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Strong correlation between the antifungal effect of amphotericin B and its inhibitory action on germ-tube formation in a *Candida albicans* URA⁺ strain

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Summary. The hypothetical capacity of amphotericin B to suppress the formation of germ-tubes, which is the first step of yeast-to-hypha conversion in *Candida albicans*, has been investigated in the wild-type strain CEY.1 (CAI.4-URA⁺). Exponential cells exposed to concentrations of amphotericin B below or around the MIC₉₀, exhibited a weak reduction in the percentage of human serum-induced germ-tube formation at 37°C compared with a non-exposed control. However, the dimorphic transition was drastically suppressed after addition of potentially lethal doses of amphotericin B, which also caused severe cell killing. In contrast, an identical experimental approach carried out with the fungistatic compound 5-fluorocytosine had no significant effect on the level of the germ-tube formation. Together, these results strongly point to a close correlation between the fungicidal action of amphotericin B and its ability to impair morphogenetic conversion in *C. albicans*. [Int Microbiol 2015; 18(1):25-31]

Keywords: *Candida albicans* · amphotericin B · 5-fluorocytosine · germ-tube · cell killing

Introduction

Antifungal therapy is less developed than the antibacterial equivalent. However, the striking increase in morbidity and mortality caused by invasive mycosis, which mainly affects the human immunocompromised population, demands new effort in this pharmaceutical field [22,24,25]. Together with the development of safer, more potent and less toxic compounds, another line of work involves improving the currently

available arsenal of fungicidal antibiotics [1]. A good example of this pursuit is the polyene macrolide amphotericin B (AmB). Since its discovery in the 1950s [20] AmB has been the predominant compound utilized in clinical practice for the treatment of systemic infections caused by species of *Candida*, *Aspergillus*, *Cryptococcus* and many other pathogenic fungi. However, this extensive use has provoked some toxic side-effects (nephrotoxicity and hepatic damage, mainly), which have successfully been surmounted by new liposomal formulations, permitting its current extensive utilization against invasive candidiasis [13,19]. Interestingly, clinical resistance to AmB remains extremely rare despite 50 years of use as a monotherapy [28].

Many clinical trials have taken as a target the polymorphic opportunistic yeast *Candida albicans*, which is still the most

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prevalent fungal pathogen in humans, where it is responsible for mucosal and disseminated infections [7,19,22,25]. Furthermore, the incidence of *C. albicans* infections in neonates and patients subjected to extensive surgery therapy, as well as nosocomial bloodstream candidemia, has increased noticeably in the last two decades [7,19,22,24]. Several studies on *C. albicans* suggest that AmB may exert a complex mode of action, interacting with different cellular targets. Although the specific binding to the membrane ergosterol triggers perturbations in the selective membrane permeability that eventually lead to cell killing, the formation of pores and membrane instability has been dissociated from the fungicidal effect [21]. Other evidences gathered in *C. albicans* and *Cryptococcus neoformans* [29] involve the generation of oxidative stress through the release of reactive oxygen species, would reinforce the fungicidal effect of AmB-induced cell damage [4,6,9,16].

Candida albicans is a polymorphic organism able to grow as a unicellular budding yeast or as mycelial forms (hypha and pseudohypha). The morphological transition from yeast to hypha has been considered as a contributory factor of virulence [10,15]. It has been proposed that exposure to AmB causes a dose-dependent severe reduction in the germ-tube development, which is the first step of hypha formation [8,26]. In fact, the success obtained with early therapies that used AmB to treat oral candidiasis seemed to involve a significant reduction in the percentage of germ-tube formation, which is the first step in the yeast-to-hypha conversion of this opportunistic pathogen [8]. Oral candidiasis remains among the main manifestations of mycosis caused by *C. albicans*, including a great proportion of HIV-infected patients [18]. However, such observations are far from conclusive [7,24,25]. We have examined this suggestion in a prototypic wild-type *C. albicans* URA3⁺ strain, by testing putative effects on dimorphism and cell viability triggered by the polyene AmB and the fungistatic antibiotic 5-fluorocytosine (5-FC), a pyrimidine analogue that interferes with DNA synthesis by reducing the available nucleotide pool [12]. Our rationale was to try to dissociate morphological inhibition from killing cell processes. According to our data, the blockage of germ-tube development seems to be a direct consequence of the previous drastic fungicidal action of AmB.

Material and methods

Yeast strains and culture conditions. Because an *ura3* auxotrophy might have side effects on the physiology and virulence of *C. albicans*, a CEY.1 (CAI4-URA3⁺) strain was used throughout this study. A detailed description of the constructions and procedures followed to obtain this strain is

reported elsewhere [23]. This same strain has been used in previous research on the antifungal action of AmB [11]. Yeast cell cultures were grown at 28°C. They were shaken in a medium consisting of 2% peptone, 1% yeast extract and 2% glucose (YPD). Strains were maintained by periodic subculturing in solid YPD.

Human-serum induction of germ tube formation. For germ-tube induction, cultures were either directly supplemented with 10% human blood serum or, alternatively, samples were harvested at different stages of growth, quickly washed with water and resuspended at a density of $1-5 \times 10^6$ cells/ml in YPD prewarmed to 37°C together with 10% human serum. Before addition, serum was sterilized by filtration (0.45 µm). The appearance of germ tubes was monitored as indicated by phase contrast light microscope with a haemocytometer. When required, clumped cells were dispersed prior to microscopic examination by mild sonication (10–15 s). At least 250 cells were counted each time and the percentage of dimorphism was represented as the ratio of germ tube-forming cells to the total number of cells [3,23].

AmB and 5-FC treatments. AmB was obtained from Sigma (80% purity) and prepared in DMSO (100%). It was then maintained at room temperature for autosterilisation, since it is not possible to filter the compound. Because the solutions are all very light-sensitive, they were manipulated in dark conditions. 5-FC was also from Sigma, and was dissolved in MiliQ water.

Cultures were grown in YPD until the exponential phase ($OD_{600} = 0.3$) and then divided into several identical aliquots, which were treated with different concentrations of AmB or 5-FC and incubated at 28°C with shaking. The samples were harvested at the indicated times and viability was determined after appropriate dilution of the samples with sterile water by plating in triplicate on solid YPD. Between 30 and 300 colonies were counted per plate. Survival was normalized to control non-treated samples (100% viability).

MICs determination. The MIC₉₀ for AmB and 5-FC against CEY.1 (URA3⁺) cells was determined according to a normalized protocol for yeasts (EUCAST with minor modifications). Briefly, a microtiter plate (96 wells) (Brand 781660, Wertheim, Germany) was filled with 100 µl of different concentrations of AmB (0.0125; 0.025; 0.05; 0.1; 0.2; 0.4; 0.8; 1.6; 3.2; 6.4 and 12.8 µg/ml) in RPMI 1640 medium plus 2% glucose buffered at pH 7.0 with MOPS 0.164 M. One hundred µl of a yeast suspension in sterile saline solution (10^5 cells/ml) was added to each well. The plates were incubated at 35°C for 24 h and 48 h and read at 550 nm on a microtiter reading instrument (Asys Jupiter). In accordance with the EUCAST protocol, the strain *Candida parapsilosis* ATCC 22019 has been included as quality control. The MIC was defined as the lowest concentration which inhibited 90% of the cell growth.

Results

Effects of AmB treatments on germ-tube formation and cell killing in the *C. albicans* CEY.1 (URA) strain.

In a set of preliminary experiments, we have determined the MIC₉₀ values for CEY.1 (CAI4-URA3⁺) cells against AmB and 5-FC. They were 0.12 and 0.25 mg/l, respectively. The quality control required by the EUCAST procedure was included to ensure the validity of the calculations. These concentrations are within the range previously reported for other *C. albicans* genetic backgrounds of clinical or laboratory origin [8].

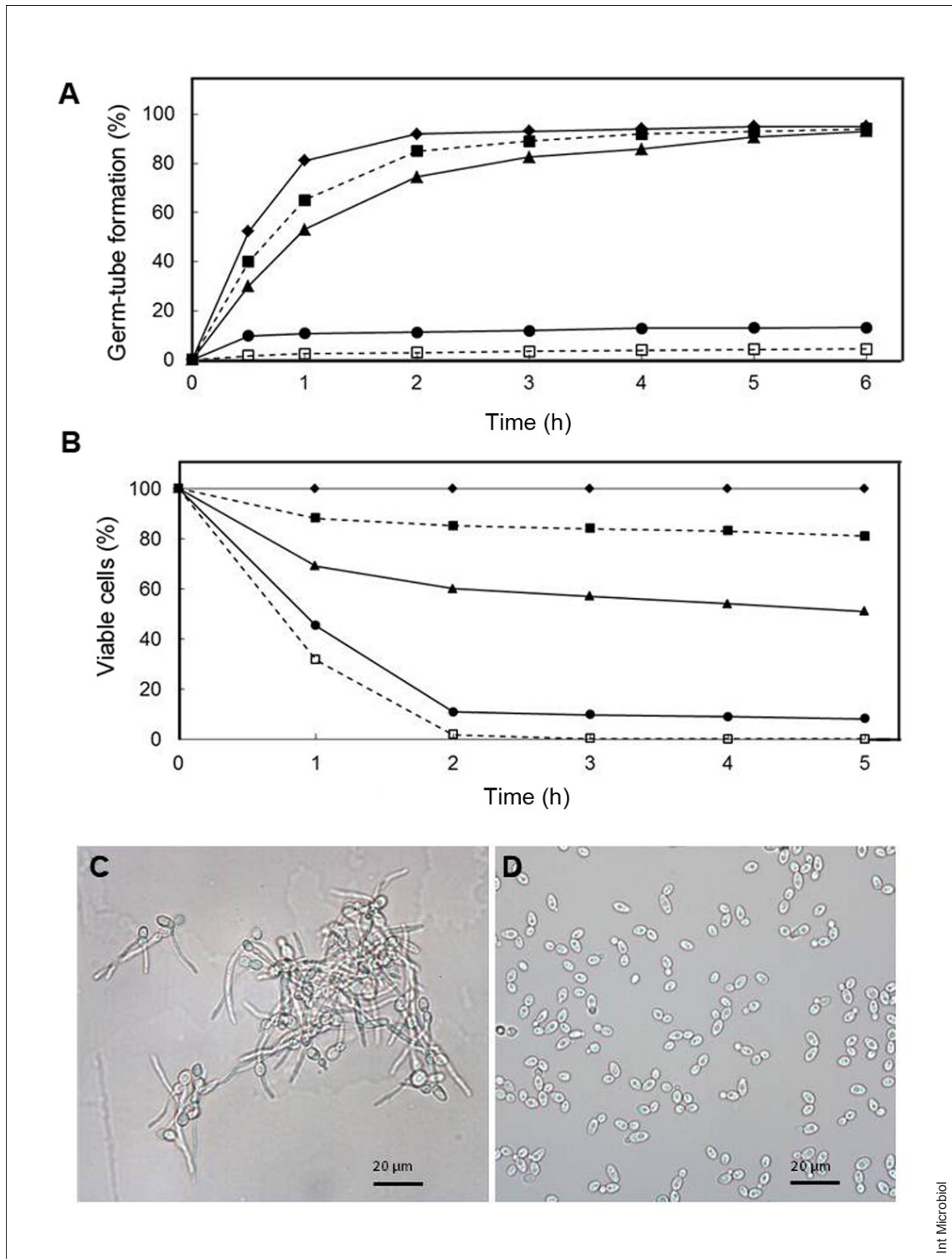


Fig. 1. Effect of rising concentrations of amphotericin B (AmB) on the time-course of germ-tube formation induced by human serum (A), the percentage of survival (B) and the microscopic cell morphologies (C and D) in the wild type strain CEY.1 (CAI-4 URA⁺) of *C. albicans*. The cultures were incubated at 28°C in YPD until early exponential phase (OD = 0.3), harvested, washed and resuspended at a cellular density of 2×10^6 cells/ml in YPD supplemented with 10% human serum and immediately transferred at 37°C. Identical samples were supplemented at time zero with the following doses of AmB (mg/l): 0 (control, ◆), 0.05 (■); 0.1 (▲); 0.5 (●) and 1.0 (□). Error bars are omitted for the sake of clarity, but the standard deviation was always lower than 12%. (A and B). Serum-induced samples were photographed after 5 h of treatment with 0.05 mg/l (C) or 0.5 mg/l (D) AmB.

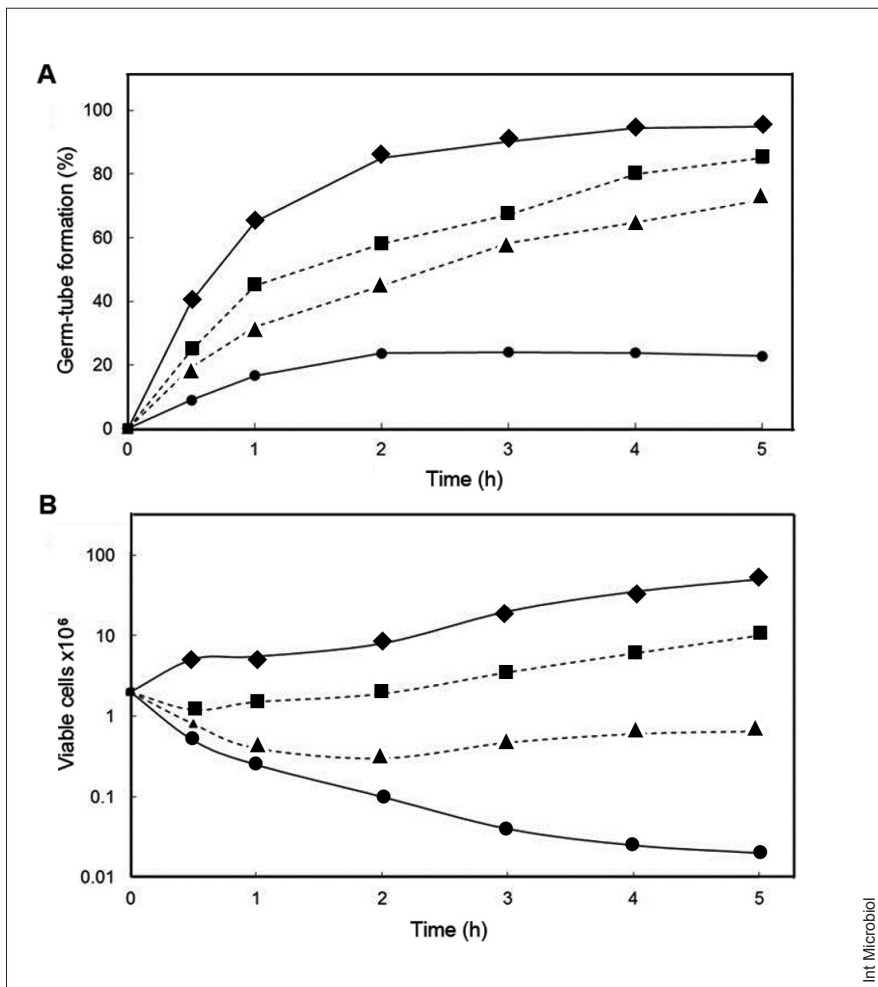


Fig. 2. Reversible action of AmB on human serum-induced germ-tube formation (A) and cell viability (B) in *C. albicans* CEY.1 strain. An exponential culture growing on YPD at 28°C was divided into identical aliquots, which were quickly supplemented with 10% human serum and transferred to 37°C. Then, they were subjected to the following treatments: addition of 0.5 mg/l AmB for 30 min (■) or 60 min (▲) followed by rapid washing out AmB and resuspension in YPD plus human serum (time zero). Alternatively, control samples were kept without AmB (◆) or with that toxic concentration of AmB throughout the assay (●). More details in Figure 1.

The addition of 10% human serum together with a temperature up-shift from 28 to 37°C has been demonstrated as an efficient procedure for inducing filamentation in *C. albicans* [3,11,23]. This was also the case for the wild-type strain CEY.1 (CAI.4-URA⁺). Exponentially-growing yeast cells (blastocidia) in a glucose-rich medium (YPD) are fully competent to enter the dimorphic program when they are transferred to the same medium prewarmed at 37°C, supplemented with human serum and further incubated at 37°C (Fig. 1A). It has been postulated that sub-lethal concentrations of AmB induce a complete suppression of the capacity to issue germ-tubes [8,26]. In the present study, the effect of several

doses of AmB on the degree of human serum-induced initial filamentation in CEY.1 growing cells was studied. As shown in Figure 1A, the addition of AmB doses below or about the MIC₉₀ value had a negligible effect on the level of germ-tube formation (about 10% reduction at 0.1 mg/l AmB; Fig. 1A). It was necessary to increase the AmB concentration to potentially lethal levels (0.5 or 1.0 mg/l) in order to bring about a significant decay of mycelial outgrowth in comparison with a control sample maintained at 37°C (Fig. 1A).

In equivalent cell aliquots withdrawn from the same cultures, the effect of those selected AmB doses on the degree of cell killing was analyzed. The time-course percentage of via-

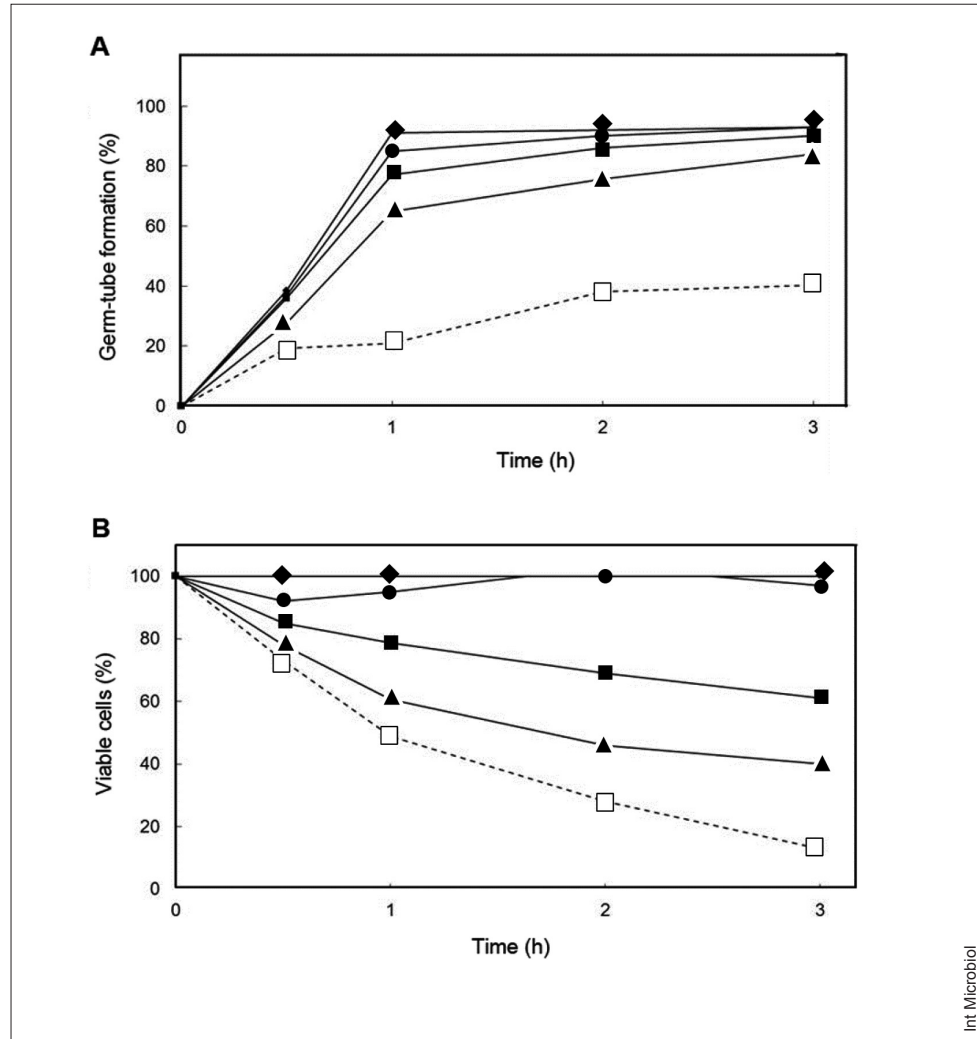


Fig. 3. Level of dimorphic conversion (A) and cell growth (B) in CEY.1 cells induced by several concentrations of the fungistatic antibiotic 5-fluorocytosine (5-FC). A growing exponential culture was processed as indicated in the legend for Figure 2. Identical aliquots were treated with the following concentrations of 5-FC (mg/l): 0 (◆), 0.25 (●), 1.0 (■) and 2.5 (▲). A sample containing 0.25 mg/l of AmB was introduced as control of the positive antifungal effect (□). Error bars are omitted for the sake of clarity, but the standard deviation was always lower than 12%.

ble CEY.1 blastoconidia underwent a minor reduction upon the addition of tolerated concentrations of AmB (ca. 20% at 0.05 mg/l and 40% at 0.1 mg/l) (Fig. 1B), whereas fungicidal doses of the polyene ($4 \times \text{MIC}_{90}$ and $8 \times \text{MIC}_{90}$) caused a more drastic and progressive loss of viable cells (Fig. 1B). Optical examination of samples treated for 1 h with 0.05 mg/l AmB showed normal serum-induced germ-tube emergence (Fig. 1C), whereas a 10-fold increase in the AmB concentration (0.5 mg/l) completely prevented the dimorphic transition (Fig. 1D). Hence, these data strongly suggest that the suppressive action on germ-tubes emergence in *C. albicans* after AmB supply might likely be an off-side indirect action de-

rived from its intrinsic fungicidal power rather than a direct target. Interestingly, AmB triggers a conspicuous individual reduction of cell size in yeast cultures [11].

In order to confirm this putative relationship between the antifungal power and suppression of hypha formation exerted by AmB, the following experiment was carried out. Growing CEY.1 blastoconidia were adjusted to a similar cell density (2×10^6 cells/ml) and preincubated with a toxic concentration of AmB (0.5 mg/l) for 30 or 60 min. Then, the antifungal was removed by washing the cells, which were quickly resuspended in human serum (10%) at 37°C at an identical cell density. The degree of germ-tube development and the percentage of

cell survival were determined in parallel. According to the results presented in Figure 2, pretreatment with 0.5 mg/l AmB caused a time-dependent loss of viable cells (30 or 60 min) (Fig. 2B). After that, however, the surviving fraction was still able to resume active growth to a certain extent as well as to enter the dimorphic program by producing conspicuous germ-tubes (Fig. 2; results not shown). This dimorphic conversion markedly increased as the incubation period was prolonged (Fig. 2A). As expected, a sample subjected to the permanent presence of AmB lost its capacity to form germ-tubes, the reduction in cell viability being drastic and the subsequent level of survival extremely low (Fig. 2A and B). In turn, in a control culture treated with human serum at 37°C, the degree of myceliation was higher than 80% after 2 h (Fig. 2A). In this set of experiments, a control sample was maintained at 37°C without serum addition, and the level of germ-tube formation was about 10–20% (results not shown).

Analysis of the action of 5-FC. The suppressive action triggered by fungicidal exposure of AmB on the yeast-to-hypha conversion capacity in *C. albicans* was also reinforced by studies carried out with the fungistatic compound 5-fluorocytosine (5-FC). In exponential CEY-1 cells, the addition of concentrations around the MIC_{90} (0.25 mg/l) had no relevant effect on the percentage of filamentation or cell viability (Fig. 3A and 3B). A moderate increase ($4 \times MIC_{90}$) in the dose of 5-FC only caused a partial and sustained decrease in the level of survival and had an irrelevant effect on dimorphic conversion, which was manifested by the rather similar ability to produce germ-tubes (Fig. 3B). The effect of 5-FC was not dose-dependent, since an additional rise ($10 \times MIC_{90}$) only caused a weak additional decrease in the level of survival and the percentage of germ-tube formation (Fig. 3). In this assay, an aliquot exposed to a lower concentration of AmB (0.25 mg/l) was introduced as a positive control of the fungicidal action. As expected, the polyene provoked a severe degree of cell killing and, simultaneously, a pronounced inhibition in the ability to issue germ tubes (Fig. 3).

Discussion

The morphological conversion from yeast cells (blastozoonidia) to mycelial structures (hypha and/or pseudohypha) in *C. albicans* has been considered a factor of virulence, although the matter is open to dispute [10]. The genetic evidence is not entirely conclusive: whereas some mutants unable to filament are avirulent [15], homozygous null mutants deficient in the

MAP kinase HOG1 and in the transcriptional repressor factor TUP1 show a phenotype of hyperfilamentation but lack infectivity in a murine model for systemic candidiasis [2,5]. Furthermore, in other dimorphic fungi with great incidence in pathogenesis, e.g. *Histoplasma capsulatum* or *Blastomyces dermatitidis*, yeast-like cells instead of mycelia are the predominant invasive morphology in clinical mycoses [10]. These fungi are very susceptible to both AmB and azoles [13,14]. In turn, *Candida glabrata*, which displays high clinical incidence due to its inherent resistance towards usual antifungals, shows a typical growth as oval yeast cells [17].

In this context, a strong and inhibitory action of the polyene AmB on the ability to produce germ-tubes in *C. albicans* has been proposed [8,26], although the mechanism for this suggested targeted inhibition remains unknown. However, the evidence provided in this study seems to dismiss this proposal and strongly points to the fungicidal action triggered by AmB as the main cause of the reduced formation of germ tubes in *C. albicans*. Thus, exponential blastozoonidia of the wild type CEY.1 strain (URA⁺) did not show significant loss of cell growth upon addition of sublethal concentrations of AmB (Fig. 1B) and were able to enter the dimorphic program, issuing conspicuous germ tubes (Fig. 1A and 1C), although with a certain delay compared to the untreated samples (Fig. 1A). Only the treatment with toxic doses of AmB impeded the formation of evident mycelial structures (Fig. 1D), probably due to the halt in cell growth (Fig. 1B).

On the other hand, these harmful effects on cell growth and hypha formation are reversible, conditioned to the presence of the antifungal in the culture broth. When a $4 \times MIC_{90}$ concentration of AmB was applied for a short time (30 or 60 min) and then quickly removed from the medium, the cells suffered an initial decay in cell survival with no production of germ-tubes as long as the antifungal was present (Fig. 2). But, afterwards, they were able to resume active growth (Fig. 2B) and showed a capacity to undergo a noticeable dimorphic transition, which had remained unaffected during the transitory exposure to AmB (Fig. 2A). Similar experiments performed with the fungistatic 5-FC provided equivalent results and confirmed the validity of this proposal (Fig. 3).

The relationship between fungicidal action and hypha formation might be relevant for clinical therapeutic purposes, particularly in the light of the ability of *C. albicans* to develop biofilms as a main pathogenesis mechanism [27]. Laboratory studies have proven that *C. albicans* biofilms have high intrinsic resistance to several antifungals, including polyenes, 5-fluorocytosine (5-FC) and azoles [27], although they still remain susceptible to the application of echinocandins and

some lipid preparations of AmB, in the range of doses commonly used in clinical assays. Because fungal biofilms are heterogeneous communities of blastospores, pseudohyphal and hyphal cells embedded in an extracellular complex matrix, experiments on the susceptibility of each population to AmB should be performed to clarify this matter. In conclusion, we exclude yeast-to-hypha conversion as the main target of AmB. Rather, early AmB-induced germ-tube inhibition seems to be a side effect derived from the drastic lytic action of the polyene on cell integrity.

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Competing interests. None declared.

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