In Vitro Activity and In Vivo Efficacy of Rezafungin, Anidulafungin, Caspofungin and Micafungin Against the Fifth Clade of *Candida (Candidozyma) Auris*

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Article

In Vitro Activity and In Vivo Efficacy of Rezafungin, Anidulafungin, Caspofungin and Micafungin Against the Fifth Clade of *Candida (Candidozyma) auris*

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Abstract

Objectives: The aim of our study was to investigate the in vitro activity and in vivo efficacy of rezafungin, anidulafungin, caspofungin and micafungin against *Candida auris* isolates belonging to clade V. **Methods:** Five clinical isolates were evaluated (IFRC2087, IFRC4050, MRL40, TMML616 and TMML617). Echinocandin MICs and killing activities were determined in RPMI-1640. In the survival and fungal tissue burden experiments (heart, kidney and brain), neutropenic mice were infected intravenously (10^7 CFU/mouse). Treatment was initiated 24 hours post-infection with intraperitoneal dosing of 20 mg/kg of rezafungin on days 1, 3 and 6 or once-daily dosing for 6 days with 3 mg/kg of caspofungin, 5 mg/kg of micafungin or 5 mg/kg of anidulafungin. **Results:** MIC ranges of rezafungin, anidulafungin, caspofungin, and micafungin were 0.06-0.25, ≤0.03-0.12, 0.12-0.5 and ≤0.03-0.12 mg/L, respectively. The four echinocandins at ≥1 mg/L were fungicidal only against isolate MRL40. All echinocandin regimens improved the survival in mice infected with isolates MRL40 and IFRC4050 (P-values were ≤0.0002 and 0.0006, respectively), but only rezafungin was effective against isolate TML617 (P=0.0049). All four echinocandins induced more than 3 logs mean CFU/gram decreases in the kidneys and hearts of mice infected with the three isolates compared to control mice, some of which were not statistically significant. Fungal growth, regardless of the isolate tested, was poorly inhibited by echinocandins in the brain. Histopathology showed large aggregates of pseudohyphae in the hearts, kidneys and brains in control mice. In echinocandin treated mice only blastoconidia were found. **Conclusions:** In vitro activity and in vivo efficacy of the four echinocandins against the fifth clade of *C. auris* was echinocandin- and isolate-specific. Pseudohyphal production was common in controls, but not in echinocandin treated mice. Rezafungin activity was comparable to or better than the three previously approved echinocandins.

Keywords: *Candida auris*; fifth clade; time-kill; neutropenic mouse model; pseudohypha production; rezafungin; echinocandins

1. Introduction

Candida auris as a globally distributed yeast is a member of the critical priority fungal pathogens according to the World Health Organization [1]. The first whole genome sequence (WGS) data of global clinical isolates indicated that four distinct clades (South Asian, East Asian, South African and South American) exist causing hospital outbreaks and invasive infections among critically ill patients [2]. The

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vast majority of clinical isolates are resistant to fluconazole, and to a lesser extent to amphotericin B [3]. Current guidelines recommend echinocandins (anidulafungin, caspofungin, and micafungin) as first-line therapeutic agents to treat severe *C. auris* infections [3,4]. However, the emergence of echinocandin-resistant isolates during echinocandin treatment have been reported [5,6].

Since 2018 sporadic cases of *C. auris* infections, including meningitis, chronic mucocutaneous candidiasis and otomycoses have been reported in Iranian patients. Although the isolate from the child suffering from meningitis belonged to the South Asian clade, the remaining isolates were genetically different [7–9]. Based on WGS data these isolates differed from the other clades by more than 200,000 single-nucleotide polymorphisms, confirming that the Iranian isolates form a separate cluster (Iranian or fifth clade). Two of five of these isolates showed high MICs to fluconazole and voriconazole, but amphotericin B, posaconazole, isavuconazole, anidulafungin and micafungin MIC values were low for all strains [10,11].

Rezafungin (Rezzayo™) is the first new drug approved to treat candidemia and invasive candidiasis in more than 10 years. Rezafungin is a once-weekly, next-generation echinocandin with excellent in vitro and in vivo activity against clinically important *Candida* species. Rezafungin received approval for the treatment of candidaemia and invasive candidiasis in patients older than 18 years who have limited or no alternative treatment options [12–18]. However, data on in vitro activity and in vivo efficacy of the four approved echinocandins against Iranian clade *C. auris* are lacking.

The aim of our study was to investigate the in vitro activity of rezafungin, anidulafungin, caspofungin and micafungin using time-kill methodology against *C. auris* isolates belonging to the Iranian lineage. We also determined the efficacy of the four echinocandins in a neutropenic murine model.

2. Materials and Methods

2.1. In Vitro Experiments

2.1.1. Isolates

The five clinical isolates used in this study are available from the CBS Westerdijk collection (Table 1) and described in previously [11]. None of the isolates produced aggregates in saline. The *C. auris* type strain (NCPF 13029=CBS 10913; East Asian clade II) and *C. parapsilosis* ATCC 22019 were also included in MIC assays (Table 1). Two days before the experiments, isolates were sub-cultured on Sabouraud dextrose agar and screened on CHROMagar Candida (Becton Dickinson) to ensure purity of the isolates [14].

Table 1. *MIC (mg/L) values of anidulafungin (ANI), caspofungin (CAS), micafungin (MICA) and rezafungin (REZA) in RPMI-1640 against C. auris Iranian isolates, and C. auris type strain (CBS 10913) and C. parapsilosis ATCC 22019 strain. MICs were determined at least twice using the CLSI broth microdilution method.*

Strain	Place	Body site	ANI	CAS	MICA	REZA
IFRC2087 (CBS 18598)	Babol	ear	≤0.03	0.5	≤0.03	0.12-0.25
IFRC4050 (CBS 18600)	Babol	ear	0.12	0.12	≤0.03	0.12
MRL40 (CBS18599)	Isfahan	ear	≤0.03	0.06	≤0.03	0.06
TMML616 (CBS18601)	Shiraz	skin	≤0.03	0.25	0.12	0.12
TMML617 (CBS18602)	Shiraz	ear	≤0.03	0.25	0.12	0.12
C. auris type strain	Japan	ear	≤0.03	0.5	0.06	0.12
ATCC 22019	-	-	0.5	0.5	0.5	0.5

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2.1.2. Antifungal Susceptibility Testing

Anidulafungin, caspofungin and micafungin were obtained from Molcan Corporation (Ontario, Canada). Rezafungin was provided by Cidara Therapeutics (San Diego, USA). Antifungals were dissolved in 100% DMSO and further diluted in RPMI-1640 (Sigma, Budapest, Hungary) to final concentrations between 0.015-8 mg/L. The starting inoculum was ~10^3 CFU/mL [14]. Plates were incubated at 35 °C and MICs were read visually after 24 hours using the partial inhibition criterion [19–21]. MICs were determined at least twice. For susceptibility categorization, tentative MIC breakpoints as suggested by the Centers for Disease Control and Prevention (CDC) were used: susceptible ≤2 mg/L for both anidulafungin and micafungin, and ≤1 mg/L for caspofungin [3]. For rezafungin, a provisional CLSI susceptible breakpoint of ≤0.5 mg/L was used [22].

2.1.3. Phase-Contrast Microscopy

Microscopic morphology was examined in isolates IFRC4050, MRL40 and TML617 after 24 hours at 30 ^{0}C and 37 °C in RPMI-1640. Echinocandin-induced morphological alterations were determined at 0.25 and 16 mg/L with the same isolates with all echinocandins after 24 hours of incubation at 37 °C in RPMI-1640 [19]. We used a Zeiss Axioskop 2 mot microscope coupled with a Zeiss Axiocam 212 camera using phase contrast technique (Zeiss, Jena, Germany). Image acquisition was performed, using Zeiss ZEN lite 3.13 software. The total volume examined was 10 µL.

2.1.4. Time-Kill Studies

Anidulafungin, caspofungin, micafungin and rezafungin killing curves were determined with all isolates in RPMI-1640 at 0.03, 0.25, 1, 8, 16 and 32 mg/L [19]. The starting inocula were 2-3.3x10^5 CFU/mL. Samples were removed at 0, 4, 8, 12 and 24 hours, serially diluted tenfold, plated (4x30 mL) onto a single Sabouraud dextrose agar and incubated at 35 °C for 48 hours. The limit of detection was 50 CFU/ml. Fungicidal or fungistatic activity was defined as ≥3-log CFU/mL or <3-log CFU/mL changes in viable cell count compared to the starting inoculum [19]. All experiments were performed twice; means of the resulting data are presented. Killing kinetics at the tested concentrations were calculated as described previously. Positive killing rate (k) values indicate killing, and negative k values indicate growth. The mean times to achieve 99.9% reduction of the starting inoculum ($T_{99.9}=3/k$) were calculated from the k values for each isolate and concentration [19]. One-way ANOVA with Tukey's post-testing was used to determine significant differences in killing kinetics among isolates and concentrations [19].

2.2. In Vivo Experiments

2.2.1. Lethality Experiments

BALB/c female mice (23-25 g) were given cyclophosphamide (Endoxan, Baxter, Hungary) 4 days before infection (150 mg/kg) and 1 day before infection (100 mg/kg). Immunosuppression was continued by administration of 100 mg/kg cyclophosphamide every third day until the end of the experiment on the 21st day [23]. The Guidelines for the Care and Use of Laboratory Animals were strictly followed during maintenance of the animals; experiments were approved by the Animal Care Committee of the University of Debrecen (permission no. DEMÁB 09/2024).

For in vivo experiments isolates MRL40, IFRC4050 and TML617 were used. Mice (groups of ten mice/isolate) were infected intravenously through the lateral tail vein (day 0). The infectious dose was 10^7 CFU/mouse, administered in volumes of 0.2 mL. Inoculum densities were confirmed by plating serial dilutions on Sabouraud dextrose agar plates [23].

Rezafungin was dosed at 20 mg/kg on days 1, 3 and 6. The other echinocandins were dosed daily for 6 days at 3 mg/kg (caspofungin) or 5 mg/kg (micafungin and anidulafungin). All treatments were started 24 hours post-infection [24]. These doses mimic the human once-weekly dosing regimen for rezafungin, and the daily dose regimens for anidulafungin, caspofungin and micafungin [24]. Control groups were given saline. Mice were monitored at least twice a day for survival for 21 days. Animals

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that became immobile or showed signs of severe illness were terminated and recorded as dying on the following day. Survival rates were compared using the Kaplan-Meier log rank test. Statistical tests were performed in GraphPad Prism 6.03 [24].

2.2.2. Fungal Tissue Burden Experiments

The tissue bioburden experimental design was similar to that employed in the survival experiments. Each group included 6 mice. On day 7, mice were sacrificed; both kidneys, the heart and the brain were removed from each animal, weighed and homogenized aseptically in 1 mL of saline; the resulting tissue suspension was serially diluted. Fungal tissue burden was determined by quantitative culturing. The lower limit of detection was 100 CFU/g of tissue. Mean fungal tissue burdens observed in the same organs were compared using the Kruskal-Wallis test with Dunn's post-test [23,24].

2.2.3. Histopathology

Two mice from both the treatment and control groups of the fungal tissue burden experiments were used for histopathological examination on day 7. Moreover, on day 1 and 4, two-two mice infected with isolate MRL40 were used to determine the early heart, kidneys and brain involvement. Additionally, five surviving mice (control and echinocandin treated mice) infected with isolate MRL40 from the lethality experiment on day 21 were dissected and analysed similarly. Organs (heart, both kidneys, and brain) were fixed in formalin and embedded in paraffin. Tissue sections (4 μm) were stained with haematoxylin–eosin and periodic acid–Schiff [23,24].

3. Results

3.1. MIC Values of the Echinocandins Against C. auris

MICs of anidulafungin, caspofungin, and micafungin were not higher than the suggested tentative CDC breakpoints for *C. auris* (Table 1) [3]. Rezafungin MICs were lower than a provisional CLSI susceptible breakpoint (≤0.5 mg/L) [22].

3.2. Phase-Contrast Microscopy

Fungal cells of the three isolates used grew as separate, rounded or slightly elongated cells at 30 °C in RPMI-1640; the longest pseudohypha were produced by isolate MRL40 (88.2 μm) (Figure 1). Pseudohypha production was prominent at 37 °C with all three isolates, especially in the case of isolate IFRC4050 (115 μm) (Figure 1). Yeast aggregates were not observed. In contrast, echinocandin-treated yeasts at 37 °C showed small rounded (~20 μm) and large (120x ~80 μm) aggregates at both (0.25 and 16 mg/L) echinocandin concentrations (Figure 2). The smallest aggregates (~10 cells) were observed in anidulafungin treated cells at both concentrations, while more than 100-cell aggregates were seen with micafungin at 16 mg/L (Figure 2). Pseudohyphae were never observed in echinocandin treated isolates.

3.3. Time-Kill Studies

The four echinocandins showed concentration dependent killing activities against isolate MRL40 (Figure 3). T99.9 value ranges against isolate MRL40 for anidulafungin at 0.03-32 mg/L, for caspofungin at 1-32 mg/L, for micafungin at 0.25-32 mg/L and rezafungin at 0.25-32 mg/L were 1.99-3.84 hours, 2.72-4.72 hours, 3.23-5.48 hours and 2.58-3.30 hours, respectively. The four echinocandins used against isolates IFRC2087 and IFRC4050 produced <0.5-log CFU decreases with prominent re-growth and almost always negative *k* values (Figure 4). Against the remaining two isolates (TML616 and TML617), anidulafungin and micafungin at ≥0.25 mg/L generated positive *k* values at all tested concentrations (Figure 4); the highest CFU decreases (2.40-2.48 log CFU) were observed at 16-32 mg/L with anidulafungin for isolate TML617 (Figure 2). For isolates TML616 and TML617, caspofungin and rezafungin produced only transient CFU decreases after 4-8 hours, followed by regrowth after 24 hours (Figure 3); killing rate values were rarely positive (Figure 4). Anidulafungin at 1 and 8 mg/L showed

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greater (*k* values were 0.36 and 0.34 1/h, respectively) mean killing activities compared to 16 or 32 mg/L (*k* values were 0.21 and 0.25 1/h, respectively) against isolate TML617 (mini-paradoxical growth) (Figure 4). The similar phenomenon was observed with micafungin against the same isolate [19].

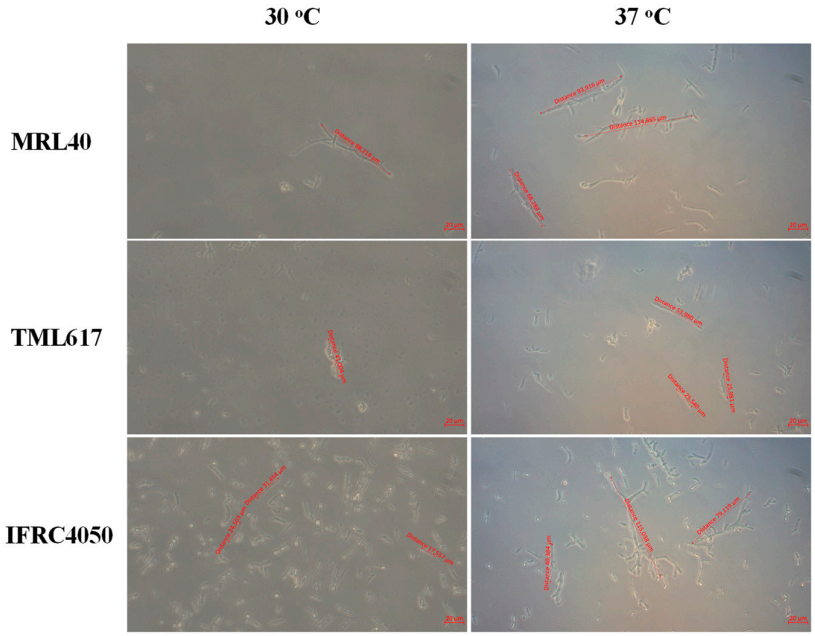

Figure 1. Phase-contrast microscopy images of *C. auris* cells in RPMI-1640 with isolates MRL40, TML617 and IFRC4050 at 30 °C and 37 °C after 24 hours incubation. At 30 °C single and budding cells or elongated cells were detected in all three isolates. The highest number of fungal cells was noticed in the case of isolate IFRC4050, and the longest pseudohypha was visible in the case of isolates MRL40. At 37 °C prominent pseudohyphae production was noticed with all three isolates; isolate TML617 produced up to 53.9 μm long pseudohyphae, while in the cases of isolates MRL40 and IFRC4050 the pseudohyphae were longer than 100 μm. Fungal cell aggregates were not noticed. Bar: 20 μm.

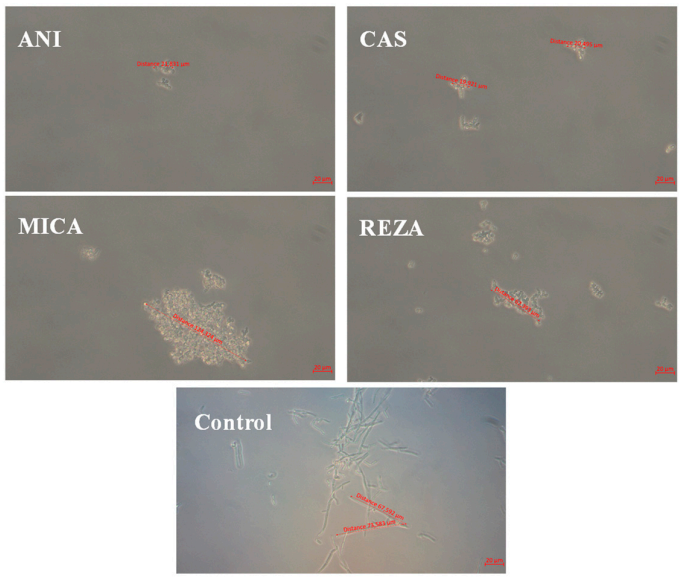

Figure 2. Phase-contrast microscopy images of *C. auris* IFRC4050 cells treated with 16 mg/L anidulafungin (ANI), 16 mg/L caspofungin (CAS), 16 mg/L micafungin (MICA) and 16 mg/L rezafungin (REZA) in RPMI-1640 against

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isolate IFRC4050. Micrograph was taken after 24 Micrograph was taken after 24 hours. Echinocandin treated fungal cells never produced pseudohyphae. In the cases of ANI and CAS treated cells small aggregates (~20 μm in diameter) are visible. MICA and REZA treated fungal cells produced larger aggregates (~120 μm and ~60 μm in diameter, respectively). Bar: 20 μm.

3.4. Lethality Experiments

All echinocandin regimens improved the survival in mice infected with the MRL40 strain (P values for anidulafungin, caspofungin, micafungin and rezafungin were 0.0002, 0.0001, 0.0001 and 0.0001, respectively, Figure 5). In contrast, in mice infected with isolate TML617, only rezafungin significantly improved survival (P values for anidulafungin, caspofungin, micafungin and rezafungin were 0.1606, 0.2121, 0.2286 and 0.0049, respectively, Figure 5). Two micafungin treated mice infected with isolate TML617 showed ataxia 2-3 days before their death (died on days 17 and 21). For isolate IFRC4050, all four echinocandins increased the survival compared to the untreated control mice (P values for anidulafungin, caspofungin, micafungin and rezafungin were 0.0006, 0.0001, <0.0001 and 0.0006, respectively, Figure 5) and ataxia was not observed.

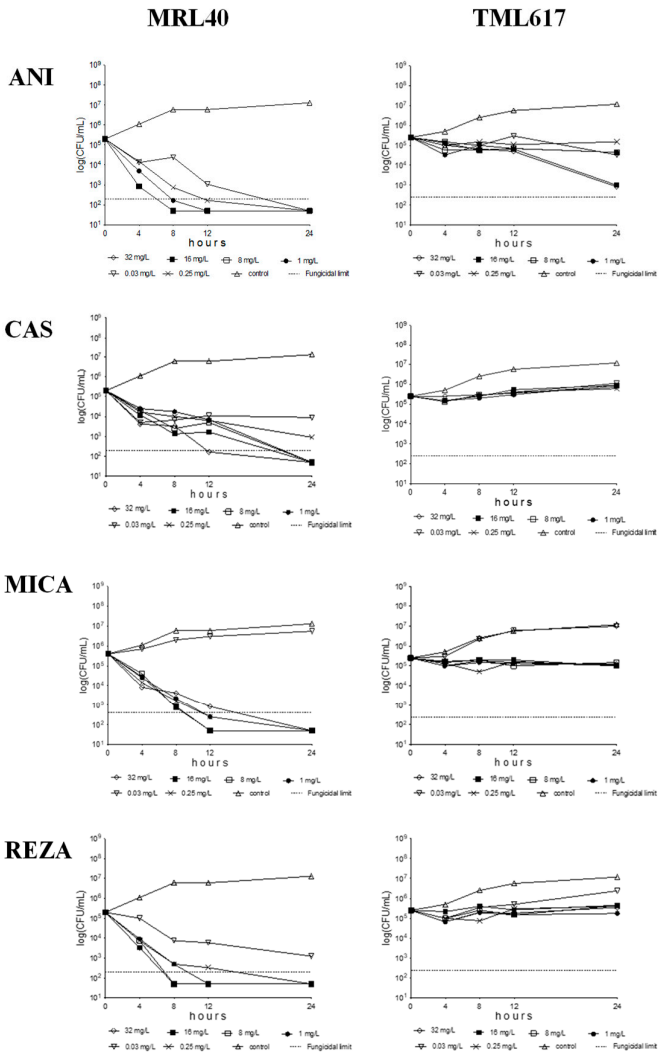

Figure 3. Time-kill plots of anidulafungin (ANI), caspofungin (CAS), micafungin (MICA) and rezafungin (REZA) in RPMI-1640 against isolates MRL40 (left) and TML617 (right) in RPMI-1640. Dotted lines indicate the fungicidal limits (≥ 3 log CFU/mL changes in viable cell count compared to the starting inoculum).

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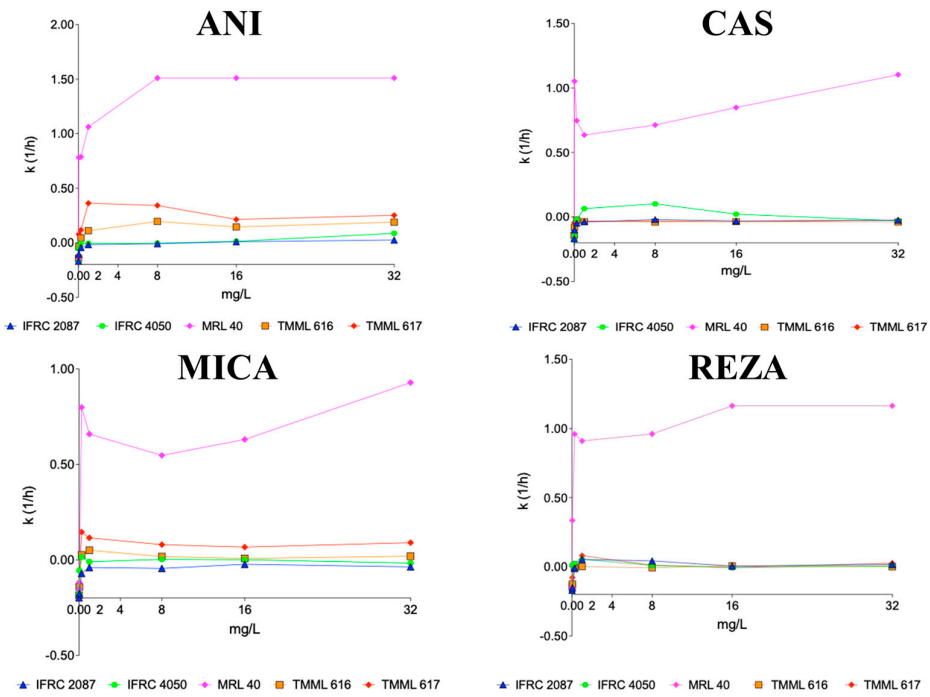

Figure 4. Mean killing rate values of anidulafungin (ANI), caspofungin (CAS), micafungin (MICA) and rezafungin (REZA) in RPMI-1640 against isolates IFRC2087, IFRC4050, MRL40, TML616 and TML617. Echinocandins killing rate values (*k*=1/h) were determined at 0.03, 0.25, 1, 8, 16 and 32 mg/L. Positive and negative *k* values indicate the decrease and increase, respectively in viable cell numbers. Error bars were omitted for better visualization of the graphics.

3.5. Fungal Tissue Burden Experiments

The four echinocandins produced more than 5 and 4 logs mean CFU/gram decreases in the kidneys (P<0.001 for all echinocandins) and hearts (P<0.001 for all echinocandins), respectively in mice infected with isolate MRL40 compared to control mice on day 7 (Figure 5). Moreover, in the kidneys and hearts the CFU decreased at least 2 and 1 logs CFU/gram, respectively compared with controls on day 1 with all four echinocandins. Only caspofungin showed statistically significant CFU decreases in the brains (P<0.05). However, in echinocandin treated mice the mean fungal burden in brain tissue was always higher than 10^5 CFU/gram (Figure 5).

In mice infected with isolate TML617, rezafungin (P<0.01), caspofungin (P<0.05) and micafungin (P<0.05) generated >3 logs, >2 logs and >2 logs CFU/gram decreases, respectively in the kidneys on day 7, some of which were not statistically significant. In the hearts rezafungin (P<0.001) and caspofungin (P<0.05) treatment showed more than 4 and 3 logs mean CFU/gram decreases, respectively (Figure 5). However, only rezafungin treatment decreased the tissue burden in the heart and kidneys (at least with 1 log CFU/gram) compared to controls at day 1. None of the echinocandins induced CFU decreases in the brain (P>0.05 for all echinocandins); the mean fungal burden in the brain tissue was higher than 10^6 CFU/gram (Figure 5).

In mice infected with isolate IFRC 4050 the four echinocandins produced <3-log CFU mean fungal kidney and heart burden decreases on day 7 (Figure 5). Caspofungin and micafungin treatments generated statistically significant CFU decreases in the brains (<1 log). However, on day 7 the fungal tissue brain burden in echinocandin treated mice was higher (~1 log CFU/gram) compared with controls on day 1 (Figure 5).

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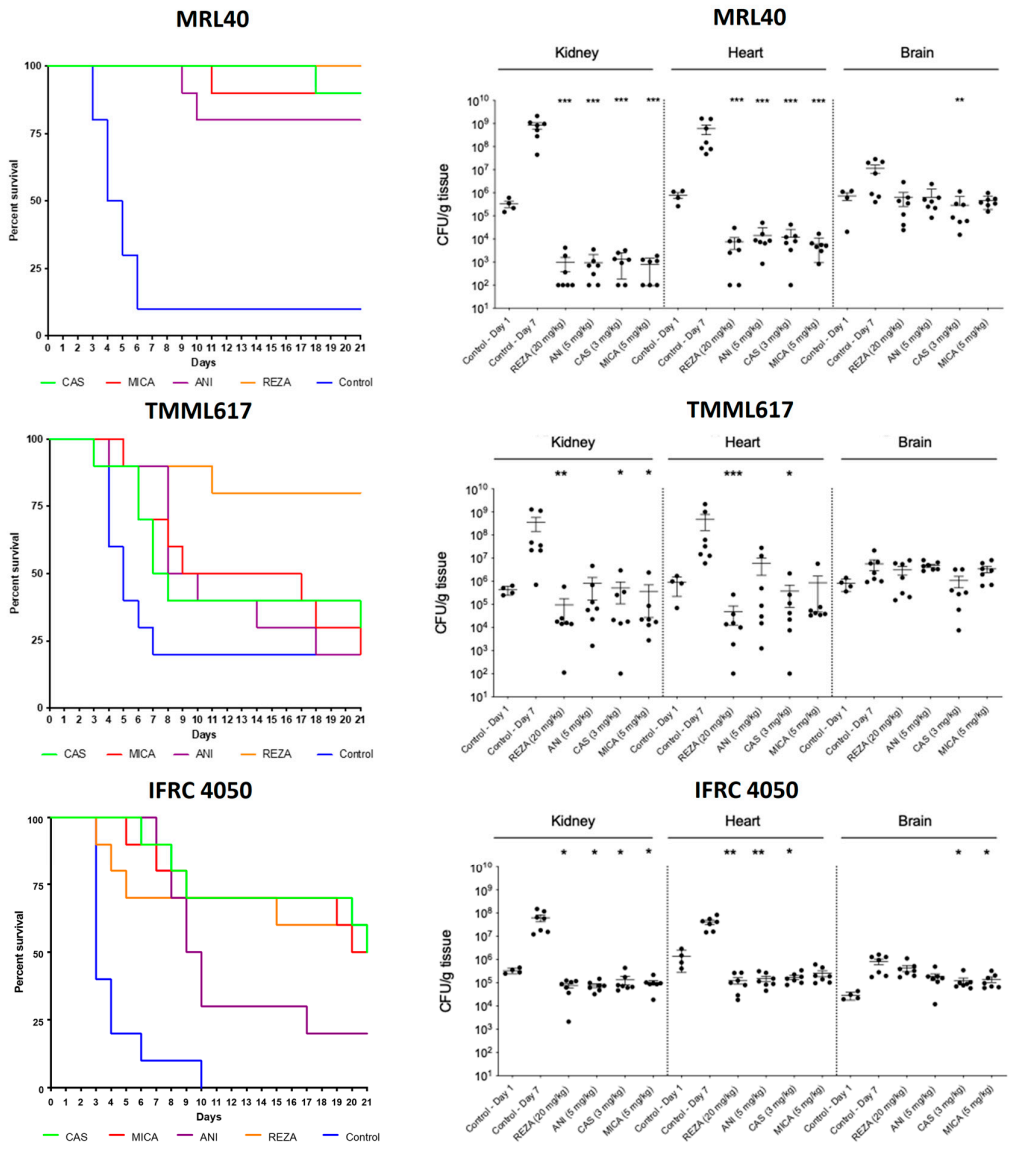

Figure 5. Survival (left) and kidney, heart and brain fungal burdens (right) of rezafungin (REZA), anidulafungin (ANI), caspofungin (CAS) and micafungin (MICA) treated and control neutropenic BALB/c mice infected with the isolates MRL40, TML617 and IFRC4050. The infectious dose was 10^7 CFU/mouse. A 20-mg/kg dose of rezafungin was administered on days 1, 3, and 6. Additionally, beginning 24 hours post-infection, the mice received 3 mg/kg caspofungin (Cancidas®), 5 mg/kg micafungin (Mycamine®), and 5 mg/kg anidulafungin (Eraxis®) once a day for 6 days. After 21 days, survival rates were compared using the Kaplan–Meier log rank test. In the fungal tissue burden experiments, the kidney, the heart, and the brain burdens were determined on day 7. The bars represent the medians. Asterisks indicate level of significance (* $p<0.05$, ** $p<0.01$, *** $p<0.0001$).

3.6. Histopathology

Histopathology showed small and large aggregates of predominantly pseudohyphae in hearts, kidneys and brains in control mice on day 7. Pseudohyphae were visible in the heart and kidneys as early as day 1, but were only detected in the brain from day 4 (Figure 6). The sizes of the aggregates in the heart, kidney and brain were ~20-50, ~50-60 and ~20 μm in diameters, respectively. Echinocandin treatment, especially rezafungin, showed sporadic or no fungal cells in hearts and kidneys, but blastoconidia were always visible in cerebrum or cerebellum. Pseudohyphae were not found in echinocandin treated mice (Figure 6). The largest aggregate (~40 μm in diameter) was found

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in the heart in caspofungin treated mouse (Figure 6). The heart of the survived control mouse dissected at the end of the lethality experiment (day 21) showed coagulative necrosis with scattered fungal cells, but large and small aggregates of rounded cells (blastoconidia) were detected in the kidney and brain, respectively. In the echinocandin treated groups only in the micafungin treated mouse, large and small aggregates of blastoconidia were observed in the heart and kidney, respectively. However, in the brains aggregates of blastoconidia (~20 μm in diameter) were always visible in echinocandin treated mice (Figure 7).

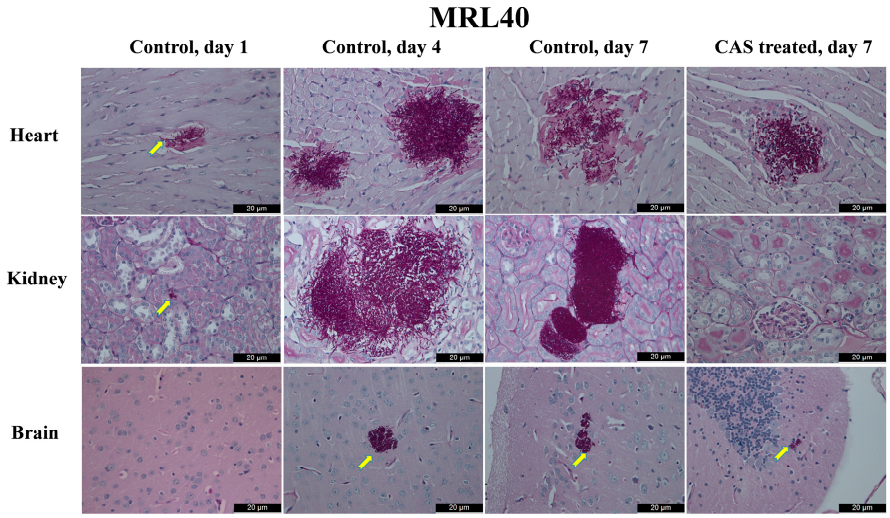

Figure 6. Histopathological findings of the heart, kidney, and cerebrum with periodic acid–Schiff staining in control (days 1, 4 and 7), and caspofungin (CAS) treated (3 mg/kg once daily for 6 days) mice infected with isolate MRL40. Fungal lesions are indicated by yellow arrows. In control mice, pseudohyphae were visible in the heart and kidneys starting from day 1, but were not detected in the brain.

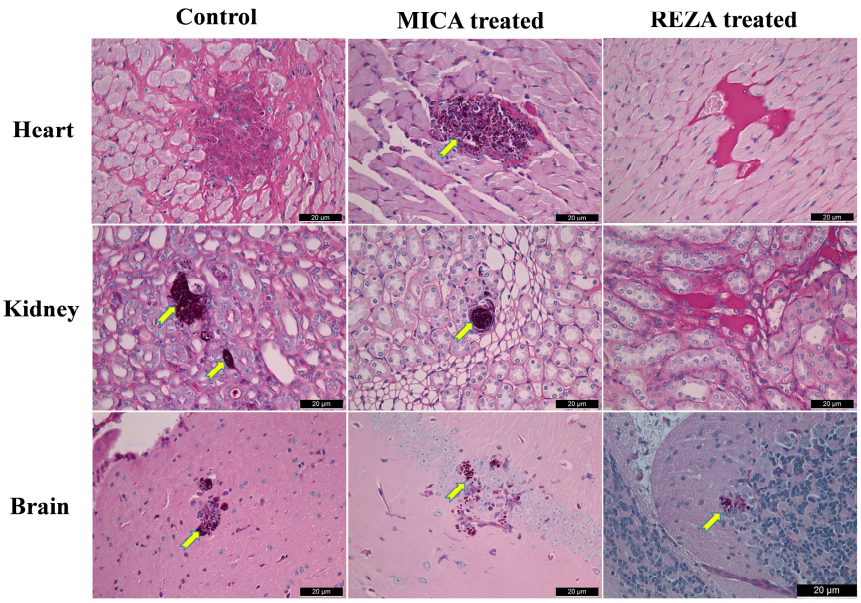

Figure 7. Histopathological examination of the hearts, kidneys and brains using periodic acid–Schiff staining in survived neutropaenic mice infected with isolate MRL40 on day 21. Fungal lesions are indicated by yellow

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arrows. In the control mouse, in the heart within the coagulative necrotic area scattered fungal cells were noticed, but the kidney and brain showed dense aggregates of blastoconidia. In the case of micafungin (MICA) treated mouse (5 mg/kg once daily for 6 days) in the heart one large, in the kidney one small, but dense aggregate of blastoconidia were visible, while in the brain numerous very small aggregates of blastoconidia were seen. In rezafungin (REZA) treated (20 mg/kg on days 1, 3, and 6) mouse only in the brain were detected fungal cells. Magnification, ×100.

4. Discussion

MIC values of the four echinocandins against all five Iranian isolates did not exceed the tentative breakpoints proposed by CDC for anidulafungin, caspofungin and micafungin or the provisional CLSI susceptible breakpoint of rezafungin [3,22]. In time-kill studies, all four echinocandins showed rapid fungicidal activities against isolate MRL40 and a fungistatic effect with frequently negative killing rate values against the remaining four isolates. Mini-paradoxical effect was noticed in the cases of anidulafungin and micafungin against isolate TML617 (Figure 4). All three strains produced pseudohyphae at 30 °C and 37 °C in vitro, but pseudohyphae were eliminated even by 0.25 mg/L of echinocandins at 37 °C. Our in vivo results correlated with the in vitro killing studies only in the case of isolate MRL40. In the lethality experiments all echinocandin regimens improved the survival of mice infected with isolates MRL40 and IFRC 4050, but only rezafungin proved to be effective against mice infected with isolate TML617. The four echinocandins in mice infected with isolate MRL40 induced larger mean CFU/gram decreases in the kidneys and hearts compared to mice infected with isolates TML617 and IFRC 4050. Fungal growth in the brain, regardless of the isolate tested, was poorly inhibited by echinocandins (Figure 5). Histopathology showed large aggregates of pseudohyphae in hearts, kidneys and brains in control mice (Figure 6). Echinocandin treatment, especially rezafungin, showed sporadic yeast cells in hearts and kidneys (blastoconidia), but yeasts were always visible in cerebrum or cerebellum. Pseudohyphae were not detected in echinocandin treated mice (Figures 6 and 7).

Data on echinocandin in vitro killing kinetics and in vivo efficacy against the Iranian *C. auris* clade are lacking. However, Barough and co-workers determined the in vivo pathogenicity of a single Iranian isolate (IFRC4050) and amphotericin B efficacy in an immunocompromised mouse model [25]. They found 60% mortality rate at the end of the 21 days experiment that was lower compared with our current results with the same isolate (100 % mortality after 10 days). In their fungal burden experiments the highest and lowest fungal load were detected in the hearts and kidneys, and in the brains, respectively in control mice. Their histopathology analyses indicated that the heart and kidney were the most severely affected organs, followed by the spleen, liver, lung, and brain [25]. The lack of studies evaluating echinocandin activity against Iranian clade V *C. auris* precludes comparative discussion of our results.

A notable strength of this study is that the in vitro efficacy of the four approved echinocandins were tested against the recently described Iranian isolates. Pseudohyphae were visualized in all three isolates with phase-contrast microscopy in RPMI-1640 at 30 and 37 °C. A limitation of this work is that in vivo experiments were not performed with all five isolates. Although, all four approved echinocandins were tested against 1 of 2 the Iranian isolates derived from different cities (Babol and Shiraz) in vivo. Histopathology also revealed pseudophyphae in the kidneys, heart and brain in mice infected with the three isolates from the three different Iranian cities. The fungal burden was determined only in the kidneys, heart and brain which may be regarded as another limitation. However, other authors as well as our previous studies indicated that the heart and kidneys are the most important target organs in cases of *C. auris* candidemia [23–25].

C. auris was considered a species that produces cellular aggregates in vitro but does not produce pseudohypha neither in vitro nor in vivo [26,27]. Other authors found that changes of the environmental conditions and genotoxins (hydroxyurea, methyl methane sulfonate and 5-fluorocytosine) could provoke pseudohyphal formations [28]. Yue et al. observed filament formation after passage in the mouse model with the four main clades [29]. In a recent study Gifford et al.

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developed a fish embryo yolk-sac microinjection model using *Aphanius dispar* (Arabian killifish) at human body temperature to studying the virulence of the five *C. auris* clades [30]. Clades I-IV showed budding yeast morphologies both in vitro and in vivo, but natural filamentation was observed with the Iranian isolate in vivo. Our current results with the in vitro and in vivo observed pseudohyphae formation at 37 °C was consistent with the findings by Gifford et al [30]. Moreover we found pseudophyha production even at 30 °C, at the temperature where *C. auris* typically is causing human skin colonization and infections [7–10]. The pseudohypha production seems to be more prominent at 37 °C than at 30 °C (Figure 1), suggesting a higher virulence of the fifth clade at body temperature.

In vivo efficacy against the Iranian isolates was echinocandin- and isolate-dependent. Although all four echinocandins were highly effective against isolate MRL40 both in vitro and in vivo, the in vitro and in vivo correlation was poor for the other two isolates. Despite the weak fungistatic activity of the four echinocandins against isolate IFRC4050, all four antifungals significantly increased survival and decreased fungal tissue burdens. In contrast, in the case of isolate TML617 anidulafungin produced the largest CFU reduction in the killing study, but showed very poor activity in the lethality and fungal tissue burden experiments. Interestingly, histopathology showed no fungal cells in these echinocandin treated mice. It is notable that against isolates TML617 both anidulafungin and micafungin produced better killing activities at lower than at higher concentrations (mini-paradoxical growth). Echinocandin exposure induces a variety of stress adaptation pathways with increased cell wall chitin amount coupled with aggregate formation in the internal organs, that may explain the weak in vivo efficacy of anidulafungin, caspofungin and micafungin against some Iranian isolates [31].

With the exception of isolate MRL40, the most recently approved echinocandin, rezafungin, showed weak in vitro fungistatic activity against the Iranian isolates belonging to *C. auris* clade V. In contrast, rezafungin had similar or better in vivo activity than anidulafungin, caspofungin, and micafungin against the three isolates from different geographical areas in Iran. It is notable that only rezafungin was effective in the lethality experiment in mice infected with isolate TMML617. Our current results align with previous observations that correlations do not always exist between in vitro activity and in vivo efficacy [19,24]. A possible explanation for the increased in vivo efficacy of rezafungin against the *C. auris* isolates is its differentiated pharmacokinetic profile (i.e. front-loaded dosing rapidly achieves high concentrations in plasma and tissues) [15,16].

It is well known that the South Asian, the South African and the South American clades are frequently associated with invasive, life threatening infections and large outbreaks, while the East Asian clade is mainly responsible for ear infections. The fact that three isolates from Korea related with bloodstream infections proved to be genetically similar to isolates producing ear infections suggests that in special clinical situations the East Asian clade could become invasive [32]. The same approach may be true for the fifth *C. auris* clade as its virulence in our study was comparable with the previously described four main clades [23,24]. The special microenvironment on the intact skin or external ear allows to produce pseudohyphae in Iranian isolates at 30 °C. In colonized patients disruption of integrity of the skin or mucous membrane may lead to invasion of the deeper tissues and blood due to the increased pseudohyphae production at 37 °C as described in the present study.

In this study the isolates of the fifth clade without echinocandin exposure universally showed pseudohyphae production both in vitro and in vivo, sharply distinguishing this clade from the previously described other four clades [23,24].However, in the presence of echinocandins, isolates belonging to the fifth clade behaved similar as clades I-IV, producing large and small aggregates of blastoconidia in vitro and in vivo. Our current and previous in vivo results suggest that the recently approved rezafungin, and to a lesser extent caspofungin, regardless of the *C. auris* clades are highly effective in the treatment of invasive fungal infections, including myocarditis and pyelonephritis due to *C. auris* [24].

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