocandins

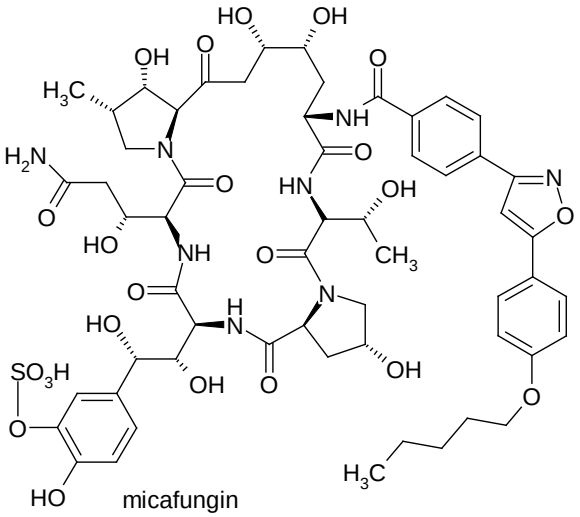
After the discovery of the penicillin which specifically inhibits bacterial cell wall synthesis, achieving similar drug to target the fungal cell wall has become the center of focus of the antifungal drug discovery [78, 79]. Given that the cell wall is uniquely vital for the fungal cell and considering that its components are lacking in the mammalian host, the fungal cell wall provides an excellent target for antifungal agents [80].

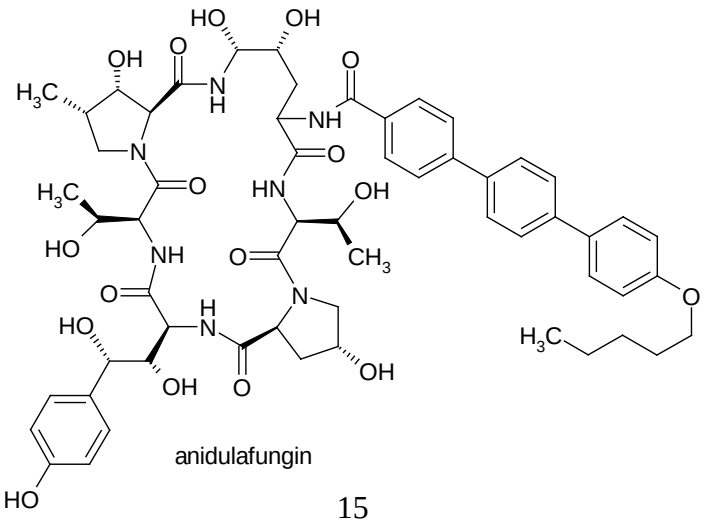
Due to substantial difference among various species, the most important elements of the cell wall of the majority of fungi consist of alpha- or beta linked glucans, chitin, along with a number of mannoproteins [78]. The characteristics of the fungal cell wall are directly coordinated with cell growth and cell division, and therefore the main purpose of the cell wall is towards the control of internal turgor of the cell [79]. Dysfunction of the cell wall composition results in osmotic instability and can eventually cause the lysis of the fungal cell [81].

The second generation semisynthetic echinocandins with extended spectrum against *Candida* and *Aspergillus* species have been introduced during the last decade [82]. Echinocandins are preferred because of their excellent safety profile, efficacy and preferred pharmacokinetic characteristics in clinical studies [83]. These echinocandins include caspofungin (Cancidas™), micafungin (Mycamine™), and anidulafungin (Ecalta™ and Eraxis™) [83]. With specific exceptions, existing records show that these three compounds are not basically different in connection with pharmacodynamics, pharmacokinetics, spectrum, safety, and antifungal efficacy [84] figure. 1.

Fig. 1. Chemical structure of caspofungin, micafungin, and anidulafungin (courtesy of G. Kardos)







All three compounds possess poor oral bioavailability and are for intravenous use only [85]. The potential disadvantages of this new class of antifungal agent include their higher cost than the other FDA approved antifungals, lack of oral formulations, and lack of *in vitro* activity against emerging pathogens like *Fusarium, Scedosporium*,, and *Zygomycetes* [37].

2.3.3.1. Mode of action

The echinocandins generally exhibit concentration dependent activity, non-competitive inhibitors of 1,3-β- and 1,6-β-D-glucan synthase [37], and is encoded by two homologous FKS1 and FKS2 genes [37].

Glucan is a major component of the fungal cell walls, and is consisting of 30 to 60 percent in *Candida* and *Saccharomyces* species cell walls [86]. Changes in the components of the cell wall can cause osmotic fluctuations and cell lysis and death [86]. Due to the lack of 1,3-β-D-glucan in human cells, the echinocandins avoid direct human cell toxicity [80].

The glucan proportion of the fungal cell wall differs greatly among various species of fungi [80]. The target site, 1,3-beta-D-glucan is much more predominant in the cell walls of *Candida* and *Aspergillus* species (particularly *C. albicans* and *A. fumigatus*) in comparison to yeast forms of dimorphic fungi [87]. In addition, the cell walls of mycelial forms *of Paracoccidioides braziliensis, Histoplasma capsulatum*, and *Blastomyces dermatitidis* consist of considerable amount of 1,3-beta-D-glucan, whereas *Zygomycetes* do not have such specific component [88]. But, these factors do not necessarily predict echinocandins activity [37]. As an example, the cell wall of *C. neoformans* possesses 1,3-beta-D-glucan while, the echinocandins show little activity against this pathogen [87]. This suggests that there are probably additional or alternate aspects of the mechanism of action for the echinocandins [37].

Early studies confirmed that echinocandins show a species dependent mechanism of action [89]. *In vitro* studies support this conclusion and reveal echinocandins fungicidal activity against a large number of *Candida* species and fungistatic activity against *Aspergillus* species [90].

2.3.3.2. Spectrum of activity

The three echinocandins have potent *in vitro* activity against all clinically important isolates of *Candida* species [91]. The three *Candida* including *C. parapsilosis, C. guilliermondii* and *C. lusitaniae* in comparison with remaining *Candida* species exhibit higher MICs for all echinocandins [92].

Echinocandins have shown activity against *Saccharomyces cerevisiae* but almost no *in vitro* activity against *Trichosporon asahii* and *C. neoformans* [92]. The echinocandins also have potent and broad spectrum activity against *Aspergillus* species [85]. They possess variable activity against dematiaceous and endemic molds [93], and are considered inactive *in vitro* against most *Hyalohyphomycetes* as well as the *Zygomycetes* [94].

Echinocandins efficacy against invasive infections due to *Candida* and *Aspergillus* species has been confirmed in several experimental models in immunocompetent and immunocompromised animals [79]. Interestingly, all the echinocandins were preventive and had therapeutic activity against *pneumonia* caused by *Pneumocystis jirovecii* in animal models [95].

Generally, the echinocandins have MIC_{90} values of $\leq$2 mg/L against *Candida* species [96]. A concise review of the existing data has analyzed the MIC values of the echinocandins, and demonstrated that normally, anidulafungin displays the lowest MIC values against the vast majority of *Candida* species, follows by micafungin and caspofungin [87].

But, all these results should be confirmed with clinical data due to the absence of relationship of MICs with treatment success [37]. Additionally, the presence of human serum reduces the *in vitro* effectiveness of all the echinocandins and reduces the effects of the *in vitro* MIC superiority of micafungin over caspofungin [37].

2.3.3.3. Antifungal susceptibility testing of echinocandins

2.3.3.3.1. *Candida species*

In order to monitor changing trends in the antifungal susceptibility patterns of invasive *Candida* isolates, the Clinical and Laboratory Standards Institute (CLSI) has developed key standardized methods for *in vitro* susceptibility testing of these pathogens [97].

The CLSI recently suggested that *Candida* species having MICs ≤2 mg/L are regarded as being susceptible to echinocandins [98]. The guidelines also recommend performing the MIC determination for echinocandins after 24 hours of incubation [99]. The method employs RPMI-1640 broth medium, incubation at 35°C for 24 h, and a MIC endpoint criterion of prominent decrease in growth (50% inhibition compared to control growth) [100].

In many studies, the MIC_{90} for *C. parapsilosis* for any of the echinocandins has been 2 mg/L, which is at the breakpoint of susceptibility [99]. The clinical relevance of this finding among patients with invasive *Candida* species infections is not known. A naturally occurring proline-to-alanine replacement in the region of FKS1p may possibly describe the higher MICs experienced with *C. parapsilosis* and echinocandins [101].

2.3.3.3.2. *Aspergillus species*

The CLSI has also developed a reproducible broth microdilution method (document M38-A2) for the antifungal susceptibility testing of filamentous fungi (molds) to a number of antifungal agents, including the echinocandins [102].

Because of the fungistatic activity of echinocandins against mold infections, it is challenging to determine a precise MIC at which point mold is inhibited; thereby the minimum effective concentration (MEC) rather than the MIC is chosen to verify the activity of echinocandins against molds [103]. Echinocandins significantly inhibit growth of *A. fumigatus* and several other filamentous fungi *in vitro*. Cultures from treated hyphae with echinocandins show an aberrant morphology, with swollen, highly branched germ tubes and evidence of lysis at the growing tips under the microscope. [104, 105].

The CLSI standard recommends the use of RPMI-1640 medium and an inoculum of 0.4×10^4 to 5×10^4 CFU/ml [106]. Microdilution trays are incubated at 35°C. The MEC can be determined, after 48 h of incubation [106].

2.3.3.4. Caspofungin

Caspofungin (Cancidas) was the first member of the echinocandins family, approved in 2001 by the FDA for the treatment of invasive fungal infections in adults and in 2008 for use in children ≥3 months of age [87].

Caspofungin (caspofungin acetate, MK-0991; L-743872) is a semi-synthetic, water soluble lipopeptide antifungal produced from a fermentation product from the fungus *Glarea lozoyensis* [107]. It belongs to the echinocandin family, and is a derivative of the natural product pneumocandin B_0 [107].

After single intravenous administration of 5 to 100 mg caspofungin dosages to healthy subjects, linear pharmacokinetics with a beta half-life of 9 to10 h and an average plasma clearance of 10 to 12 mL/min was demonstrated over the dose range [108]. At higher dosages, an additional, prolonged gamma half-life from 40 to 50 h was evident. In plasma, caspofungin is bound to proteins (97.5 %) [108].

Multiple dose studies at doses of 15, 35, and 70 mg daily for 2 and 3 weeks revealed dose-related accumulation of drug in plasma as high as 50% [108]. A loading dose of 70 mg, followed by 50 mg daily, maintained plasma concentrations higher than 1 mg/mL from day 1 in advance; this is above the documented MIC values for the majority of susceptible fungi [108].

Data from higher dosages studies of caspofungin displayed constant pharmacokinetics following by single doses of 150 and 210 mg and keeping with twenty-one days of 100 mg; peak concentrations were 29.4 and 33.5 mg/L, respectively [109].

Tissue distribution experiments in murine models revealed preferential exposure of liver, kidney, and large intestine, but exposure for lung, spleen and small intestine was comparable to that relating to plasma. Organs having a lower intensity of exposure consisted of the heart, brain, and thigh [110]. Excretion of caspofungin in humans happens to be slow, with 41% and 35% of the dosed radioactivity being recovered in urine and feces, respectively, during twenty-seven days [110]. Caspofungin is slowly metabolized through peptide hydrolysis and N-acetylation [110]; just a small percentage of caspofungin (approximately 1.4% of dose) is excreted unchanged in urine [111].

Dosage adjustment is not essential for consumers with chronic kidney disease and end-stage renal dysfunction. While patients with mild hepatic impairment do not need a dosage adjustment, a dosage of 35 mg daily and the starting loading dose of 70 mg is required for patients with moderate hepatic impairment as a result of an average increase of 76% in caspofungin AUC [112]. There is no clinical or pharmacokinetic data about patients with severe hepatic insufficiency [113]. No dosage adjustment based on age, weight, serum albumin concentration, gender or on the basis of race is required [114].

2.3.3.5. Micafungin

The clinical efficacy of micafungin has been assessed for the treatment of *Candida* and *Aspergillus* infections [115]. In the United States, the FDA has approved micafungin to be used for the management of esophageal candidiasis as well as for the prevention of candidiasis during the pre-engraftment duration in hematologic stem cell transplant recipients [115].

Micafungin stands out as the second licensed antifungal agent in the echinocandin list and is currently used worldwide for the treatment of life-threatening fungal infections [116]. It is a water-soluble and is semi-synthesized compound derived from the acylated cyclic hexapeptide FR901379, and a natural product of this fungus *Coleophoma empetri* F-11899, via a selective enzymatic deacylation of FR901379, followed by chemical reacylation with the optimized N-acyl side chain [116].

Micafungin demonstrates linear plasma pharmacokinetics with doses between 12.5 to 200 mg [117]. Micafungin displays minimal systemic accumulation following repeated high concentration dosing [118], and steady phase is normally achieved in approximately 4 to 5 days [118, 119]. During single dose of 100-mg of micafungin the mean peak plasma volume reached 8.8 mg/mL, the $AUC_{0-\infty}$ 125.9 mg.h/mL, and total metabolic clearance was estimated to be 9.8 mL/h/kg, with a half-life of 14.6 h [120].

Micafungin extensively binds (>99%) to proteins in plasma, mainly to albumin and it widely distributes into tissues [121]. The highest tissue concentrations were measured in the lungs, liver, spleen, and kidney in animals. Micafungin was undetectable in cerebrospinal fluid, but the drug concentration in brain tissue exceeded MIC_{90} values in a dose dependent manner.

Micafungin is metabolized through the liver [121, 122]. But renal insufficiency or hemodialysis has no related effect on the pharmacokinetics of micafungin [121].

The pharmacokinetics of micafungin in febrile neutropenic pediatric patients between 2 and 17 years of age dosed ranging from 0.5 to 4 mg/kg, were linear and generally comparable to those found in adults [117]. However, in 2 to 8 years old patients, clearance was about 1.35 times that relating to patients ≥9 years of age [117]. Existing pharmacokinetic data in premature neonates show a significantly higher clearance level in comparison with other pediatric age categories and adults and the prospective need to receive higher doses in these infants [123-125].

2.3.3.6. Anidulafungin

Anidulafungin (Eraxis; Pfizer) is the most recently released echinocandin antifungal which licensed by the US Food and Drug Administration [126] for the management of candidemia, esophageal candidiasis, and deep-tissue candidiasis [127].

Anidulafungin is a semi-synthetic lipopeptide compound synthesized from fermentation products of *A. nidulans* [127]. The product is insoluble in water and soluble to some degree in ethanol [128].

In healthy subjects, upon intravenous dosages of 35 to 100 mg, anidulafungin exhibited linear pharmacokinetics having mean peak plasma concentration between 1.71 to 3.82 mg/mL, and mean $AUC_{0-\infty}$ values ranging from 37.46 to 104.81 mg.h/mL [128]. The mean amount of circulation was from 0.72 and 0.90 L/kg, additionally, the half-life for the terminal phase was an estimate of 40 h [128]. Population dependent studies of concentration data received from 225 patients that had life-threatening fungal infections obtained during four phase II/III clinical studies showed no relevant differences in pharmacokinetics in comparison to healthy individuals [126].

Anidulafungin is not metabolized through the liver but steadily degraded chemically to inactive substances. Only negligible renal contribution during the drug's elimination was noticed [122]. In laboratory animals, tissue concentrations at trough right after several dosing were superior in lung and liver, and then spleen and kidney. Measurable concentrations in

brain tissue were observed exclusively at the higher end of the dosage scale. No associated inter-species differences were detected. The pharmacokinetics of anidulafungin are not changed in people with mild, moderate and severe renal dysfunction, nor in end-step renal problems or while having hemodialysis; in addition, hepatic impairment does not result in clinically relevant differences in the pharmacokinetics [128, 129].

In a cohort, sequential dose-escalation study, age-stratified in pediatric patients concentrations and AUC were comparable throughout subjects, and, in comparison with caspofungin and micafungin, weight modified clearance rates were consistent throughout their age. Pharmacokinetic variables following 0.75 or 1.5 mg/kg/day were comparable to those found in adult patients given 50 or 100 mg/day, respectively [130].

2.4. Paradoxical growth

Paradoxical growth is a phenomenon initially described in vitro, though some reports suggest that it may also be detected *in vivo* [131]. Paradoxical growth is defined by efficient inhibition or killing by echinocandins at the MIC and supra-MIC concentrations, but lack of activity at concentrations well above the MIC. Paradoxical growth at present is considered echinocandin-specific [87, 132].

Cell wall content analysis detected increased chitin concentrations in strains surviving in very high echinocandin concentrations [133]. This enhanced chitin synthesis is the most probable explanation of paradoxical growth. As the concentrations at which paradoxical growth is usually detected are far above those that occur in patients with the recommended clinical regimen [133], the clinical importance of this phenomenon is still unclear.

The observation of the paradoxical effect of caspofungin *in vitro* at supra-MIC concentrations has been linked to upregulation of FKS1, GSL2, MKC1, and GSC1 gene expression, as a possible cause of phenotypic drug resistance [133].

To date, the paradoxical effect could not be demonstrated reproducibly in animal models [134]. But, there has been no signal in subjects treated with high doses of these three antifungals. The *in vitro* paradoxical effects tend to occur at concentrations greatly above those that are safely achieved in plasma may describe the lack of an *in vivo* correlation [135].

3. Aims of the study

The therapy of invasive fungal infections, though having advanced enormously in the past decade, is still a therapeutic challenge. Only three major group of antifungal agents can be used against the most frequent infections candidiasis and aspergillosis. For these reasons, understanding the ins and outs of antifungal chemotherapy is one of the most important field of research at present in clinical mycology. This is especially true for the newest class of antifungals, echinocandins, which, as a consequence of the novelty, are the presently less understood group of antifungals.

Specific aims of this work are:

1. To test the efficacy of various clinically relevant caspofungin doses (single 6 mg/kg, two times 3 mg/kg and 1 mg/kg) on a mouse model of infection and their therapeutic efficacy on tissue burden in disseminated candidiasis due to *C. albicans* infection. (First study, see section 5.1. Experiments with *Candida albicans*)

2. To find an effective drug concentration of caspofungin for treating an isolate of *C. tropicalis* showing paradoxical growth *in vivo* in an intraperitoneal abscess of model of infection and its potential implication in clinical use for treating the disseminated candidiasis due to infection to this *Candida* species. (Second study, see section 5.2. Experiments with *Candida tropicalis*)

4. Materials and Methods

4.1. Animals

We have used female BALB/c mice weighing from 26 to 28 g and 18 to 20 g in the experiments with *C. albicans* and *C. tropicalis*, respectively. In the lethality experiments mice received ceftazidime (5 mg/day subcutaneously) during the experiments to prevent bacterial superinfection and its potential confounding effect. The experiments were approved by the local Animal Care Committee (permission no. 12/2008).

4.2. Immunosuppression of mice

All BALB/c mice were immunosuppressed intraperitoneally using three doses of 200 mg/kg of cyclophosphamide at four days prior to infection, one day after receiving the infectious organisms and finally four days after the initiation of therapy in the experiment with *C. albicans*. In the second set of studies with *C. tropicalis*, the mice were immunosuppressed intraperitoneally with two doses of 200 mg/kg of cyclophosphamide four days prior to and one day after the infection.

4.3. Models

4.3.1. Intravenous infection with *C. albicans*:

We used three *C. albicans* bloodstream isolates (10920, 4780 and 17471), derived from our previous study, with caspofungin MICs uniformly 0.03 mg/L. Isolate 17471 was resistant to fluconazole (MIC=64 mg/L).

In the lethality experiments the infectious dose was set at 10^5 CFU/mouse (in a 0.2-ml volume) based on preliminary studies.

Tissue burden experiments were performed with isolates 10920 and 17471. To ensure 100% survival in the control groups, we used $4x10^4$ CFU/mouse for the tissue burden determination. Inoculum density was confirmed by plating serial dilutions on Sabouraud agar plates. At this stage, the kidneys were removed in a sterile manner, then homogenized. The homogenates were diluted by 1 ml sterile saline, and serial tenfold dilutions were prepared in saline. Aliquots of 100 µl of these dilutions were plated onto Saboraud

dextrose agar plates. After incubation of the plates for 48 h at 30 ºC the resulting colonies were counted and used to determine the CFUs/kidney pairs.

4.3.2. Intraperitoneal abscess model of study with *C. tropicalis*:

The *C. tropicalis* isolate tested showed a caspofungin MIC of 0.024 mg/L; 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 hours (paradoxical growth).

We used the intraperitoneal abscess model described by Ninomiya et al. in 2005 [136]. Autoclaved caecal content from mice were mixed with equal amount of fungal suspension (0.25-0.25 ml) and inoculated into mice intraperitoneally (final inoculum 10^7 CFU/mouse). Inoculum density was confirmed by plating serial dilutions on Sabouraud agar plates (see above).

4.4. Treatment

To assess the therapeutic efficacy of caspofungin against the *Candida* species, the commercial preparation of caspofungin (Cancidas) was used. Caspofungin was dissolved in sterile saline for the *in vivo* experiments.

4.4.1. Intravenous infection with *C. albicans*:

Mice were assigned randomly into the study groups (ten mice/group) as follows; no treatment, 1 mg/kg daily dose for 6 days, 3 mg/kg two times and a single dose of 6 mg/kg of caspofungin (Cancidas, commercial preparation). Intraperitoneal treatment (0.5 ml of caspofungin) was started 10 hours postinfection. Mice were followed up for six days to observe the effect of treatment on early lethality.

Survival rate was analyzed by Kaplan-Meier test; the effect of different caspofungin doses was compared using logrank test.

In tissue burden experiments, treatment groups were assigned as in the lethality experiment, one treatment group included 45-60 mice. Kinetics of drug efficacy was monitored by determining CFU average per kidney pair in seven to ten mice on each study

day for six days postinfection. Two mice from each group were sacrificed and analyzed at the start of therapy to verify the fungal replication initiation in the kidneys.

Drug efficacy was compared by determination of the CFU numbers in every kidney pair among the treatment groups every day using Kruskal-Wallis test (with Dunn's post-testing). P values of <0.05 were regarded as significant. For statistical analysis GraphPad Prism (Windows version 4.03) was used.

4.4.2. Intraperitoneal abscess model of study with *C. tropicalis*:

In the first set of the *in vivo* experiments, we have used seven treatment groups with single doses of caspofungin treatment (0.12, 0.25, 1, 2, 3, 5 and 15 mg/kg) besides their control group. Treatment groups consisted of 5 mice. Caspofungin treatment was started one hour after the inoculation. We have followed the experiment for seven days, and the survival rate was also measured.

In the second set of experiments, we administered caspofungin with daily doses for 5 days, using the same doses 0.12, 0.25, 1, 2, 3, 5 and 15 mg/kg. Treatment groups consisted of 5 to 8 mice. This experiment was performed twice.

All mice were monitored twice daily and those who became immobile and showed signs of severe illness were euthanized and recorded as death on the same day. Those mice who survived till the end of the experiment were sacrificed and the colony numbers of viable fungi were determined from an abdominal lavage sample taken by washing the peritoneal cavity with 1 ml of sterile saline. Additionally, the total number of abdominal abscesses and the fungal colony numbers in each abscess were also determined.

To analyze the relationship between treatment and survival of mice, we have used chi-square test. Colony forming unit of peritoneal lavage and peritoneal abscesses were compared by Kruskal-Wallis test with Dunn's post-testing. Values of $p<0.05$ is considered to be significant. For statistical analysis GraphPad Prism (Windows version 4.03) was used.

5. Results

5.1. Experiments with *C. albicans*

5.1.1. Lethality

BALB/C mice have been approved to have disseminated candidiasis after intravenous infection with all three strains of *C. albicans*. All caspofungin regimens used in this study improved the survival of mice infected with isolates 17471, 10920 and 4780 (Figure 2, 3, and 4; P values were <0.0001, p=0.0014, and p=0.0003, respectively). 50% was the lowest survival rate among the treated groups, found in the 2x3 mg/kg dose group with the 10920 isolate. However, differences between groups in efficacy of caspofungin were not statistically significant (p>0.05).

Figure 2. Survival of mice infected by *C. albicans* isolates 17471. After 6 days, the survival rate was analyzed by Kaplan-Meier test. Values of p<0.05 were considered to be significant. (P value <0.0001)

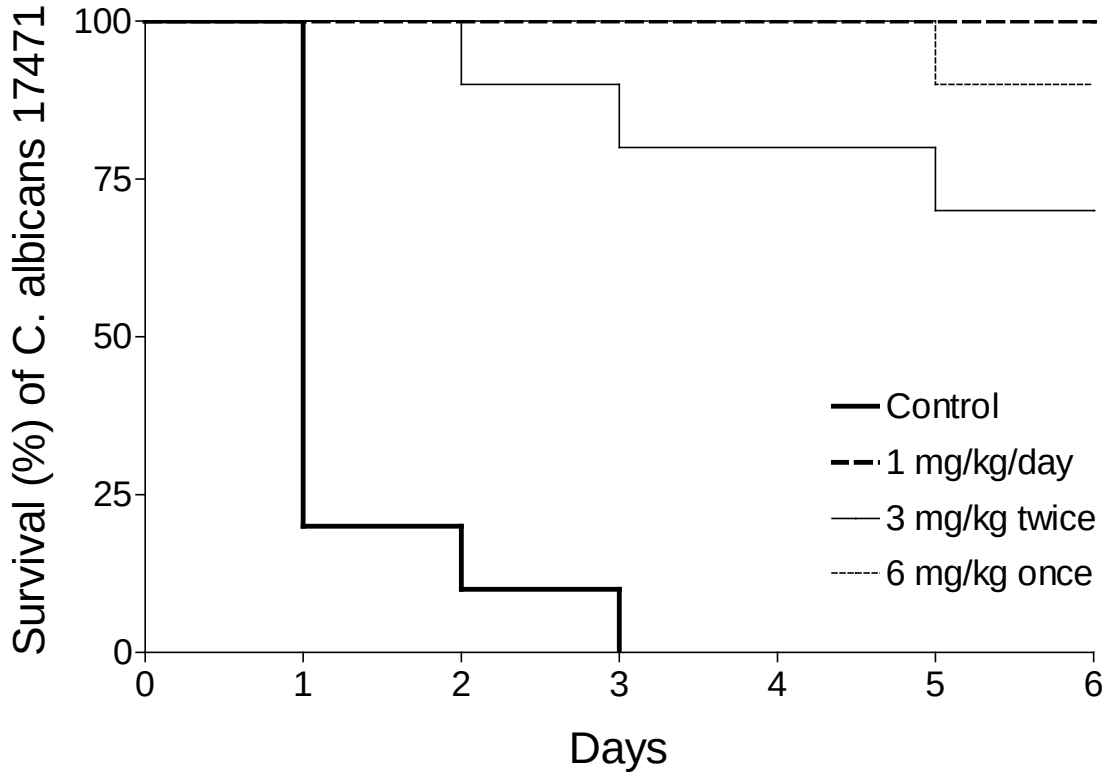


Figure 3. Survival of mice infected by *C. albicans* isolates 10920. After 6 days, the survival rate was analyzed by Kaplan-Meier test. Values of $p<0.05$ were considered to be significant. (P value =0.0014)

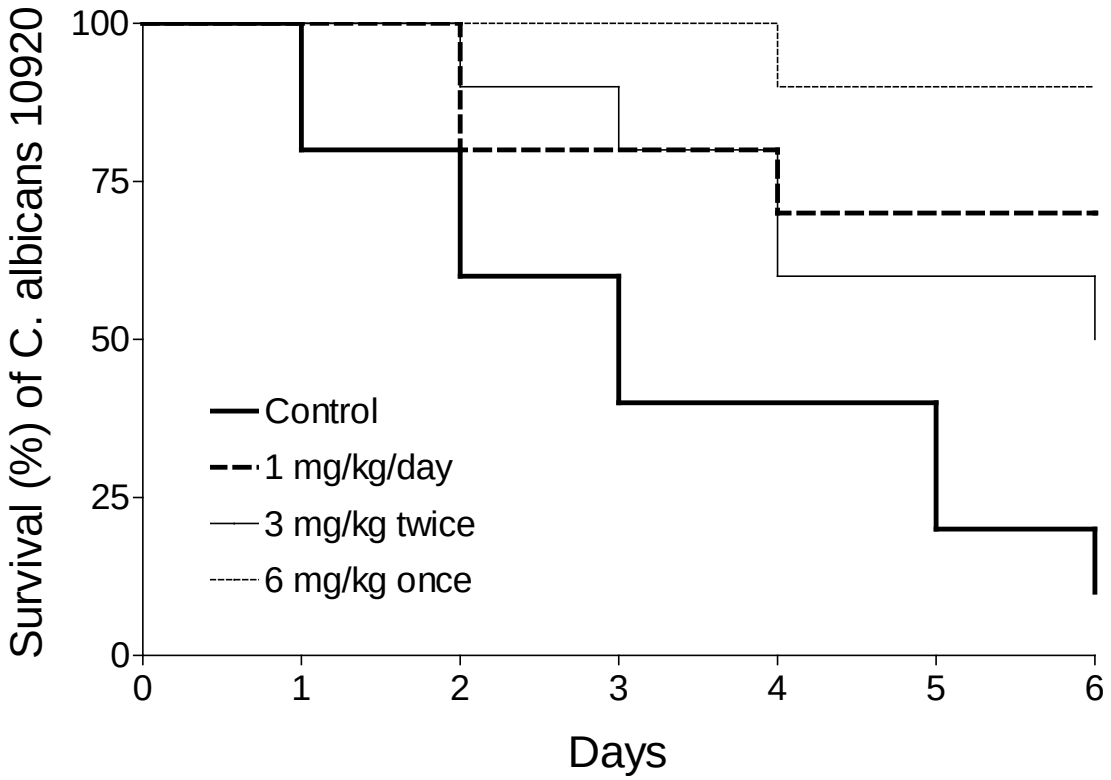
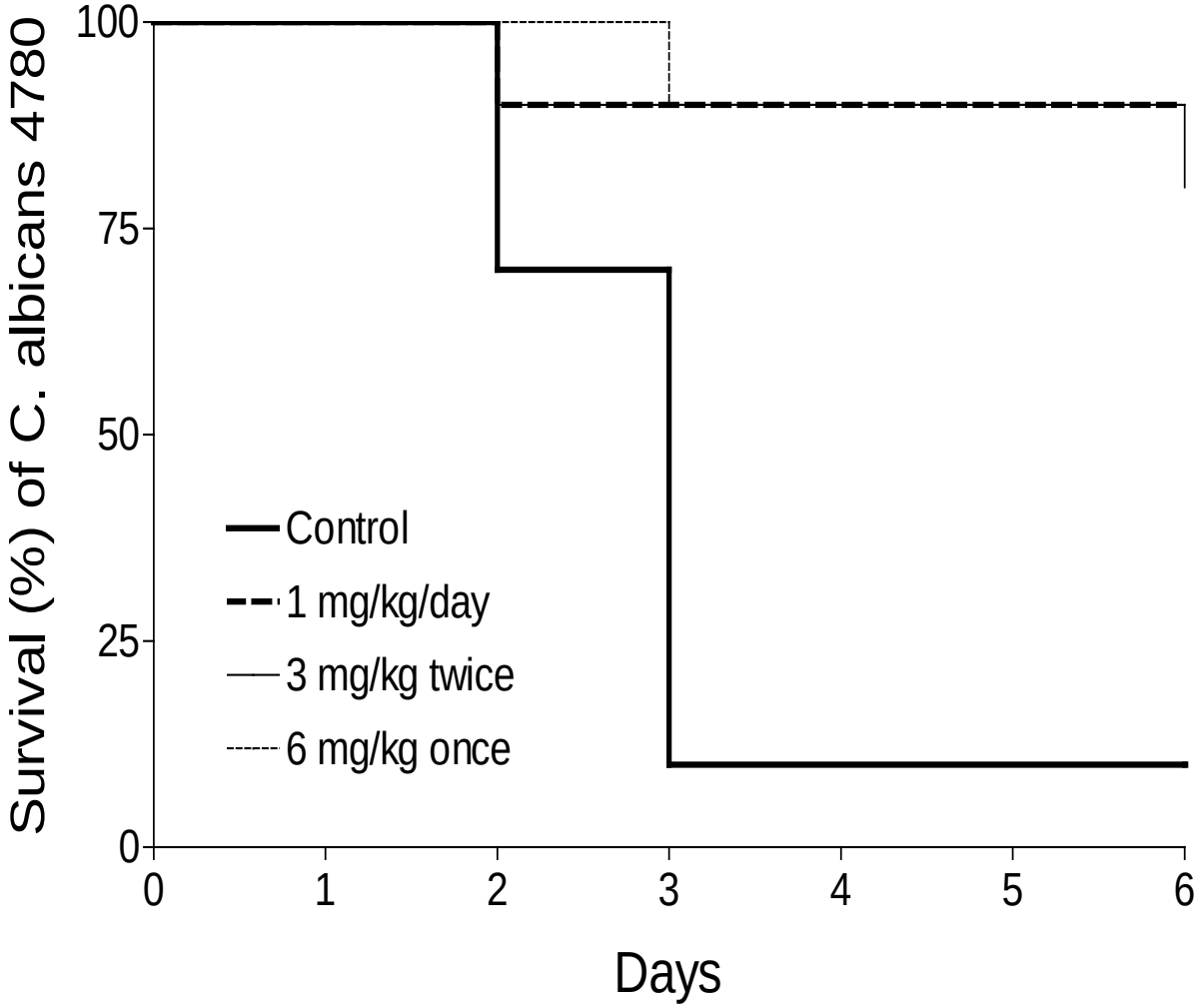


Figure 4. Survival of mice infected by *C. albicans* isolates 4780. After 6 days, the survival rate was analyzed by Kaplan-Meier test. Values of $p<0.05$ were considered to be significant. (P value =0.0003)



5.1.2. Fungal tissue burden

The activity of the three *C. albicans* isolates in the tissue burden experiment were 3.32 ± 2.53 and 3.37 ± 2.46 log10 CFU/kidney at the beginning of therapy for isolates 10920 and 17471 (Fig. 5. and Fig. 6.). All treatment regimens decreased the tissue burden in comparison to the control group. We did not observe any paradoxical growth at the higher doses.

All treatment regimens except the 1 mg/kg on the second day ($p>0.05$) (Fig. 5B) and 2x3 mg/kg on the third day ($p>0.05$) (Fig. 5C), were found to be beneficial in reducing the tissue burden for each day ($p<0.05-<0.001$; Fig. 5) in case of isolate 10920. The single 6 mg/kg dose significantly decreased the fungal tissue burden on each day in comparison to the all days results of the control group ($p<0.05-0.001$). Between different treatment groups there were no staticstically significant differences.

For the *C. albicans* isolate 17471, all doses except 2x3 mg/kg caspofungin on its first day (Fig. 6A) proved to be effective in clearing the tissues ($p<0.05-0.001$). Three mice in the 2x3 mg/kg caspofungin group on day 4-6 showed higher than 1000 CFU/kidney, indicating an incomplete eradication of infection (Fig. 6.D-F). Differences between groups treated with different regimens were not significant statistically.

Figure 5. Kidneys tissue burden of neutropenic BALB/c mice infected intravenously with *C. albicans* isolates 10920. Tissue burden experiments were performed on postinfection days 1(A), 2(B), 3(C), 4(D), 5(E) and 6(F). Median with interquartile ranges is represented for each data set. Level of statistical significance is indicated at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). (No significant differences between the treatment groups.)

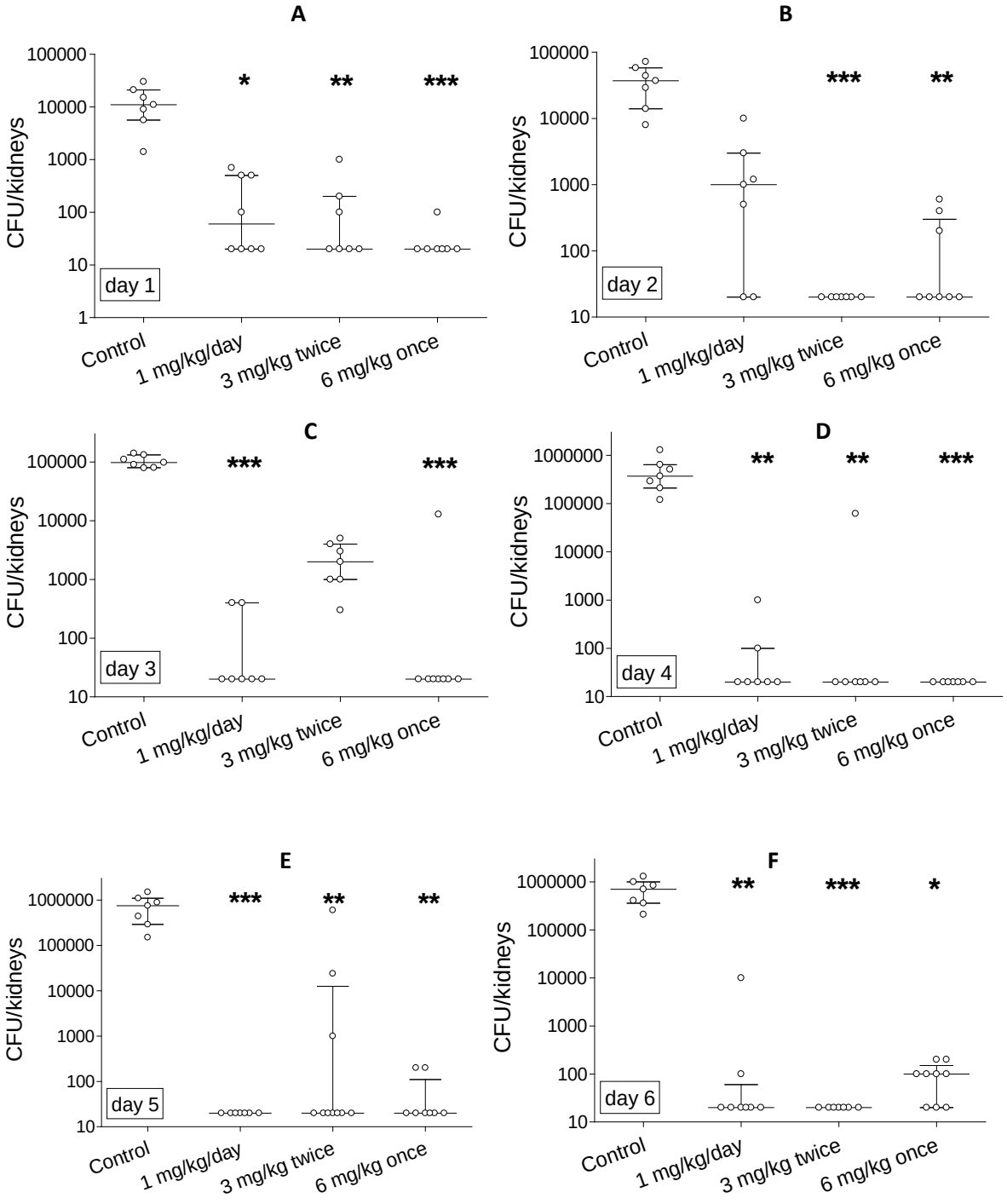
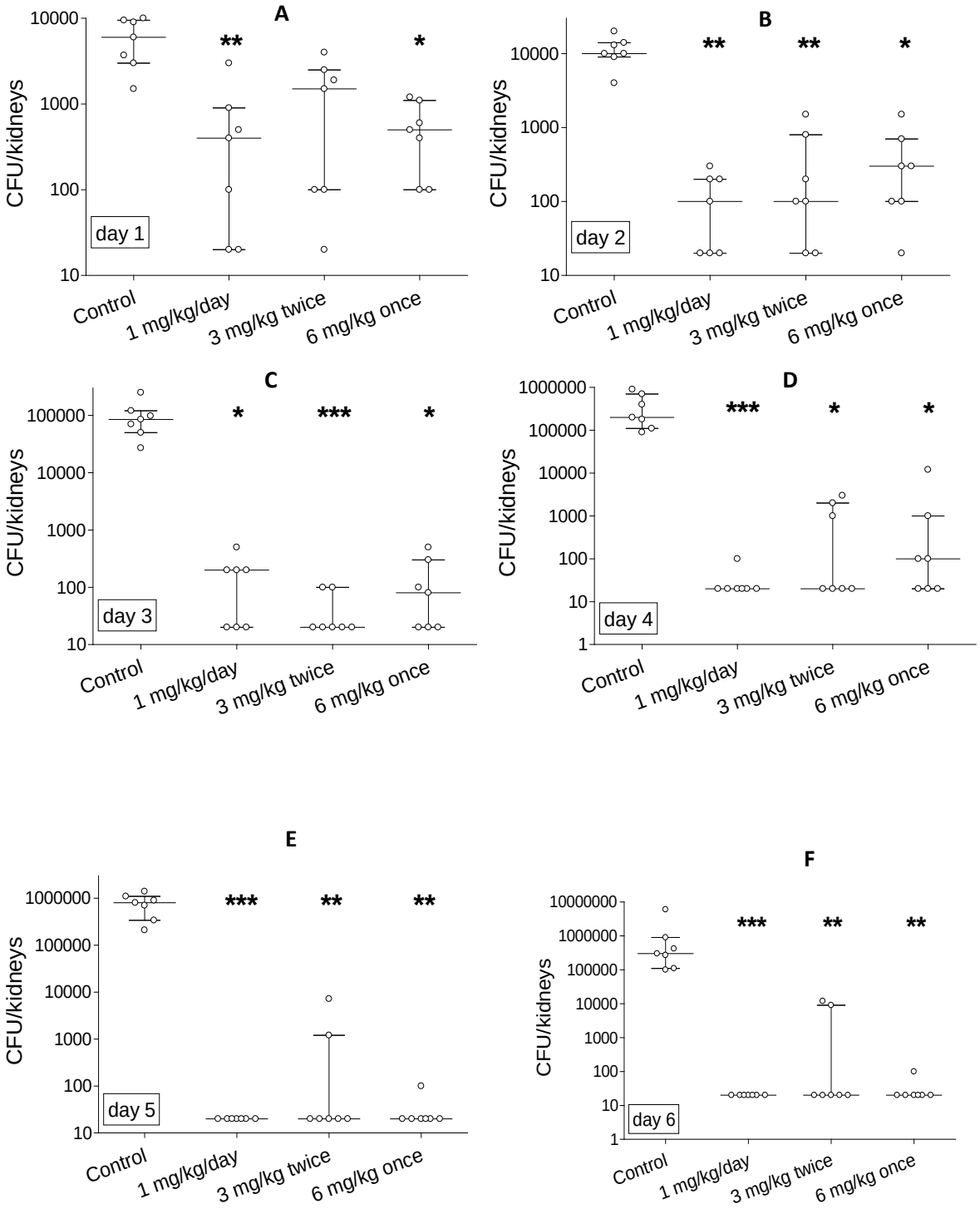


Figure 6. Kidneys tissue burden of neutropenic BALB/c mice infected intravenously with *C. albicans* isolates 17471. Tissue burden experiments were performed on postinfection days 1(A), 2(B), 3(C), 4(D), 5(E), and 6(F). Median with interquartile ranges is represented for each data set. Level of statistical significance is indicated at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). (No significant differences between the treatment groups.)



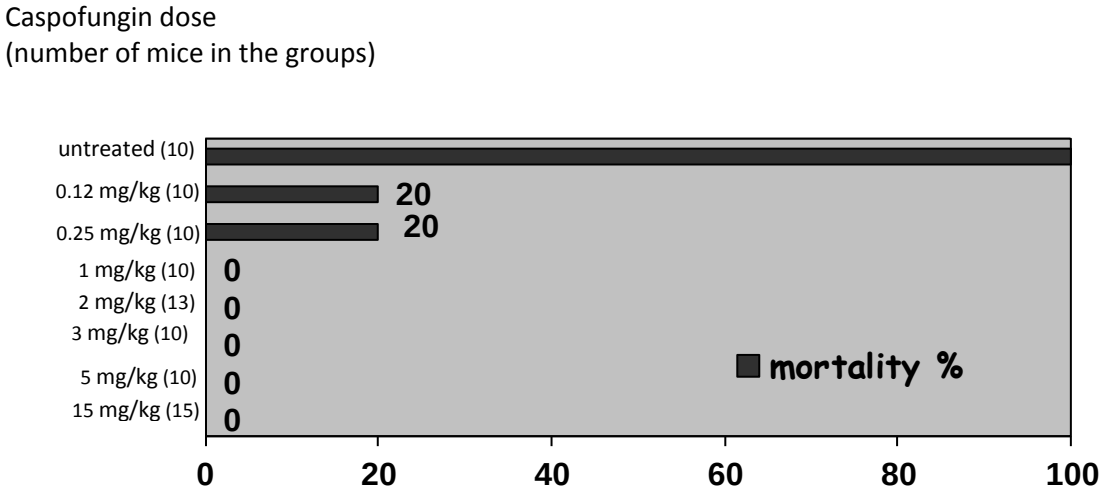
5.2. Experiments with *C. tropicalis*

5.2.1. Lethality

After the intraperitoneal infection with *C. tropicalis*, all mice in the control group succumbed to infection within five days. Treatment with single caspofungin doses of 0.12, 0.25, 1, 2, and 3 mg/kg 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 regardless of the dose ($p<0.0001$); differences among the caspofungin-treated groups were statistically not significant ($p>0.05$) (Fig. 7).

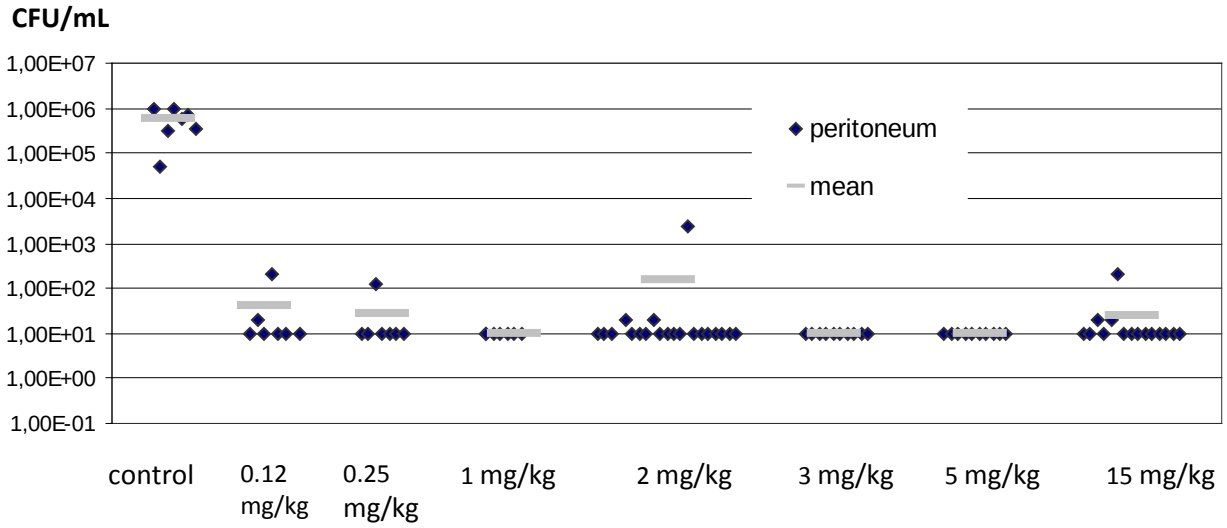
Figure 7. Cumulative mortality of mice after infection with *C. tropicalis* (10^7 CFU/mouse) in the untreated controls and the caspofungin treated groups.



5.2.2. Peritoneal lavage

Caspofungin treatment significantly decreased the number of viable yeasts in the peritoneal lavage samples in 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. 8.).

Figure 8. 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. This figure shows the number of viable yeast number obtained from peritoneal lavage.



5.2.3. Intraperitoneal abscess model

The vast majority of abscesses were found in the liver (Fig. 9). The number of abscesses containing viable yeasts as well as yeast CFU numbers in the abscesses decreased significantly in the 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 to the control group (Fig. 10). There was no difference between the groups treated with the two lowest doses of 0.12 and 0.25 mg/kg of caspofungin and the control group ($p\geq0.05$ in all cases) (Fig. 9 and Fig. 10.). Viable yeast CFU numbers significantly decreased in groups treated with 1, 3, 5 and 15 mg/kg of 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. 9).

In the two experiments using 2 mg/kg of 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 (20000 CFU/mL in the first and 180 and 560 in the second experiment in the single liver abscesses, respectively, Fig. 10). The yeast CFU numbers in

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 of caspofungin group, we repeated the experiment with this dose for third time. The third experiment has led to similar results (one of eight mice remained infected, carrying 6300 CFU/ml in a single abscess). The results of this third experiment were statistically comparable to the former ones.

Figure 9. 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. The figure shows the number of sterile and infected formed abscesses.

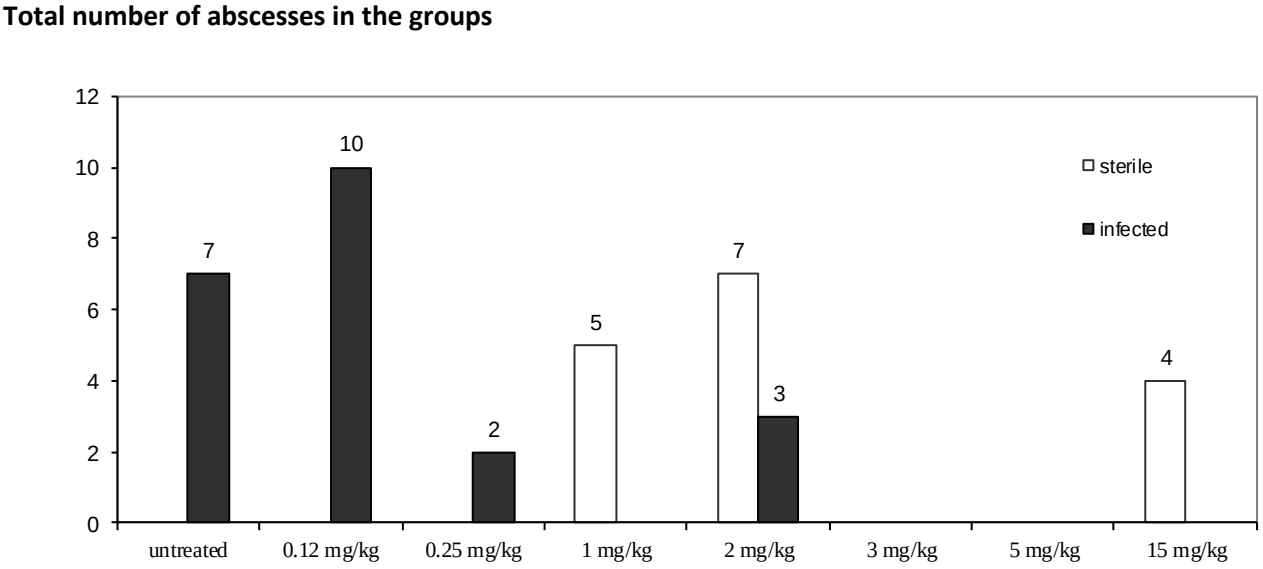
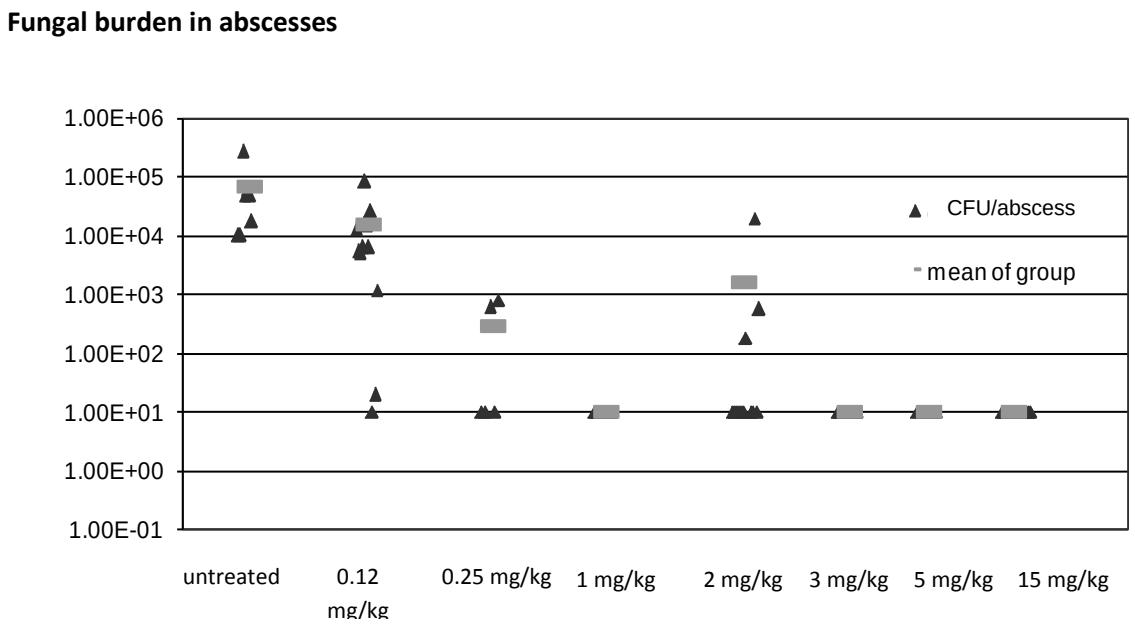


Figure 10. Therapeutic efficacy of caspofungin against *C. tropicalis* in neutropenic mice determined in an intraperitoneal abscess model. Caspofungin was administered intraperitoneally at doses of 0 (control), 0.12, 0.25, 1, 2, 3, 5, and 15 mg/kg/day. Treatment once daily was started 1 h after fungal inoculation, and continued for 5 days. This figure shows the viable yeast number in the infected formed abscesses.



6. Discussion

Due to the high level of reduced fluconazole susceptibility of *Candida* species, the echinocandin antifungals are now considered first line for the treatment of infections caused by these pathogens [137]. All three agents (caspofungin, micafungin, and anidulafungin) have been approved by the U. S. Food and Drug Administration for the treatment of esophageal candidiasis and invasive candidiasis, including candidemia [138]. Echinocandins are fungicidal against *Candida* species and actively growing tip of *Aspergillus* hyphae and have been proved to be highly effective both in animal models and in clinical trials [84, 87, 139, 140].

In 2007, the CLSI Antimicrobial Susceptibility Testing Subcommittee recommended 2 mg/L of concentration as the latest susceptible breakpoint for every three echinocandin member against *Candida* species [138, 141]. But the subcommittee has not defined resistant breakpoint for the echinocandins due to lack of enough resistant isolates in the population at that time. Isolates which are showing higher than 2 mg/L of MIC values have been defined as non-susceptible organisms [138].

Following increased use of echinocandins, sporadic cases of failures associated with elevated MICs have been reported [142]. Arendrup, M.C., et al. [142] has compared the ability of the EUCAST Edef 7.1, agar dilution, Etest and disk diffusion with RPMI-1640+2% glucose (2G) and IsoSensitest agar-2G media and CLSI M27A-3 methods to detect caspofungin, anidulafungin and micafungin resistant to detect FKS hot spot mutation in the selected *Candida* strains [142]. Also of note, it has become evident that *Candida* strains with mutations in fks1 and/or fks2 do not necessarily have MICs above the CBP of 2 mg/L [92]. These evidences have led the researchers to assume lower the susceptibility breakpoint for echinocandins and *Candida* species [143]. In addition, the new and species-specific echinocandins breakpoints are probably more sensitive to detect resistant strains [138].

Despite the introduction of the new class of antifungal agents, echinocandins, into the clinical practice, the morbidity and mortality resulting from invasive *Candida* infection still remains high, especially among patients with neutropenia [144]. In animal studies, higher echinocandins concentrations have provided greater serum and tissue concentrations, which was associated with increased fungal killing and clearance [145].

Dose escalation is one of the echinocandins treatment strategies for invasive *Candida* infection. The maximum tolerated dose of caspofungin is unknown, but caspofungin at two and three times the standard 50 mg/day of dosing regimen were well tolerated in adult non-candidemic and candidemic patients [146, 147]. High daily doses of caspofungin have produced a higher favorable overall response rate in the patients infected with *C. albicans* and *C. parapsilosis* when compared to the standard dosing regimen [146, 148].

In a study with a murine model, caspofungin concentrations have reached in a range of cca. 6 and 9 mg/L in the liver in the subjects received 1 mg/kg of caspofungin [149]. In another study with healthy adult participants following multiple 100-mg doses of caspofungin, on day 21 geometric mean AUC_{0-24} was 227.4 mg·h/L, peak concentration was 20.9 mg/L, and trough concentration was 4.7 mg/L. These results confirm that higher daily dose of echinocandins lead to higher serum and probably tissue concentration at the infected site of body, therefore a better treatment and tissue clearance can be expected [109].

In our work, caspofungin proved to be highly effective in an immunocompromised murine model of infection with a *C. tropicalis* strain with a proof of showing paradoxical growth *in vitro* in our preliminary studies [second study, [139]]. In this work, the subtherapeutic daily doses of 0.12 and 0.25 mg/kg did not improve the survival significantly, and were not able to eradicate the infection in the tissue sites. Among the evaluated dosages, the caspofungin standard daily dose of 1 mg/kg, cleared and eradicated the infection from the peritoneal cavity and has led to 100% survival in the lethality experiment. Other supratherapeutic caspofungin doses (including 2, 3, 5 and 15 mg/kg/day) were also effective in decreasing lethality and in preventing abscess development [139]. These results are in concordance with previous studies which produced excellent therapeutic outcome using higher echinocandins daily dosages [139]. But, in our *C. tropicalis* experiment, three out of 13 mice which were receiving the 2 mg/kg of daily dose did not recover from the infection. These results were confirmed in a third repeated experiment.

Attenuated activity of echinocandins antifungals at high concentrations, recognized as the "*paradoxical growth*" [150]. Paradoxical growth is echinocandin-specific, its occurrence and frequency is species- and strain-dependent [150]. The time-kill method produces results that are typically more sensitive than the BMD [151, 152].

Candida exposure to high concentration of caspofungin leads the organisms to induce the chitin synthesis. This stress-induced chitin synthesis is thought to be the mechanism for paradoxical growth [133, 153]. Clinical relevance of paradoxical growth is unknown, but a number of reports suggest that paradoxical growth may be associated with therapeutic failure in clinical situations [154].

The caspofungin efficacy has been investigated in the treatment and prophylaxis of invasive pulmonary aspergillosis due to *A. fumigatus* in persistently neutropenic rabbits. In this experiment, the animals treated with caspofungin 3 and 6 mg/kg/day demonstrated a paradoxical trend toward increased residual fungal burden in the lungs [155]. Another study has experienced similar results [156]. In that study, the investigators have observed an increase in fungal viability at 16-32 mg/L of caspofungin concentrations along with the expected decline in the viability at lower caspofungin concentrations, in both cases of *A. fumigatus* and *C. albicans* [156].

The occurrence of paradoxical growth was varied between 10 to 90% among the tested *Candida* strains [157]. The only species was able to grow in the presence of high concentration of the three marketed echinocandins, (caspofungin, micafungin and anidulafungin) was *C. tropicalis* [157].

High daily doses of caspofungin used in clinics have produced lower favorable overall response rate in patients infected with *C. tropicalis* in contrast to the standard daily dose of caspofungin regimen. These differences were not statistically significant and were not considered to be associated with the phenomenon of paradoxical growth [146, 147].

In the experiment with *C. tropicalis*, in contrast to 1 mg/kg in sterilizing the abscesses, the 2 mg/kg of caspofungin dose did not eradicate the infection in 3 out of 13 mice; which the paradoxical growth may speak in these cases. The reduction in infection burden was statistically significant at both 1 and 2 mg/kg of body weight per day and there was not a significant difference among the two groups [157].

Other researchers have also found a paradoxical growth with *C. albicans* strains *in vivo* at 20 mg/kg of caspofungin dose in examination the kidneys of the infected mice. But, they could not reproduce their results and they have concluded that paradoxical growth had role limited in an *in vivo* murine model [134].

These results suggest that supratherapeutic doses can result in incomplete sterilization in some animals and it could be the source of relapse after the discontinuation of therapy. Therefore, the role of paradoxical growth in the late clinical failure cannot be excluded [146, 148].

These findings can challenge the conclusions of Cornely, O.A., et al. [65] and Betts, R.F., et al. [146], who discarded the *in vivo* role of paradoxical growth on the basis of the lack of statistical differences between the groups treated with normal and with supratherapeutic doses of caspofungin, in case of less successful therapeutic experience with the supratherapeutic doses regimens in treating *C. tropicalis* [139].

Very recent studies suggest that paradoxical growth did not play an important role in the clinical failures among patients with candidemia. A study [158] has evaluated 5 to 60 minutes exposure of *C. albicans* to 8 mg/L of caspofungin in RPMI-1640. During this time paradoxical growth was eliminated for all *C. albicans* isolates showing paradoxical growth in time-kill experiments and showing that prolonged caspofungin exposure is required for paradoxical growth *in vitro* [158]. First, paradoxical growth was eliminated when serum was added to the RPMI-1640 medium [158]. Second, inhibition and killing by caspofungin against *Candida* species dramatically decrease in 50 % serum using time-kill methodology as determined by Szilagyi, J., et al. (unpublished data).

Although, high daily doses of caspofungin did not lead to statistically decreased therapeutic effect against immunocompromised mice infected with *C. tropicalis* isolate, the most important finding in this work was the excellent survival rate (100 %) elicited by a single dose (5 and 15 mg/kg) of caspofungin. This result shows that these dosing regimens can be useful for further evaluation in treating the infections caused by other *Candida* species as well.

Pharmacokinetic studies indicated that caspofungin distributes to and accumulates in tissues [159]. These tissues act as slow release of drug reservoirs which may explain the good efficacy of larger single and divided echinocandin doses in animal models. These facts raised the possibility that larger echinocandin doses used infrequently may be beneficial in certain clinical situations [159].

Recent findings have shown that echinocandins can prolong *postantifungal effect* (PAFE) against *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. lusitaniae* [160, 161]. Clancy, C.J., et al. [160] has found high killing rates of caspofungin against *C. albicans*, *C. glabrata* and *C.*

parapsilosis in simultaneous PAFE and time-kill experiments [160]. Yet, a correlation between *in vitro* and *in vivo* PAFE is not clearly defined, although the prolonged growth inhibition may provide a new approach in the treatment of invasive *Candida* infection by the echinocandins. This effect has inspired some investigators to hypothesize that administration of the same cumulative dose of echinocandins in less frequent large doses might be at least as efficacious as smaller daily doses [162-164].

Other observations also support that less frequent large doses of echinocandins may be effective for the treatment of invasive candidiasis [165]. Pharmacokinetic and pharmacodynamic analyses suggest that echinocandins efficacy is strongly associated with the AUC/MIC (the area under the concentration-time curve/MIC) or with the Cmax/MIC (the maximum concentration of the drug in serum/MIC) [139, 159]. Either result means that intermittent dosing regimens with echinocandins are effective [166]. Another study has found in their disseminated candidiasis model that the early outcome in caspofungin therapy (up to day 3) appears to be most closely linked to the Cmax/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 [159].

The only human data with once every two days dosing of an echinocandin was published by Buell et al. (Program Abst. 45th Interscience Conference Antimicrobial Agents Chemotherapy, 2005; Abst. M-719). The authors have conducted a multicenter, multinational, double-blind, randomized, parallel group, noninferiority study of 452 patients with confirmed esophageal candidiasis. Patients were randomized (1:1:1) to intravenous micafungin 300 mg/every other day, intravenous micafungin 150 mg/day and intravenous caspofungin 50 mg/day for a minimum 14 days and for 7 days after resolution of clinical symptoms of esophageal candidiasis (maximum 28 days). The vast majority of the identified *Candida* was *C. albicans* (91.4-94 %) followed by *C. glabrata* (2.6-5.3). Their results suggest that micafungin 300 mg administered intravenously every other day was as safe and effective as intravenous daily micafungin 150 mg and caspofungin 50 mg in treating esophageal candidiasis. Moreover, micafungin 300 mg resulted in less frequent relapse at 2 and 4 weeks post-treatment in comparison to micafunngin 150 mg and caspofungin 50 mg.

Dose escalation have also been proved to be well tolerated; moreover, in an open-label study, up to 8 mg/kg of micafungin was well tolerated for seven days [167]. Because all

echinocandins show dose-dependent postantifungal inhibition, this postantifungal effect may also be enhanced by increasing the concentration of the echinocandins [168].

In our study [139], the 5 and 15 mg/kg caspofungin doses for *C. tropicalis* (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, A., et al. [159]. These results suggested us that such therapeutic approach may be applicable against the most important *Candida* pathogen, *C. albicans*.

We have evaluated the efficacy of alterative dosing regimens of caspofungin against the three *C. albicans* isolates in preventing early (first six days postinfection) lethality in a deeply neutropenic murine model [first study, [169]]. The single 6 mg/kg and 2x3 mg/kg doses of caspofungin provided comparable but not superior results in comparison to the standard 1 mg/kg daily dose of caspofungin regarding the lethality and tissue fungal burden experiments. The isolates 17471 and 10920 relatively less responded to 2x3 in both lethality and tissue burden experiments (Fig. 2, Fig. 3, Fig. 6, and Fig. 5, respectively). But the single six mg/kg of the caspofungin dose in the first three days of experiment have produced significant decrease during the study period ($p<0.05$-0.001). However, differences between the three treatment arms disappeared by 4-6 days postinfection ($p<0.05$ for all isolates during the experiment) [169]. These results strongly suggest that antifungal therapy should be started as soon as possible [170] and the higher echinocandin doses at beginning of therapy may provide a better outcome.

These results, together with those of numerous other authors [171, 172], prove that echinocandins are excellent for the treatment of *Candida* infections; early therapeutic failure is rare and usually is associated with non-susceptibility. Recommended daily dosing may be supplanted by administering infrequent large doses, but the utility of the latter approach should be further examined in future experiments.

7. Summary

Candida species are the leading cause of invasive fungal infections in humans, producing infections that range from non–life-threatening mucocutaneous disorders to invasive disease that can involve any organ. In recent years novel antifungal agents have been released, significantly increasing options for the treatment of most serious fungal infections. The most recent approved antifungal drugs include those in the echinocandin class (caspofungin, micafungin, and anidulafungin), as well as the newer generation triazoles voriconazole and posaconazole.

In this respect we have conducted studies in defining the efficacy of caspofungin in treating systemic infections with two *Candida* species. First the *in vivo* efficacy against *C. albicans* was investigated. In a study with three *C. albicans* isolates single dose of six mg/kg, two times three mg/kg, six doses of one mg/kg efficacy have been examined in the two sub-studies including lethality and tissue burden in neutropenic murine models. In lethality experiments, all treatment regimens improved survival ($p<0.0014$ for all three isolates); differences among the treated groups were not statistically significant. The kidney fungal burdens for the two of isolates were counted in every day for the six-day study period continuously. The six mg/kg dose on the first three days made a significant change in decreasing the colony numbers ($p<0.05$-0.001), but in a longer period from forth to sixth day of study there was no difference among the treated groups ($p<0.05$).

The second study investigated the efficacy of caspofungin against *C. tropicalis* using an intraperitoneal infection model using a *C. tropicalis* isolate showing paradoxical growth *in vitro*. In this study a variety of caspofungin doses (0.12, 0.25, 1, 2, 3, 5, and 15 mg/kg) were used in one and daily doses for five-day of studies. The single doses of caspofungin were effective only at 5 and 15 mg/kg concentrations (100% survival). Five-day caspofungin treatment led to 100 % survival at 1 mg/kg and higher doses. Caspofungin treatment significantly decreased the number of viable yeasts in the peritoneal lavage samples as well as in the infected abscesses at 1 mg/kg as well as higher doses in comparison to the untreated control group ($p<0.001$ in all cases), and even to the group treated with 0.12 mg/kg of caspofungin ($p<0.05$ in all cases).

These results strongly suggest that antifungal therapy should be started at earliest time possible. More studies are required to examine the beneficial and therapeutic role of

infrequently larger echinocandins doses for using in the clinical routine and determining a better dosing regimen using the echinocandins specific charateristics.

8. References

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List of publications related to the dissertation

1. **Bayegan, S.**, Szilágyi, J., Kemény-Beke, Á., Földi, R., Kardos, G., Gesztelyi, R., Juhász, B., Adnan, A., Majoros, L.: Efficacy of a single 6 mg/kg versus two 3 mg/kg caspofungin doses for treatment of disseminated candidiasis caused by Candida albicans in a neutropenic mouse model.
J. Chemother. 23 (2), 107-109, 2011.
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2. **Bayegan, S.**, Majoros, L., Kardos, G., Kemény-Beke, Á., Miszti, C., Kovács, R., Gesztelyi, R.: 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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List of other publications

3. Szabó, Z., Szilágyi, J., Tavanti, A., Kardos, G., Rozgonyi, F., **Bayegan, S.**, Majoros, L.: In vitro efficacy of 5 antifungal agents against Candida parapsilosis, Candida orthopsilosis, and Candida metapsilosis as determined by time-kill methodology.
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