Rev Iberoam Micol. 2019;36(1):37–40

Revista Iberoamericana de Micología

www.elsevier.es/reviberoammicol

Note

Antifungal susceptibility of *Saccharomyces cerevisiae* and therapy in a murine model of disseminated infection

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ARTICLE INFO

Article history:
Received 17 October 2017
Accepted 13 April 2018
Available online 11 February 2019

Keywords:
Animal model
Susceptibility
Antifungal therapy
Saccharomyces cerevisiae
Systemic infection

ABSTRACT

Background: The incidence of systemic infections by *Saccharomyces cerevisiae* has increased in recent years, especially among immunocompromised patients. Amphotericin B, voriconazole or echinocandins have been used with favorable outcome against systemic infections by this fungus. However, clinical experience is limited and no *in vivo* studies have been conducted.
Aims: We evaluated the *in vitro* activity of nine antifungal compounds against *S. cerevisiae* and the *in vivo* efficacy of those three antifungals showing the highest *in vitro* activity by using a murine model of systemic infection.
Methods: Minimal inhibitory concentrations (MICs) were determined by the microdilution method against three strains of *S. cerevisiae*. After intravenous infection with 5×10^7 CFUs, animals received liposomal amphotericin B (5 mg/kg), voriconazole (25 mg/kg) or anidulafungin (5 mg/kg). Treatment efficacy was assessed by determining of CFUs/g in liver, kidney, brain, lung and spleen.
Results: 5-Fluorocytosine was the most *in vitro* active compound followed by amphotericin B, voriconazole and anidulafungin. The *in vivo* study showed that liposomal amphotericin B was the most effective drug driving highest fungal clearance.
Conclusions: All treatments reduced the fungal load in comparison to the control group, being liposomal amphotericin B the most effective drug followed by anidulafungin and finally voriconazole.

Sensibilidad antifúngica de *Saccharomyces cerevisiae* en un modelo murino de infección diseminada

Palabras clave:
Modelo animal
Sensibilidad
Tratamiento antifúngico
Saccharomyces cerevisiae
Infección sistémica

RESUMEN

Antecedentes: La incidencia de infecciones sistémicas causadas por *Saccharomyces cerevisiae* ha aumentado en los últimos años, especialmente entre pacientes inmunodeprimidos. A pesar de que la anfotericina B, el voriconazol o las equinocandinas han dado buen resultado en infecciones sistémicas por este hongo, no se han establecido recomendaciones terapéuticas sólidas.
Objetivos: Se evaluó la actividad *in vitro* de nueve antifúngicos frente a *S. cerevisiae* y la eficacia *in vivo* de los tres fármacos con mayor actividad *in vitro* mediante un modelo murino de infección sistémica.
Métodos: Se determinaron las concentraciones mínimas inhibitorias (CMIs) frente a tres cepas de *S. cerevisiae* por el método de microdilución. Después de la inoculación intravenosa con 5×10^7 UFC, los ratones fueron tratados con anfotericina B liposomal (5 mg/kg), voriconazol (25 mg/kg) o anidulafungina (5 mg/kg). La eficacia de los tratamientos se estableció basándose en la determinación de UFC/g en hígado, riñón, cerebro, pulmón y bazo.
Resultados: La 5-fluorocitosina fue el compuesto más activo *in vitro,* seguido por la anfotericina B liposomal, el voriconazol y la anidulafungina. En el estudio *in vivo,* la anfotericina B liposomal fue el fármaco más eficaz en términos de reducción de la carga fúngica y esterilización de los órganos estudiados.

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https://doi.org/10.1016/j.riam.2018.04.004

Conclusiones: Todos los tratamientos redujeron la carga fúngica en comparación con el grupo control, y la anfotericina B liposomal fue el antifúngico más efectivo, seguido de la anidulafungina y el voriconazol.

Saccharomyces cerevisiae is a widely distributed yeast, commonly used in the production of food, alcoholic beverages and different biotechnological processes.[1,3] Despite its beneficial applications, *S. cerevisiae* can also act as a human opportunistic pathogen causing a variety of infections in immunocompromised individuals ranging from localized, genitourinary infections, esophagitis, pneumonia, liver abscess or peritonitis, to systemic infections.[12,18,21,23] The acquisition of infections by *S. cerevisiae* has been recently linked to the use of probiotics or dietary supplements, both of which could represent a source for the disease.[11,16]

According to the current guidelines, the recommended treatment for these infections consists of amphotericin B (AMB) or AMB plus 5-fluorocytosine (5FC) in the most severe cases,[2] but few studies have evaluated the efficacy of other antifungal agents. Due to the good *in vitro* activity shown by posaconazole (PSC), voriconazole (VRC) and AMB against *S. cerevisiae*,[7,14,20,26] these drugs could represent a therapeutic option, although few clinical experience exists. Similarly, echinocandins and fluconazole (FLC) in combination with AMB have shown favorable outcomes against *S. cerevisiae* infections.[9,13,17,21,24]

The aim of the present study was to evaluate the *in vitro* activity of AMB, FLC, PSC, VRC, anidulafungin (AFG), 5FC, itraconazole (ITC), caspofungin (CFG) and micafungin (MFG) against *S. cerevisiae* and to determine the time-kill kinetics as well as the *in vivo* efficacy of the most active compounds.

Three clinical strains of *S. cerevisiae* (FMR 13211, FMR 13212 and FMR 13213) isolated from patients with acute vulvovaginitis were included in the study. Species identification was confirmed by comparing the sequences of large-subunit ribosomal RNA gene of the used strains with those from the type species.

Antifungal susceptibility was assayed according to Clinical and Laboratory Standards Institute (CLSI) document M27-A3,[10] and time-kill studies were performed as previously described[6] by using four-fold serial dilutions in standard RPMI 1640 of AMB, VRC and AFG ranging from 0.06 to 32 μg/ml. At predetermined time points (0, 8, 24 and 48 h) aliquots of 100 μl diluted in sterile water were placed onto PDA plates and incubated at 35 °C for 48 h in order to determine colony forming units (CFU)/ml. Strain ATCC 22019 of *Candida parapsilosis* was used as a quality control and all assays were carried out in duplicate. A fungicidal effect was defined as a reduction of $\geq 3\log_{10}$ in viable colony counts in comparison with the starting inocula, whereas a reduction of $<3\log_{10}$ in colony counts was considered a fungistatic effect.[15]

For *in vivo* studies, male OF-1 mice weighing 30 g (Charles River; Criffa S.A., Barcelona, Spain) were immunosuppressed with cyclophosphamide given intraperitoneally.[8] Animals were housed under standard conditions and care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare Committee.

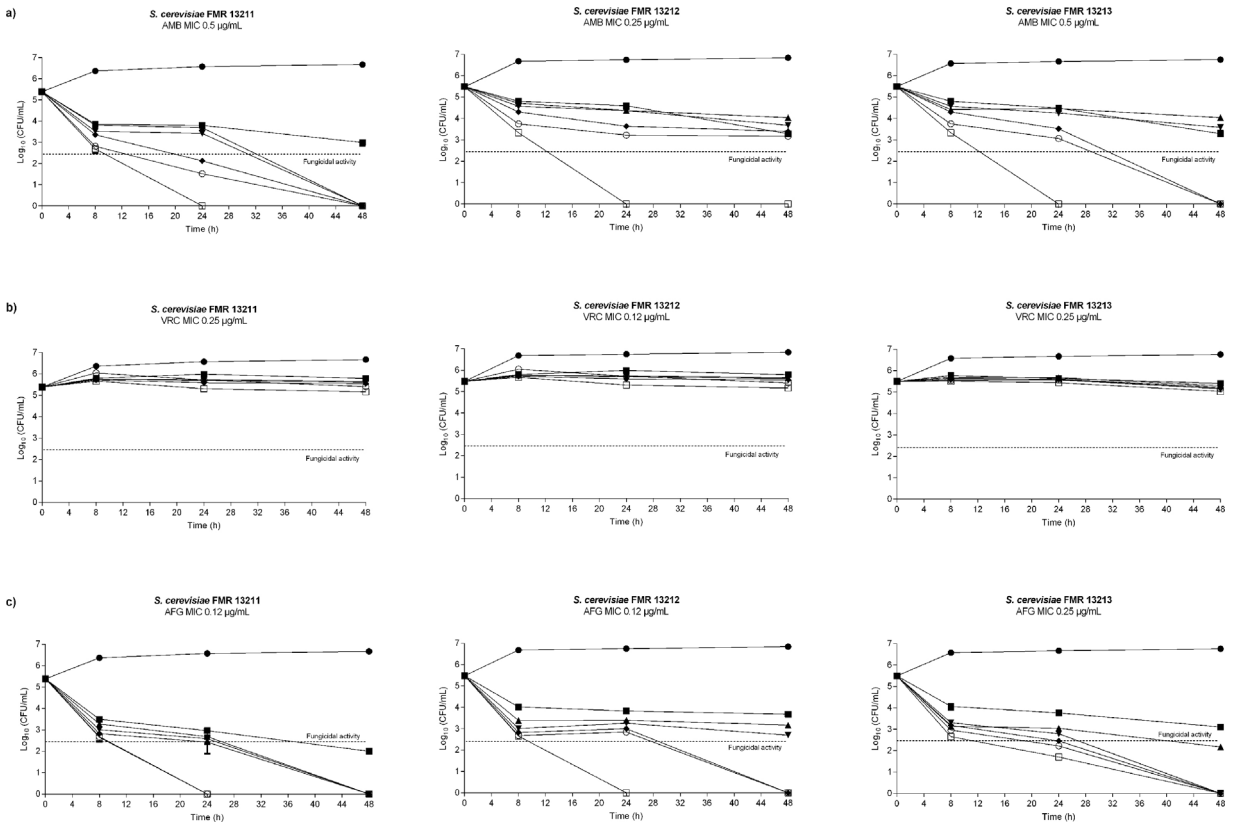

Fig. 1. Time-kill kinetic assay of a) AMB, b) VRC and c) AFG against three strains of *S. cerevisiae*.(■) 0.06 μg/ml, (?) 0.125 μg/ml, (?) 0.5 μg/ml, (♦) 2 μg/ml, (○) 8 μg/ml, (□) 32 μg/ml, (●) control.

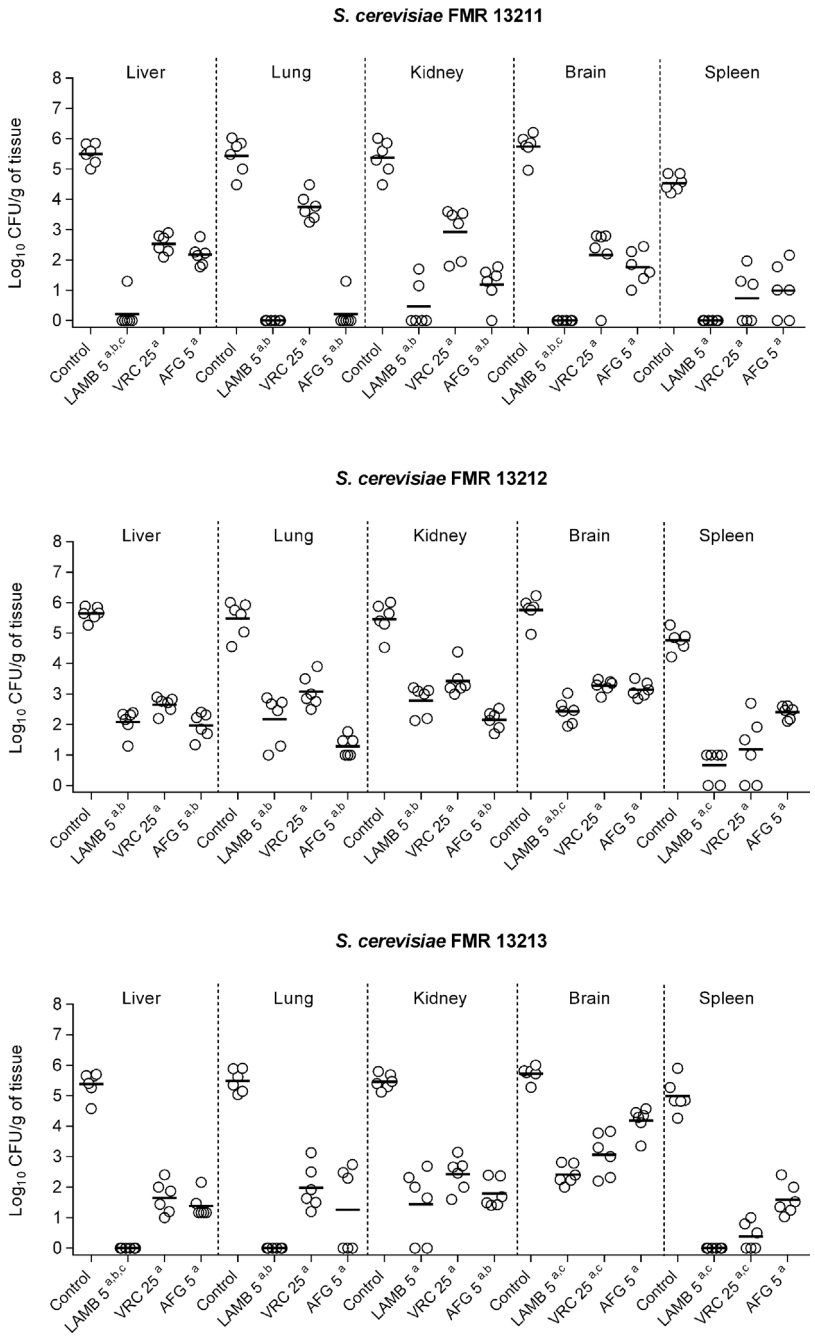

Fig. 2. Fungal load of immunosuppressed mice infected with *S. cerevisiae* FMR 13211, FMR 13212 and FMR 13213. LAMB 5, liposomal amphotericin B at 5 mg/kg i.v. QD; VRC 25, voriconazole at 25 mg/kg p.o. QD; AFG 5, anidulafungin at 5 mg/kg i.p. QD. Horizontal lines indicate median values. [a] $p \leq 0.05$ *versus* control; [b] $p \leq 0.05$ *versus* VRC 25; [c] $p \leq 0.05$ *versus* AFG 5.

For each strain and drug assayed, 8 mice were included. Mice were infected intravenously (i.v.) *via* the lateral tail vein with 5×10^7 CFU in 0.2 ml of 0.9% saline. Therapies consisted of liposomal AMB (LAMB) (AmBisome; Gilead Sciences S.A., Madrid, Spain) administered i.v. at 5 mg/kg once daily (QD), VRC (Vfend, Pfizer S. A., Madrid, Spain) at 25 mg/kg given orally by gavage (p.o.) QD or AFG (Ecalta, Pfizer S.A.) at 5 mg/kg intraperitoneally (i.p.) QD. *In vivo* assayed drugs were chosen according to the *in vitro* results obtained, and doses were selected based on previous studies.[5,27] From 3 days before infection, mice treated with VRC received grapefruit juice instead of water, as grapefruit juice is an inhibitor of cytochrome P450 enzymes, which display an extensive metabolism in mice resulting in elevated drug clearance.[25] Control groups received no treatment. Drug efficacy was evaluated according to the fungal burden reduction in brain, liver, spleen, lungs and kidneys. Despite the high *in vitro* activity of 5FC, this drug was not included into the *in vivo* study due to its known toxicity.[19] At day 8 post-infection animals were euthanized by CO_2 inhalation and organs were aseptically removed, homogenized in 1 ml of sterile saline, ten-fold diluted and placed onto PDA for CFU/g determination. Results from the tissue burden studies were analyzed using the Mann–Whitney *U*-test by Graph-Pad Prism 6.0 for Windows (GraphPad Software, San Diego California USA). A p value of ≤ 0.05 was considered statistically significant.

Despite FLC has been used in combination with AMB to treat systemic infections caused by *Saccharomyces*, this drug showed no *in vitro* activity (MIC ≥ 32 μg/ml) against the strains we tested. This has also been reported by others.[7,20] The rest of the assayed

Table 1
Results of antifungal susceptibility testing.

Antifungal agent	MIC (μg/ml)		
	FMR 13211	FMR 13212	FMR 13213
Amphotericin B	0.25	0.25	0.5
Itraconazole	1	1	0.5
Fluconazole	≥32	≥32	≥32
Posaconazole	0.5	0.5	0.25
Voriconazole	0.25	0.12	0.25
Anidulafungin	0.12	0.12	0.25
Caspofungin	0.25	0.25	0.25
Micafungin	0.25	0.25	0.25
5-Fluorocytosine	≤0.03	≤0.03	≤0.03

MIC: minimal inhibitory concentration.

antifungals showed greater activity (MIC ≤ 1 μg/ml), being 5FC the most active compound (MIC ≤ 0.03 μg/ml) (Table 1). In the time-killing assay, VRC displayed fungistatic activity while AMB and AFG showed fungicidal effect in a concentration dependent manner starting at a drug concentration of 0.06 μg/ml at 8 h in both cases (Fig. 1).

In the *in vivo* study, control animals showed high fungal load in all studied organs (ranging from 10^5 to 10^6 CFU/g tissue) with the exception of the spleen, in which fungal load was slightly lower (10^4–10^5 CFU/g) (Fig. 2). All assayed treatments were significantly effective in burden reduction from all organs in comparison with the control group regardless of the infecting strain ($p \leq 0.0043$). AMB, which is the recommended drug against *Saccharomyces* infections, showed fungicidal activity correlating with our *in vivo* results, as it displayed the highest efficacy against all strains with great clearance effect in its liposomal formulation, LAMB (Fig. 2). LAMB was especially effective against strains FMR 13211 and FMR 13213, for which fungicidal effect was observed at MIC concentration, resulting in undetectable CFUs from lung, kidney and spleen and in significant CFU reduction from liver and brain in comparison to VRC or AFG treatments ($p = 0.0022$). The obtained results with LAMB sustain clinical reports that, although scarce, have shown efficacy of AMB-based therapies.[13] In our case, AFG was the second most effective therapy corroborating previous observations of echinocandins efficacy against *Saccharomyces* in the clinical settings.[9,17,21] Both drugs, LAMB and AFG exhibited similar efficacy against all assayed strains independently of the MIC and the kill-curves obtained. Finally, as previously reported,[4,22] VRC displayed good activity although it showed lower efficacy than LAMB or AFG treatments.

Guidelines for infections by *S. cerevisiae* recommend the use of LAMB and echinocandins, as well as the discontinuation of *S. cerevisiae* as probiotic, especially in vulnerable populations.[2] Although a few strains have been tested, our study contributes with new evidence to prove LAMB treatment effectiveness against experimental invasive infection by *S. cerevisiae*, as well as the potential use of AFG (and in lesser extent, VRC) as alternative treatments for disseminated infections caused by this fungus.

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