RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2015) 18:25-31
doi:10.2436/20.1501.01.231. ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

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

José P. Guirao-Abad, Pilar González-Párraga, Juan-Carlos Argüelles*

Microbiology Area, School of Biology, University of Murcia, and IMIB-Arrixaca, Murcia, Spain

Received 22 December 2014 · Accepted 30 March 2015

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 MIC_{90}, exhibited a weak reduction in the percentage of human serum-induced germ-tube formation at 37ºC 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 polimorphic opportunistic yeast *Candida albicans*, which is still the most

*Corresponding author: J.C. Argüelles
Area de Microbiología, Facultad de Biología
Campus de Espinardo, Universidad de Murcia
30071 Murcia, Spain
Tel. + 34-868887131. Fax +34-868883963
E-mail: arguelle@um.es

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 nosocomical 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 polimorphic 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 28ºC. 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 37ºC 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 28ºC 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 MIC_{90} 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 MIC_{90} values for CEY.1 (CAI.4-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].

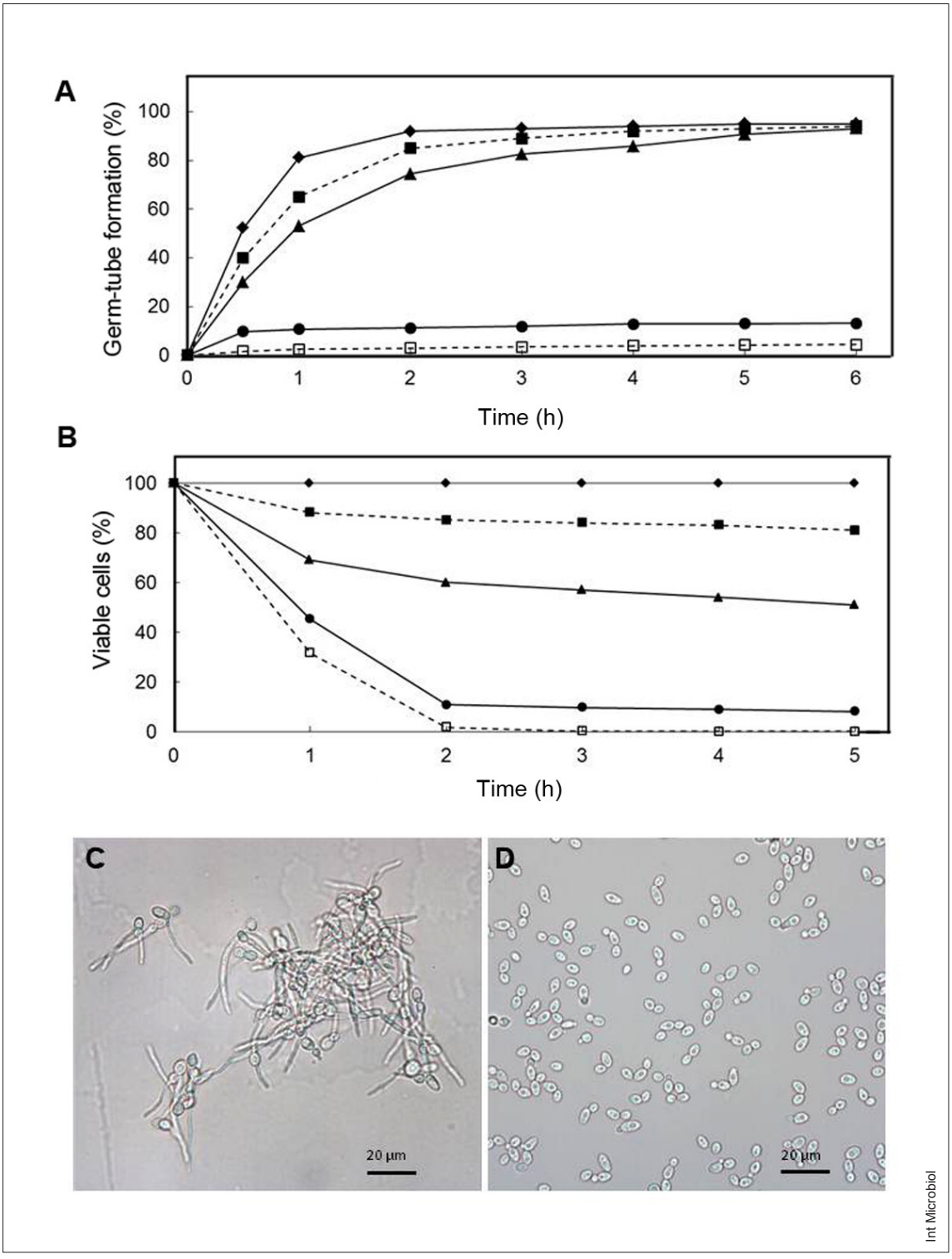

Fig. 1. Effect of rising concentrations of amphotericin B (AmB) on the time-course of germ-tube formation induced by human serum (**A**), the percentage of survival (**B**) and the microscopic cell morphologies (**C** and **D**) in the wild type strain CEY.1 (CAI-4 URA$^+$) of *C. albicans*. The cultures were incubated at 28ºC 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 37ºC. 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.

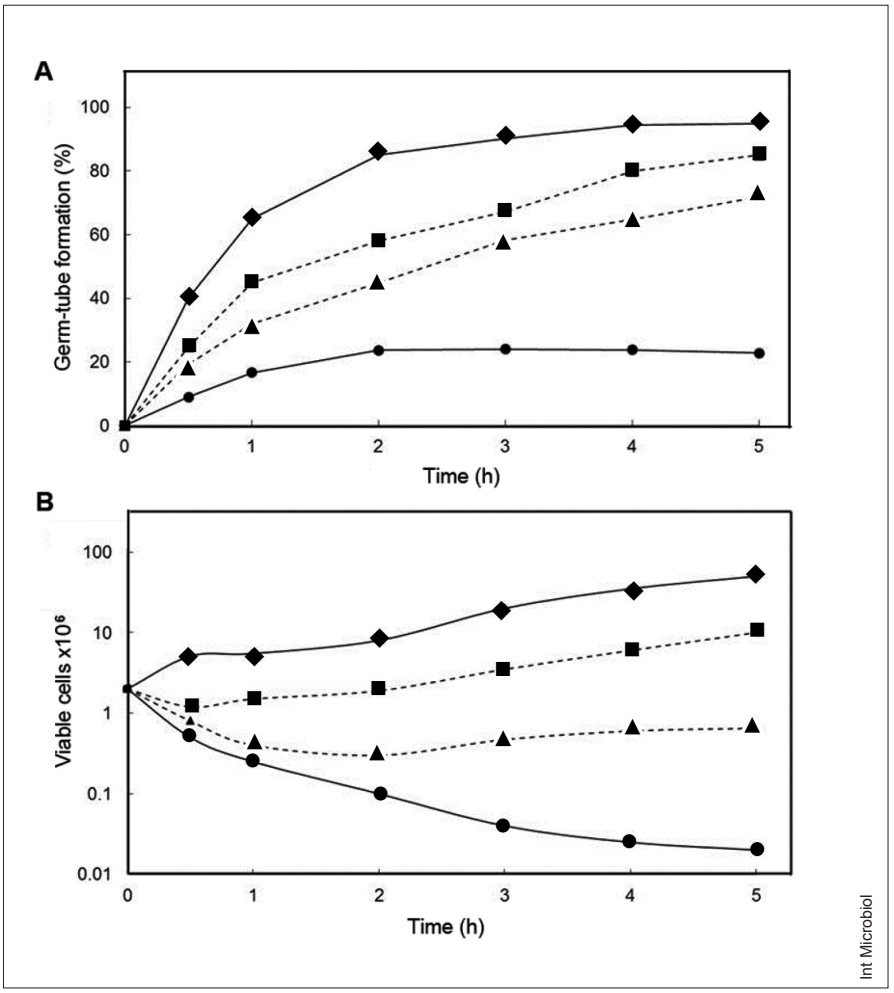

Fig. 2. Reversible action of AmB on human serum-induced germ-tube formation (**A**) and cell viability (**B**) in *C. albicans* CEY.1 strain. An exponential culture growing on YPD at 28ºC was divided into identical aliquots, which were quickly supplemented with 10% human serum and transferred to 37ºC. 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 37ºC 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 (blastoconidia) in a glucose-rich medium (YPD) are fully competent to enter the dimorphic program when they are transferred to the same medium prewarmed at 37ºC, supplemented with human serum and further incubated at 37ºC (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 MIC_{90} 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 37ºC (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-

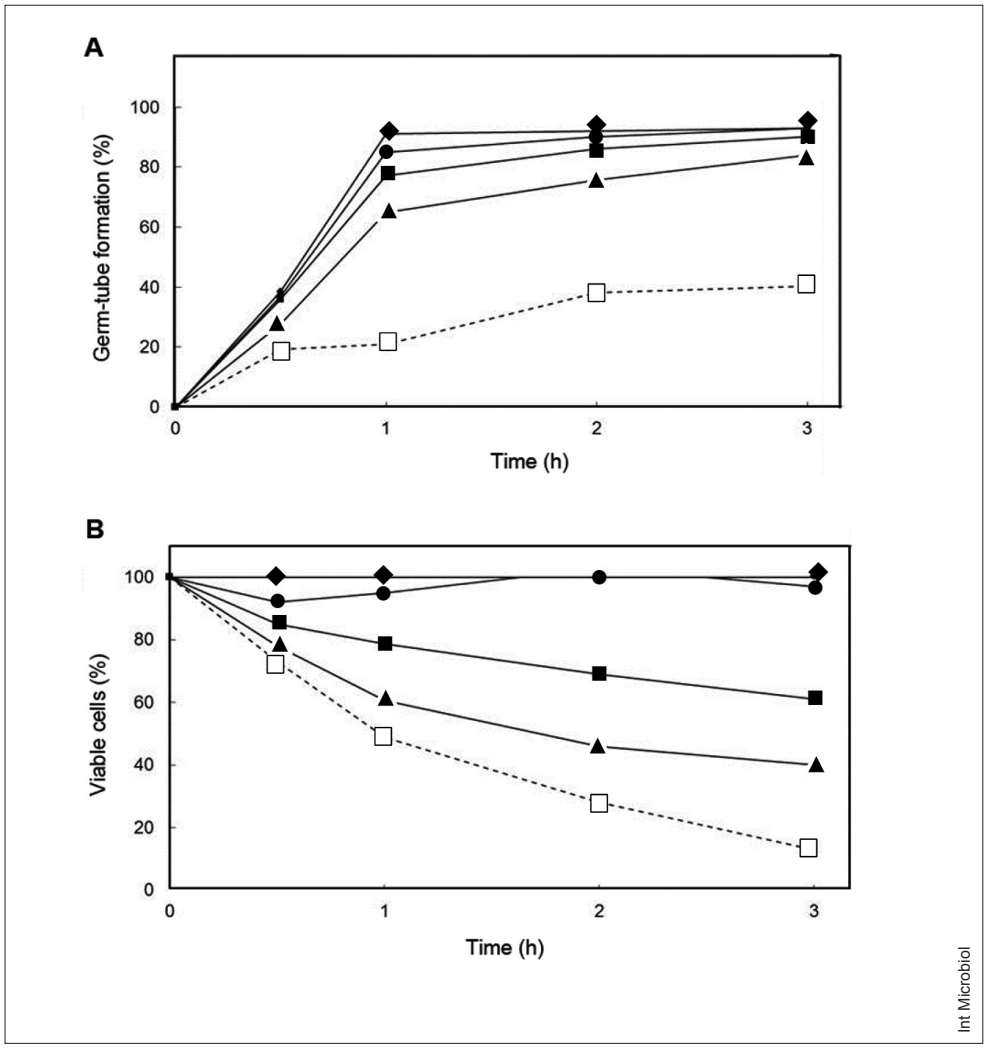

Fig. 3. Level of dimorphic conversion (**A**) and cell growth (**B**) in CEY.1 cells induced by several concentrations of the fungistatic antibiotic 5-fluorocytosine (5-FC). A growing exponential culture was processed as indicated in the legend for Figure 2. Identical aliquots were treated with the following 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 × MIC_{90} and 8 × 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 derived 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 ($2x10^6$ 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 37ºC 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). Afterthat, however, the surviving fraction was still able to resume active grow 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 37ºC, the degree of myceliation was higher than 80% after 2 h (Fig. 2A). In this set of experiments, a control sample was maintained at 37ºC 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 × 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 × 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 (blastoconidia) 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 blastoconida 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 × 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 therapeutical 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.

Acknowledgements. We thank Dr. O. Zaragoza (Instituto de Salud Carlos III, Madrid) for the critical reading of the manuscript, useful suggestions and warm support. J.P.G-A received a partial fellowship from VitalGaia, S.L. The experimental work was supported by grant PI12/01797 (Ministerio de Economía y Competitividad, ISCIII, Spain). We are also indebted to the financial contract provided by Cespa, Servicios Auxiliares de Murcia, S.A.

Competing interests. None declared.

References

1. Akins RA (2005) An update on antifungal targets and mechanisms of resistance in *Candida albicans*. Med Mycol 43:285-318
2. Alonso-Monge R, Navarro-García F, Molero G, Diez-Orejas R, Gustin M, Pla J, Sánchez M, Nombela C (1999) Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. J Bacteriol 181:3058-3068
3. Argüelles JC, Rodriguez T, Alvarez-Peral FJ (1999) Trehalose hydrolysis is not required for human serum-induced dimorphic transition in *Candida albicans*: evidence from a tps1/tps1 mutant deficient in trehalose synthesis. Res Microbiol 150:521-529
4. Belenky P, Camacho D, Collins JJ (2013) Fungicidal drugs induce a common oxidative-damage cellular death pathway. Cell Reports 3:350-358
5. Braun BR, Johnson AD (1997) Control of filament formation in *Candida albicans* by the transcriptional repressor *TUP1*. Science 277:105-109
6. Cao Y, Zhu Z, Chen X, Yao X, Zhao L, Wang H, Yan L, Wu H, Chai, Y, Jiang Y (2013) Effect of Amphotericin B on the metabolic profiles of *Candida albicans*. J Proteomics 12:2921-2932
7. Cisterna R, Ezpeleta G, Telleria O, Guinea J, Regueiro B, Rodriguez J, Esperalba J (2010). A nationwide sentinel surveillance of bloodstream *Candida* infections in 40 tertiary care hospitals in Spain. J Clin Microbiol 48:4200-4206
8. Ellepola AN, Samaranayake LB (1998) The effect of limited exposure to antifungal agents on the germ tube formation of oral *Candida albicans*. J Oral Pathol Med 27:213-219
9. González-Párraga P, Sánchez-Fresneda R, Zaragoza O, Argüelles JC (2011) Amphotericin B induces trehalose synthesis and simultaneously activates an antioxidant enzymatic response in *Candida albicans*. Biochim Biophys Acta 1810:777-783
10. Gow NAR, Brown AJP, Odds FC (2002) Fungal morphogenesis and host invasion. Curr Opin Microbiol 5:366-371
11. Guirao-Abad JP, Sánchez-Fresneda R, Valentin E, Martínez-Esparza M, Argüelles JC (2013) Analysis of Validamycin A as a potential antifungal compound against *Candida albicans*. Int Microbiol 16:217-225
12. Hope WW, Tabernero L, Denning DW, Anderson MJ (2004). Molecular mechanisms of primary resistance to flucytosine in *Candida albicans*. Antimicrob Agents Chemother 48:4377-4386
13. Klepser M (2011). The value of amphotericin B in the treatment of invasive fungal infections. J Crit Care 26:225.e1-10
14. Li RK, Ciblak MA, Nordoff N, Pasarell L, Warnock DW, McGinnis MR (2000). In vitro activities of voriconazole, itraconazole, and amphotericin B against *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum*. Antimicrob Agents Chemother 44:1734-1736
15. Lo HJ, Köhler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR (1997) Nonfilamentous *Candida albicans* mutants are avirulent. Cell 90:939-949
16. Mesa-Arango A, Trevijano-Contador N, Román E, Sánchez-Fresneda R, Casas C, Herrero E, Argüelles JC, Plá J, Cuenca-Estrella M, Zaragoza O (2014) The production of oxygen reactive species is a universal mechanism of amphotericin B against pathogenic yeasts and contributes to the fungicidal effect of this drug. Antimicrob Agents Chemother 58:6627-6638
17. Miramón P, Kasper L, Hube B (2013) Thriving within the host: *Candida* spp. interactions with phagocytic cells. Med Microbiol Immunol 202:183-195
18. Nishimura S, Shimbo T, Asayama N, Akiyama J, Ohmagari N, Yazaki H, Oka S, Uemura N (2013) Factors associated with esophageal candidiasis and its endoscopic severity in the era of antiretroviral therapy. Plos One 8:e58217
19. Ortega M, Marco F, Soriano A, Almela M, Martínez JA, López J, Pitart C, Mensa J. (2011). *Candida* species bloodstream infection: epidemiology and outcome in a single institution from 1991 to 2008. J Hosp Infect 77:157-161
20. Oura M, Sternberg ET, Wright ET (1955) A new antifungal antibiotic, amphotericin B. Antibiot Annu 3:566-573
21. Palacios DS, Anderson, TM, Burker MD (2007) A post-PKS oxidation of the amphotericin B skeleton predicted to be critical for the channel formation is not required for potent antifungal activity. J Am Chem Soc 129:13804-13805
22. Patterson TF (2005) Advances and challenges in management of invasive mycoses. Lancet 366:1013-1025
23. Pedreño Y, González-Párraga P, Martínez-Esparza M, Sentandreu R, Valentín E, Argüelles JC (2007) Disruption of the *Candida albicans ATC1* gene encoding a cell-linked acid trehalase decreases hypha formation and infectivity without affecting resistance to oxidative stress. Microbiology 153:1372-1381
24. Pfaller MA, Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 20:133-163
25. Pfaller MA, Diekema DJ (2010) Epidemiology of invasive mycoses in North America. Critical Rev Microbiol 36:1-53
26. Vale-Silva LA, Buchita V, Valentova E (2007) Effect of subinhibitory concentrations of some established and experimental antifungal compounds on the germ tube formation in *Candida albicans*. Folia Microbiol 52:39-43
27. Vediyappan G, Rossignol T, d'Enfert C (2010) Interaction of *Candida albicans* biofilms with antifungals: transcriptional response and binding of antifungals to beta-glucans. Antimicrob Agents Chemother 54:2096-2111
28. Vincent BM, Lancaster AK, Scherz-Souval R, Whitesell L, Lindquist S (2013) Fitness trade-offs restrict the evolution of resistance to Amphotericin B. Plos Biology 11:e1001692
29. Wang Y, Casadevall A (1994) Growth of *Cryptococcus neoformans* in presence of L-dopa decreases its susceptibility to amphotericin B. Antimicrob Agents Chemother 38:2648-2650