

RESEARCH ARTICLE



INTERNATIONAL MICROBIOLOGY (2013) 16:217-225

doi: 10.2436/20.1501.01.197 ISSN 1139-6709 www.im.microbios.org

Analysis of validamycin as a potential antifungal compound against *Candida albicans*

José P. Guirao-Abad,¹ Ruth Sánchez-Fresneda,¹ Eulogio Valentín,²
María Martínez-Esparza,³ Juan-Carlos Argüelles^{1*}

¹Microbiology Area, School of Biology, University of Murcia, Murcia, Spain. ²Department of Microbiology and Ecology, University of Valencia, Burjassot, Spain. ³Department of Biochemistry and Molecular Biology and Immunology, University of Murcia, Murcia, Spain

Received 18 October 2013 · Accepted 12 December 2013

Summary. Validamycin A has been successfully applied in the fight against phytopathogenic fungi. Here, the putative antifungal effect of this pseudooligosaccharide against the prevalent human pathogen *Candida albicans* was examined. Validamycin A acts as a potent competitive inhibitor of the cell-wall-linked acid trehalase (Atc1p). The estimated MIC₅₀ for the *C. albicans* parental strain CEY.1 was 500 mg/l. The addition of doses below MIC₅₀ to exponentially growing CEY.1 cells caused a slight reduction in cell growth. A concentration of 1 mg/ml was required to achieve a significant degree of cell killing. The compound was stable as evidenced by the increased reduction of cell growth with increasing incubation time. A homozygous *atc1Δ/atc1Δ* mutant lacking functional Atc1p activity showed greater resistance to the drug. The antifungal power of validamycin A was limited compared with the drastic lethal action caused by exposure to amphotericin B. The endogenous content of trehalose rose significantly upon validamycin and amphotericin B addition. Neither serum-induced hypha formation nor the level of myceliation recorded in macroscopic colonies were affected by exposure to validamycin A. Our results suggest that, although validamycin A cannot be considered a clinically useful antifungal against *C. albicans*, its mechanism of action and antifungal properties provide the basis for designing new, clinically interesting, antifungal-related compounds. [Int Microbiol 2013; 16(4):217-225]

Keywords: *Candida albicans* · *Rhizoctonia solani* · validamycin A · amphotericin B · trehalose

Introduction

Substantial progress has been achieved in the last two decades in antifungal chemotherapy, which has been increasingly employed mainly because of the worldwide rise in the immunocompromised and aging human population [23] and the fre-

quent isolation in hospitals of classical non-pathogenic fungi responsible for nosocomial outbreaks [27,28]. These isolates mainly belong to the genera *Aspergillus*, *Cryptococcus*, and “non-albicans” species of *Candida*, although *Candida albicans* remains the most prevalent infectious fungus in humans [8,9,24,27]. In recent years, the biggest problems associated with clinical fungal infections are related to the considerable increase in nosocomial bloodstream candidiasis, in which new factors of virulence [9], the rise of resistant fungal strains due to mutation, and the low selective toxicity of available antifungal therapies are characteristic [1,8,27]. This worrying scenario highlights the need for more secure and effective antifungal therapies.

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

In this context, considerable effort has been dedicated to improving conventional antimycotic drugs, e.g., the novel liposomal formulations of amphotericin B [15]. An alternative strategy requires the search for new cell targets combined with the screening of promising new antifungal compounds [23]. Our previous work supported both investigation into the enzymes involved in trehalose metabolism as potential antifungal targets and clinical trials of inhibitors specifically designed against trehalose-hydrolyzing enzymes [20,21,26]. Trehalose is a protective non-reducing disaccharide that is widely distributed in nature (bacteria, fungi, invertebrates, and plants) but absent in mammals. A loss of virulence is caused by the double disruption of the trehalose biosynthetic genes *TPS1* and *TPS2*, and the corresponding *tps1* and *tps2* null mutants show striking susceptibility to oxidative stress and heat shock. These observations are strong arguments in favor of therapeutically targeting the trehalose pathway [3,4,13,32]. Within this hydrolytic pathway, the *ATC1* gene encodes the cell-wall-linked acid trehalase (Atc1p), required to cleave off exogenous trehalose; it is also involved in the induction of filamentation and virulence in *C. albicans*, and in improved resistance against different types of stress, such as heat shock or oxidative stress [25,26].

Structural analogues of trehalose from different biological sources (e.g., validamycin A, trehalosamine, trehazoline, nojirimycin, and calistegin) act as competitive or non-competitive inhibitors of trehalase activity [10] and are therefore of interest for their putative antifungal activity. Notably, validamycin A was introduced at the beginning of the 1980s in Japan and China to control rice sheath blight, caused by the phytopathogenic fungus *Rhizoctonia solani* [5]. It has also been tested against several ascomycetes and basidiomycetes [29]. Today, it is successfully used in crop protection as a herbicide and fungicide, since it is innocuous to human and animal health [17]. The structure of this secondary metabolite from *Streptomyces hygroscopicus* consists of an aminocyclitol moiety, validoxylamine A, linked to glucose through a β -glycosidic bond [7]. Validamycin A shows strong competitive inhibition of several plant and insect trehalases, suggesting its potential as an herbicide or insecticide [6,11,22]. To our knowledge, however, the antifungal action of validamycin A for clinical purposes against human fungal pathogens has never been examined, although the compound was tested in studies on the heat- and trehalose-associated control of morphogenesis in *C. albicans* [31]. We therefore decided to investigate the putative antifungal action of validamycin A against *Candida albicans*, taking as our model the parental CEY.1 (CAI-4-*URA*⁺) strain and its isogenic homozygous *atc1Δ*/

atc1Δ mutant (deficient in acid trehalase). Our results suggest that although validamycin A has a limited antifungal action against *C. albicans*, it does merit consideration as a basis for the design of new, alternative antifungal compounds.

Materials and methods

Yeast strains and culture conditions. *Candida albicans* strain CEY-1 (CAI-4-*URA*⁺) (*ura-3 Δ::imm-434/ura-3 Δ::im-434/ RPI0::URA3*) and the homozygous acid-trehalase-deficient mutant (*atc1Δ/atc1Δ*) (*atc1Δ::hisG/atc1Δ::hisG-URA3-hisG-ura3Δ::imm434/ura3Δ::imm434*) were used throughout this study. A detailed description of the constructions and procedures followed to obtain these strains is given elsewhere [26]. The cultures were grown at 37 °C (or 28 °C for experiments on dimorphic conversion; see below) with shaking in a medium consisting of 2 % peptone, 1 % yeast extract, and 2 % glucose (YPD). The strains were maintained by periodic subculturing in solid YPD medium. Growth was monitored by measuring the OD₆₀₀ of cultures in a Shimadzu UV spectrophotometer.

Sensitivity to validamycin A and amphotericin B. Validamycin was purchased from Duchefa Biochemie (Haarlem, The Netherlands) and two stock solutions were prepared by dissolving the compound in dimethyl sulfoxide at 100 mg/ml or 10 mg/ml (as the source for final doses of 1 mg/ml or 100 μg/ml validamycin A, respectively). The stock solutions were stored at -20 °C until use and diluted in sterile distilled water. Amphotericin B (Sigma, 80 % purity) was prepared as indicated elsewhere [12]. Fungal cultures were grown in liquid YPD at 37 °C until the exponential phase (OD₆₀₀ 0.8–1.0) and were then divided into several aliquots, which were treated with the indicated doses of validamycin A or amphotericin B. After incubation for 24 h at 37 °C, viability was determined (see viability determination) and compared with a control without validamycin A. The concentration of validamycin A that induced 50 % inhibition of growth (MIC₅₀) was calculated.

Viability determination. Cultures were grown in liquid YPD overnight and then refreshed in the same medium until they reached the early exponential phase of growth (OD₆₀₀ 0.2–0.3). Then, the samples were divided into several identical aliquots, which were treated with two different validamycin A concentrations, 0.1 and 1.0 mg/ml, chosen as a function of the MIC₅₀, or maintained without treatment as a control; all cultures were incubated at 37 °C until an OD₆₀₀ of 0.8–1.0. Viability was determined in samples diluted appropriately with sterile water, plated in triplicate on solid YPD, and then incubated for 2–3 days at 37 °C. Between 30 and 300 colonies were counted per plate. Survival was normalized to that of the control samples (100 % viability).

Induction of germ tube formation. For germ-tube induction, samples were harvested at different stages of growth, rapidly washed with water, and resuspended at a density of 0.6–0.8 mg/ml (dry weight) in YPD pre-warmed to 37 °C and containing 10 % (v/v) filter-sterilized (0.45 μm, Millipore) human serum. Filamentation was also examined in colonies growing on Spider medium plates incubated at 37 °C for 7 days. The appearance of germ tubes was monitored using a phase-contrast light microscope equipped with a hemocytometer. When required, clumped cells were dispersed by mild sonication (10–15 s) prior to microscopic examination. At least 250 cells were counted for each time-point and the percentage of dimorphism was defined as the ratio of germ-tube-forming cells to the total number of cells [2,26].

Preparation of cellular extracts. After exposure to validamycin A, samples from the cultures were harvested and resuspended at known densi-

ties (10–15 mg/ml, wet weight) in *N*-morpholine ethanesulfonic acid (MES) extraction buffer (100 mM), pH 6.0, containing 5 mM cysteine and 0.1 mM phenylmethylsulfonyl fluoride. The cellular suspensions were transferred into small, pre-cooled tubes (0.5 cm diameter) containing Ballotini glass beads (0.45 mm diameter). The cells were broken by vibrating the tubes vigorously in a vortex mixer. The tubes were cooled quickly on ice, and the cell extracts were then centrifuged at $12,000 \times g$ for 10 min. The supernatant (cytosolic extract) and the pellet, resuspended in the same buffer (cell-wall extract), were preserved at 4 °C if used immediately or at –20 °C for further enzymatic analysis. The protein content in cellular extracts was estimated according to Lowry et al. [19], with bovine serum albumin as standard.

Enzymatic assays. The acid trehalase assay was performed by incubating 50 μ l of cell-wall extract with 200 μ l of trehalose (200 mM) prepared in 200 mM sodium citrate, pH 4.5, containing 2 mM EDTA. The reaction for neutral trehalase activity contained 50 μ l of cytosolic extract (25–100 μ g protein) and 200 μ l of trehalose (200 mM) prepared in 25 mM MES, pH 7.1, and 125 μ M CaCl_2 . The reactions were incubated at 30 °C for 30 min and stopped by heating in a water bath at 95 °C for 5 min. The amount of glucose released was determined by the glucose oxidase-peroxidase method. The specific activity is expressed as $\text{nmol glucose min}^{-1} (\text{mg protein})^{-1}$. Catalase activity was determined at 240 nm by monitoring the removal of H_2O_2 , as described elsewhere [12]. Specific activity is expressed as $\text{mmol min}^{-1} (\text{mg protein})^{-1}$.

Determination of endogenous trehalose. Intracellular trehalose was measured according to a previously described method [26]. Briefly, cell samples (20–50 mg, wet weight) were washed, resuspended in 1 ml of water, and boiled for 30 min with occasional shaking. The concentration of trehalose released in the supernatant was determined with commercial trehalase (Sigma). The assay contained 90 μ l of 25 mM sodium acetate buffer, pH 5.6, 100 μ l of cell-free supernatant, and 10 μ l of trehalase (2 units/ml). After incubation of the samples overnight at 37 °C, the amount of glucose produced was estimated by the glucose oxidase-peroxidase procedure. Parallel controls were run to correct for basal glucose levels.

Morphological analysis. The morphology of the cells after the different antifungal exposures was imaged with a Leica DM6000B microscope equipped with a Leica DFC280 camera connected to a PC and by using Leica Application Suite V 2.5.0 R1 software. Images were captured by bright field microscopy, using the 40 \times objective with an on-screen magnification of 920 \times , and then processed and analyzed with the public domain software ImageJ [<http://rsb.info.nih.gov/ij/>], a Java-based image processing program developed at the National Institute of Health.

Statistical analysis. Data are presented as mean \pm SD. The data were analyzed by Duncan's multiple test. The results shown are from three to five independent experiments.

Results

Effect of validamycin A on acid trehalase activity (Atc1p) in *Candida albicans*. Validoxylamine A, the aglycone fraction of validamycin A, behaves as a potent and specific competitive inhibitor of the soluble trehalase present in the pathogenic fungus *Rhizoctonia solani* [5]. Therefore, we initially analyzed whether this inhibitory action of validamycin A was also operative against the acid trehalase of *C. albicans*, a cell-wall-linked enzyme involved in the hydrolysis of exogenous trehalose [25,26]. According to the Dixon plot shown in Fig. 1, in the parental *C. albicans* strain CEY.1 (CAI-4-*URA*⁺), validamycin A also inhibited this enzymatic activity in a competitive manner, with an apparent dissociation constant (K_i) of about 1.5 μ g/ml (3.02×10^{-6} M).

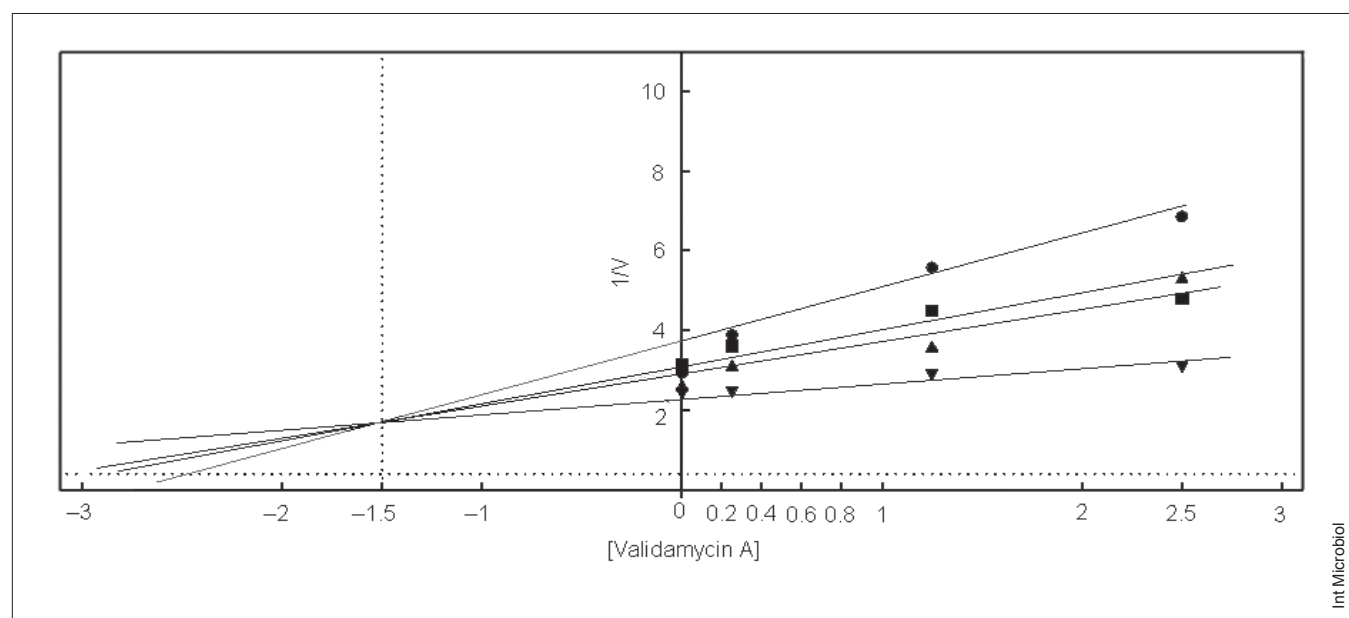


Fig. 1. Dixon plot for validamycin A inhibition of acid trehalase activity in exponentially growing cells of the *C. albicans* CEY.1 (CAI-4-*URA*⁺) strain. The incubation mixtures contained the following concentrations of trehalose: 1 mM (closed circle), 10 mM (closed square), 25 mM (closed up triangle), and 100 mM (closed down triangle).

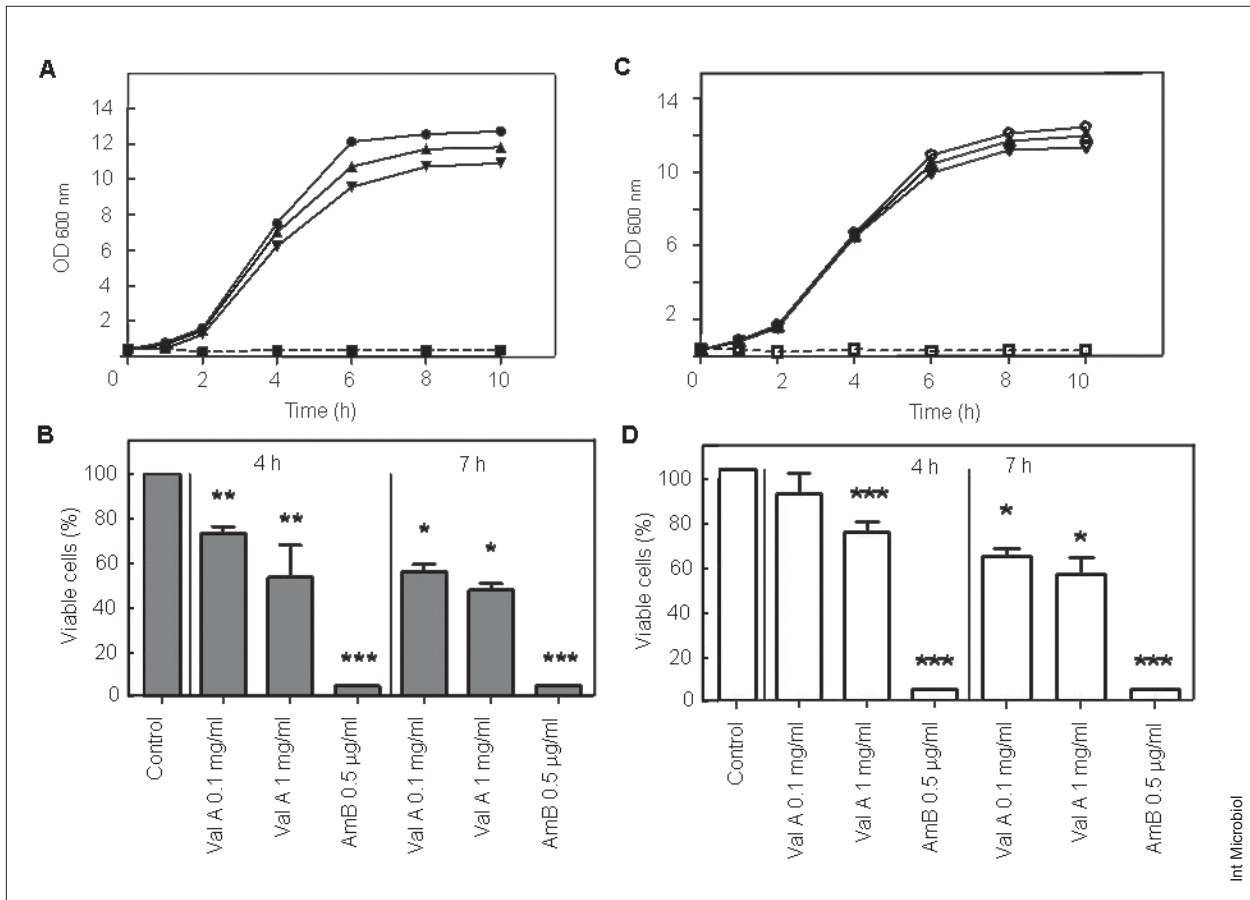


Fig. 2. Effect of validamycin A (Val A) and amphotericin B (AmB) on the growth (as determined by turbidimetric analysis) and cell viability of the parental CAI-4-*URA*⁺ strain (panels A and B, closed symbols) and the congenic mutant *atc1Δ/atc1Δ* (panels C and D, open symbols) of *C. albicans*. Cultures were grown at 37 °C in YPD liquid medium with shaking, in the absence (circles) or presence of validamycin A at concentrations of 0.1 mg/ml (up triangle) and 1 mg/ml (down triangle) or amphotericin B at 0.5 µg/ml (square). Samples were harvested in duplicate at the indicated times for OD₆₀₀ measurements in the CEY.1 (A) and *atc1Δ/atc1Δ* (C) strains. Cell viability was analyzed in CEY.1 (B) and *atc1Δ/atc1Δ* (D) strains subjected to the indicated treatments for 4 and 7 h. Identical, untreated samples were maintained at 37 °C as a control. The values shown are the mean ± SD of three independent measurements. The distinction between the values obtained for the treated samples and the controls was significant at **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, according to Duncan's multiple range test.

This value was higher than the K_i recorded for validoxylamine A in *R. solani* [5,6], although the two cannot be strictly compared because we used cell-wall extracts, rather than partially purified samples, as the enzymatic source. Furthermore, only acid trehalase activity can be measured in the crude homogenates, as demonstrated by specific antibody binding [30], which excludes hypothetical residual cross-contamination with the cytosolic fraction containing neutral trehalase.

Effect of validamycin A on cell growth and the viability of the CAI-4 and *atc1Δ* null strains of *Candida albicans*. In *C. albicans*, the lack of a functional *URA3* gene affect the growth and virulence capacity of the fungus [16]. Therefore, in this study we used two *URA3*⁺

strains previously employed in studies on dimorphism and infectivity [26]. To test the putative antifungal role of validamycin A, we measured the response of parental CEY.1 cells to the addition of two concentrations of this inhibitor, chosen according to the corresponding MIC₅₀ and calculated in advance (500 mg/l) as described in the Materials and methods section. Turbidimetric inspection (Fig. 2A) showed that 0.1 mg validamycin A/ml caused a slight decrease in cell growth while the reduction was more prominent in samples exposed to a concentration of 1000 mg/l (2× MIC₅₀) (Fig. 2A). The determined cell viability (Fig. 2B) was consistent with the previously obtained absorbance data (Fig. 2A). After 4 h of exposure, 0.1 mg validamycin A/ml caused a weak reduction in the number of viable cells compared with the control (Fig. 2B), and only the

highest concentration resulted in a significant level of cell death (ca. 50%). Prolonged incubation (7 h) in the presence of the compound produced only a slight additional increase in the fungicidal activity, which was dose-dependent (Fig. 2B). These results also suggested that validamycin A remained stable for long periods.

We additionally analyzed the putative antifungal action of validamycin A on the homozygous null mutant *atc1Δ/atc1Δ*, which lacks a functional acid trehalase, the enzyme inhibited by the drug (Fig. 1). As expected, turbidimetric analysis revealed that the mutant was more resistant to the inhibitory action of the drug than the parental strain (Fig. 2C). This resistant phenotype was unequivocally confirmed through a count of viable cells in cultures incubated for 4 and 7 h in YPD liquid medium (Fig. 2D). Exposure to 1.0 mg validamycin A/ml for 7 h resulted in a higher survival percentage of the mutant than of the parental cells (55 vs. 46 %, respectively)

(Fig. 2B and 2D). Throughout this experimental work, the antifungal action of validamycin was referred to that of amphotericin B (0.5 μg/ml) in an identically treated sample, which was used as a positive control. The presence of the polyene provoked a dramatic amount of cell killing in both cell types (Fig. 2B and 2D). Taken together, our results suggest that validamycin A causes only a partial loss of cell viability and that, compared with other well-tested compounds currently available, it behaves as a weak antifungal agent.

By contrast, optical microscopy analysis of the yeast morphology seemed to suggest a possible direct effect of the two tested antifungal agents (validamycin A and amphotericin B) in the reduction of individual cell size, which, in the case of validamycin A, was more noticeable after prolonged treatment (Fig. 3). Thus, the results from five independent measurements in exponential CEY.1 cultures showed a direct correlation between the specific antifungal treatment applied for

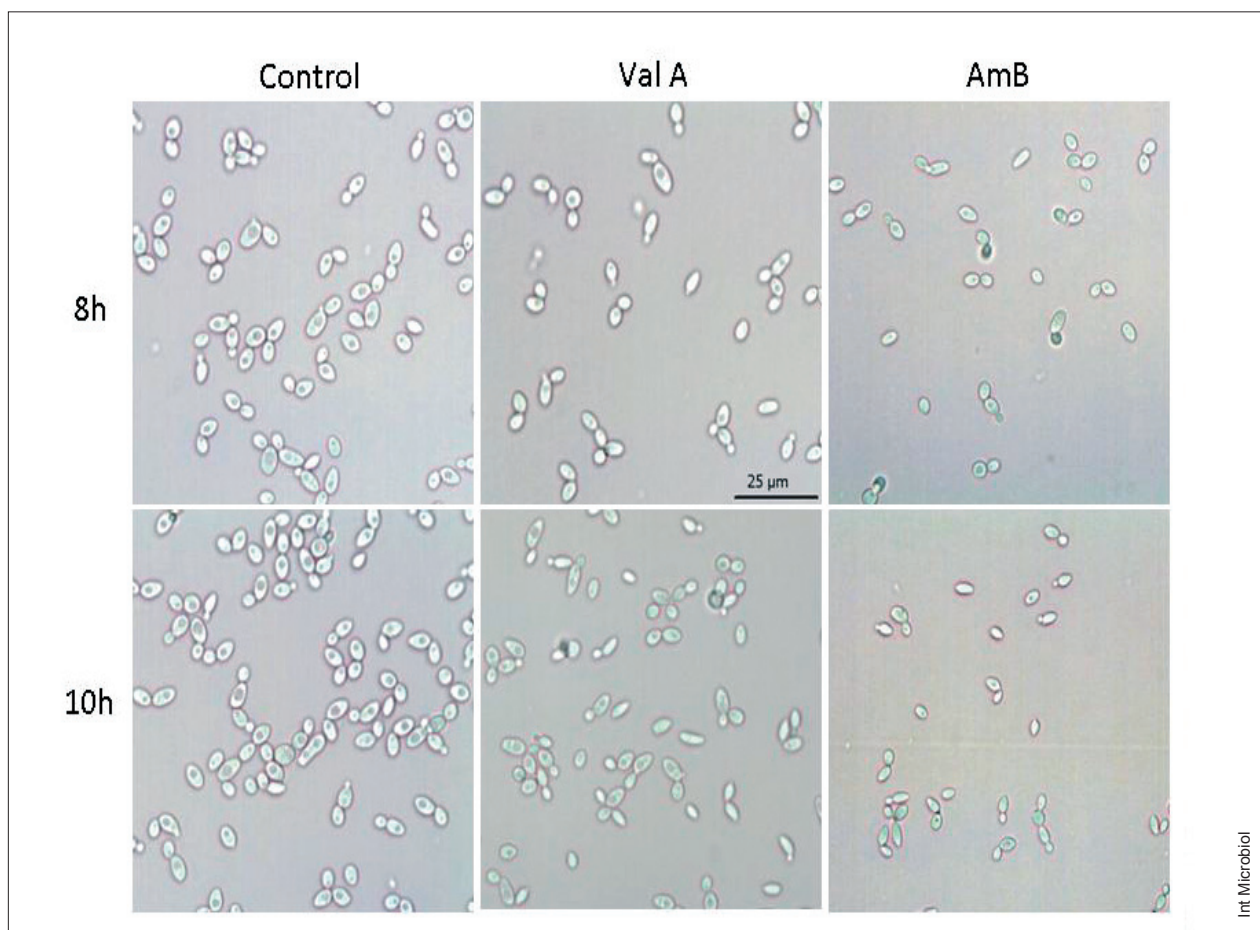


Fig. 3. Morphological changes induced by validamycin A and amphotericin B in *C. albicans* strain CAI-4. YPD-grown CAI-4-*URA*⁺ cells (OD₆₀₀ 0.3) were treated with 0.1 mg validamycin A/ml or 0.5 μg amphotericin B/ml for 8 and 10 h. Identical, untreated samples were maintained at 37 °C as a control.

Table 1. Changes in endogenous trehalose storage measured in response to different concentrations of validamycin A in the *C. albicans* URA⁺ strains CAI-4 and the *atc1Δ/atc1Δ* mutant

Treatment	Trehalose ^a	
	CAI-4	<i>atc1Δ/atc1Δ</i>
Control	5.9 ± 0.7	6.8 ± 0.9
Validamycin A (0.1 mg/ml)	13.8 ± 1.5 ^c	15.3 ± 2.4 ^c
Validamycin A (1 mg/ml)	7.3 ± 1.1	9.1 ± 1.3
Amphotericin B (0.5 µg/ml)	12.7 ± 2.1 ^c	14.5 ± 2.2 ^c

^anmol trehalose/mg wet weight.

^b*P* < 0.05, ^c*P* < 0.01 and ^d*P* < 0.001, according to Duncan's multiple range test.

Cultures were grown at 37 °C in YPD, harvested (OD₆₀₀ = 0.3), and exposed to the test concentrations of validamycin A for 4 h and to amphotericin B for 1 h. Control samples were maintained at 37 °C.

The results are the mean ± SD of three independent measurements.

10 h and the subsequent cell size observed, the cell area of the control samples being larger than that of cells treated with 0.1 mg validamycin A/ml (25.42 ± 6.3 vs. 21.73 ± 4.8 µm²). As expected, the smallest cell sizes occurred in samples treated with 0.5 µg amphotericin B/ml (17.52 ± 2.8 µm²).

Changes in trehalose content during validamycin A treatment. In *C. albicans*, the non-reducing disaccharide trehalose acts as a specific protectant against oxidative stress [3] and is also synthesized in response to amphotericin B exposure [12]. Because the addition of validamycin A to several plant species triggers the rapid synthesis of the trehalose required to confront abiotic stresses [11,18,22], we measured the content of stored trehalose in CEY.1 and *atc1Δ*

null cells under our experimental conditions. As shown in Table 1, endogenous disaccharide accumulation was greatest in the samples exposed to a concentration of validamycin A (100 mg/l) lower than its MIC₅₀, whereas a 10-fold increase in the validamycin A dose triggered lower trehalose storage, probably due, at least in part, to the moderate toxicity caused by acute exposure of the cells to the drug. The strong antifungal effect of amphotericin B caused similar damage to cell viability over short periods of time (1 h), as previously observed (Fig. 2B and 2D). Thus, taken as a whole, the residual population that survived the antifungal challenge synthesized trehalose de novo to a lesser extent [12]. Consistent with previous findings, in the congenic *atc1Δ* null cells the intracellular disaccharide content was slightly higher [26].

Table 2. Levels of enzymatic activities

Treatment	URA ⁺ strains CAI-4			<i>atc1Δ</i> (null mutant of <i>C. albicans</i>)		
	Atc1p nmol glucose · (mg protein) ⁻¹	Ntc1p nmol glucose · (mg protein) ⁻¹	Catalase mmol min ⁻¹ · (mg protein) ⁻¹	Atc1p nmol glucose · (mg protein) ⁻¹	Ntc1p nmol glucose · (mg protein) ⁻¹	Catalase mmol min ⁻¹ · (mg protein) ⁻¹
Control	2.3 ± 0.3 (1.0)	18.5 ± 1.9 (1.0)	0.77 ± 0.05 (1.0)	<0.3	19.1 ± 1.8 (1.0)	1.2 ± 0.3 (1.0)
Validamycin A (0.1 mg/ml) for 4 h at 37 °C	1.7 ± 0.5 (0.74)	12.9 ± 1.6 (0.7) ^c	0.65 ± 0.07 (0.84)	<0.3	16.9 ± 1.7 (0.88) ^a	0.95 ± 0.5 (0.79)
Amphotericin B (0.5 mg/ml) for 1 h at 37 °C	2.7 ± 0.8 (1.17)	27.2 ± 2.5 (1.47) ^c	2.8 ± 0.92 (3.63) ^c	<0.3	29.5 ± 1.4 (1.54) ^c	4.3 ± 0.2 (3.6) ^c

Numbers in parentheses are the relative activity normalized in relation to the control for each parameter, with the control treatment taken as 1.0. The results are the mean ± SD of three independent measurements.

^a*P* < 0.05, ^b*P* < 0.01, and ^c*P* < 0.001 according to Duncan's multiple range test.

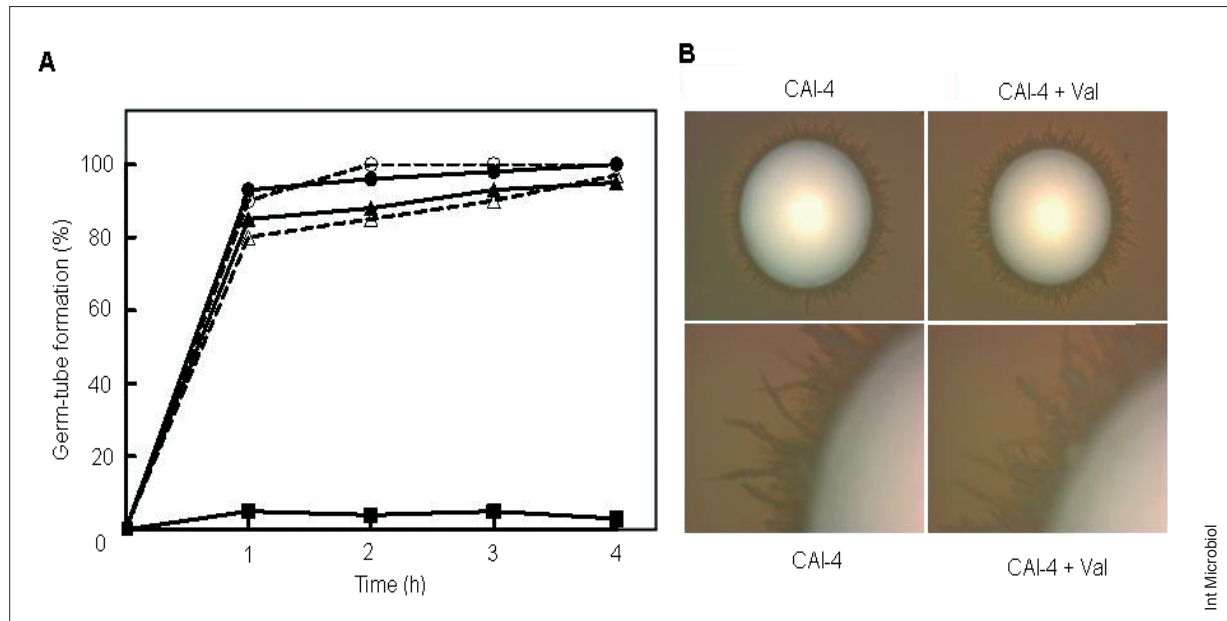


Fig. 4. Effect of validamycin A on germ-tube formation. **(A)** Percentage of germ-tube formation induced by human serum. Exponentially growing cultures of CEY.1 (circles) and *atc1Δ/atc1Δ* (triangles) strains were incubated in YPD medium at 28 °C in the presence (open symbols) or absence (closed symbols) of 0.1 mg validamycin A /ml. At time zero, they were supplemented with sterile human serum (10 %) and incubated at 37 °C. CAI-4 cells grown at 28 °C without serum were run in parallel (filled squares) as the control. Values are the average of two determinations. **(B)** Germ-tube formation induced in colonies growing on Spider medium. The colony morphologies of strain CAI-4-*URA*⁺ on solid Spider medium were analyzed after a 7-day incubation at 37 °C in the presence or absence of 0.1 mg validamycin A /ml. The upper panel shows a whole colony and the lower panel a detail of the border.

Enzymatic activity of the two trehalases and catalase in validamycin-A-treated *Candida albicans*.

To complement these studies, we tested a number of enzymatic activities that may play an important role in protecting the cellular integrity of *C. albicans* exposed to antifungal treatments [12,26], including in response to validamycin A exposure. In terms of possible changes in the enzymatic pathway involved in trehalose hydrolysis, Atc1p activity showed a certain degree of competitive inhibition in CAI-4-*URA*⁺ cells treated with 0.1 mg validamycin A/ml (Table 2), although basal enzymatic levels were very low because the *ATC1* gene is subjected to catabolic repression by glucose in YPD medium [25]. As expected, acid trehalase was virtually undetectable in the *atc1Δ* null mutant (Table 2). Cytosolic Ntc1p activity was also diminished in the presence of validamycin A in CEY.1 cells and, albeit to a lesser extent, in the *atc1Δ* strain (Table 2). Conversely, amphotericin B caused an increase in this activity, probably because the cells obtained the energy necessary to withstand stress through trehalose degradation. These data also point to a possible inhibitory action of amphotericin B on this cytosolic neutral trehalase.

The induction of catalase activity has also been cited as a component of cellular defenses against antifungal exposure [12].

However, there were no significant differences in the activity of this antioxidant between control samples and validamycin-A-treated cells in the two strains analyzed, with higher basal levels in the *atc1Δ* cells. This is consistent with the observation that the double disruption of *ATC1* in *C. albicans* confers superior resistance to various environmental stresses [25,26]. In turn, in exponentially growing cultures of the studied strains subjected to amphotericin B (0.5 μg/ml) there was a clear activation of catalase, which likely reflected the capacity of the polyene to induce oxidative damage in *C. albicans* [12]. Therefore, this antioxidant activity seems to be a sensitive mechanism to withstand antifungal treatments that might seriously compromise cell integrity, but it does not appear to act against weak antifungal exposure. In addition, catalase activity was not induced after the addition of validamycin A, at least at the doses used in this study.

Effect of validamycin A on germ-tube formation in *Candida albicans*.

The addition of human serum (10 %) to cultures of *C. albicans* and the simultaneous transfer of the cells from 28 to 37 °C is a rapid and reliable procedure to induce germ-tube formation in this fungus [2]. We therefore analyzed whether validamycin A exerted some influence on the serum-induced dimorphic transition of the

atc1A null mutant compared with the isogenic parental CEY.1 cells. As shown in Fig. 4, the addition of validamycin A (0.1 mg/ml) did not induce any significant variation in the number or kinetics of human-serum-induced germ-tube formation (Fig. 4A). Furthermore, the level of filamentation in CEY.1 colonies that developed on Spider medium was not modified upon the addition of the inhibitor (Fig. 4B). Hence, these data suggested that validamycin A did not exert any conspicuous influence on the yeast-to-hypha conversion of *C. albicans*. Interestingly, the percentage of germ-tube formation recorded after 4 h of induction was similar in CEY.1 and *atc1A* cells, although in the latter strain dimorphic conversion was initially slower (Fig. 4A). This behavior might have well been due to structural perturbations in the intact cell wall that occurred as a consequence of the double disruption of *ATC1* [26].

Discussion

The analysis of commercially available validamycin A leads us to propose that this compound is a weak antifungal drug against the prevalent opportunistic pathogen *C. albicans*, as opposed to its customary application against the phytopathogenic fungus *R. solani* in China and Japan [5,6]. However, in both fungal species, validamycin A acts as a potent competitive inhibitor of cell-wall-linked acid trehalase, Atc1p (Fig. 1, Table 2) [5]. Notably, this compound also caused a significant reduction in cytosolic neutral trehalase activity (Ntc1p) in *C. albicans* (Table 2), although we do not presently regard Ntc1p inhibition as the main cause of the weak fungicidal effect recorded. This observation suggests that either validamycin A per se or its aglycone fraction (validoxyamine A) is able to cross the cell wall and plasma membrane in order to inhibit cytosolic hydrolases, although more experiments on specific transport are required to confirm this hypothesis.

When provided in concentrations lower than the MIC₅₀ (100 mg/l), the drug had less effect on the viability of the CEY.1 strain of *C. albicans*, while cell damage was virtually negligible in an *atc1A/atc1A* mutant lacking functional Atc1p activity. An evident, but still partial, reduction in the level of survival required an increase in the dose of validamycin A to two-fold the MIC₅₀, but this was still only achieved in the parental cells. As expected, *atc1A* null cells were less sensitive to the drug. The usefulness of validamycin A as a clinical antifungal was thus clearly restricted compared with the important amount of cell killing recorded in assays performed with the classical polyene amphotericin B, at least with respect to *C. albicans* as a pathogenic yeast model (Fig. 2).

In addition, validamycin A appeared to play no significant role in the induction of an intracellular antioxidant state promoted by catalase activity (Table 2), which might be relevant for counteracting drastic antifungal exposure [12,15]. Furthermore, the content of endogenous trehalose increased after the addition of 0.1 mg validamycin A/ml (Table 1), in agreement with similar results obtained in distinct genetic backgrounds of *C. albicans* [31]. However, the degree of accumulation was lower in the presence of higher doses and after amphotericin B addition, probably because of the toxic effect provoked by these antifungal treatments (Table 1), whereas there was a slight decrease in antioxidant catalase activity in the presence of validamycin A (Table 2). Of note is the fact that *ATC1*, which encodes the acid trehalase activity, is not essential in *C. albicans*. However, it is clearly involved in virulence, since both heterozygous and homozygous mutants have a lower infectivity, as shown in a mouse model [25,26]. Thus, *ATC1* is a potential target in the search for and design of new antifungals, although the specific inhibition of Atc1p by validamycin A only caused a partial reduction in cell viability and failed to affect the formation of hyphal structures (Figs. 2 and 4).

Changes in the morphological and physical properties of fungal cells (shape, size, height, roughness or stiffness) are among the less well known toxic effects caused by the action of several antifungals, but they have been clearly demonstrated for amphotericin B and 5-flucytosine [14]. In our case, the addition of non-lethal doses of validamycin A caused a small but consistent diminution in cell size with respect to a control sample (Fig. 3). This effect on cell size was obviously dependent on the antifungal power of the compound used; hence, a greater reduction was achieved in cells treated with amphotericin B (Fig. 3). In turn, neither the percentage of serum-induced germ-tube formation by blastospores nor the filamentation recorded in macroscopic colonies grown on Spider medium was modified by exposure to validamycin A (Fig. 4). On the other hand, although trehalose hydrolysis does not seem to be a preferential energy source for serum-induced morphogenesis in *C. albicans* [2], recent evidence collated from a mutant deficient in the glucose sensing receptor Gpr1 indicated that trehalose acts as negative regulator of filamentous development by counteracting the inhibitory effect of the HSP90 protein [31].

Thus, we conclude that, although validamycin A cannot presently be considered as a suitable clinical antifungal, it is likely to be a promising substrate in the design of new compounds directed against the trehalose metabolism pathway as an antifungal target. This potential should promote future re-

search into the development and testing of new drugs to more effectively combat life-threatening systemic infections caused by *C. albicans*.

Acknowledgements. We thank Dr. J. Tudela (University of Murcia) for helpful suggestions. 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 Urbanos de Murcia, S.A.

Competing interests: None declared.

References

- Almirante B, Rodríguez D, Park BJ, et al. (2005) Epidemiology and predictors of mortality in cases of *Candida* bloodstream infection: Results from population-based surveillance, Barcelona, Spain, from 2002 to 2003. *J Clin Microbiol* 43:1829-1835
- Álvarez-Peral FJ, Argüelles JC (2000) Changes in external trehalase activity during human serum-induced dimorphic transition in *Candida albicans*. *Res Microbiol* 151:837-843
- Álvarez-Peral FJ, Zaragoza O, Pedreño Y, Argüelles JC (2002) Protective role of trehalose during severe oxidative caused by hydrogen peroxide and the adaptive oxidative stress response in *Candida albicans*. *Microbiology* 148:2599-2606
- Argüelles JC (2000) Physiological roles of trehalose in bacteria and yeast: a comparative analysis. *Arch Microbiol* 174:217-224
- Asano N, Yamaguchi T, Kameda Y, Matsui K (1987) Effect of validamycins on glycohydrolases of *Rhizoctonia solani*. *J Antibiot* 40:526-532
- Asano N, Takeuchi M, Kameda Y, Matsui K, Kono Y (1990) Trehalase inhibitors, validoxylamine A and related compounds as insecticides. *J Antibiot* 43:722-726
- Asamizu S, Yang J, Almabruk KH, Mahmud T (2011) Pseudoglycosyltransferase catalyzes nonglycosidic C-N coupling in validamycin A biosynthesis. *J Am Chem Soc* 133:12124-12135
- Cowen LE, Anderson JB, Kohn LM (2002) Evolution of drug resistance in *Candida albicans*. *Annu Rev Microbiol* 56:139-165
- Eggimann P, Garbino J, Pittet D (2003) Epidemiology of *Candida* species infections in critically ill non immunosuppressed patients. *Lancet Infect Dis* 3:685-702
- El Nemr A, El Ashry SH (2011) Potential trehalase inhibitors: synthesis of trehalozin and its analogues. *Adv Carbohydr Chem Biochem* 65:45-114
- Goddijn OJM, Verwoerd TC, Voogd E, et al. (1997) Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. *Plant Physiol* 113:181-190
- 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
- Iturriaga G, Suárez R, Nova-Franco B (2009) Trehalose metabolism: From osmoprotection to signaling. *Int J Mol Sci* 10:3793-3810
- Kim KS, Kim YS, Han I, Kim MH, Jung MH, Park HK (2011) Quantitative and qualitative analyses of the cell death process in *Candida albicans* treated by antifungal agents. *PLoS One* 6:e28176
- Kim JH, Faria NC, Martins ML, Chan KL, Campbell BC (2012) Enhancement of antimycotic activity of amphotericin B by targeting the oxidative stress response of *Candida* and *Cryptococcus* with natural dihydroxybenzaldehydes. *Front Microbiol* 3:261
- Lay J, Henry LK, Clifford J, Kotlin Y, Bulawa CE, Becker JM (1998) Altered expression of selectable marker Ura3 in gene-disrupted *Candida albicans* strains complicates interpretation of virulence studies. *Infect Immun* 66:5301-5306
- Liao Y, Wei ZH, Bai L, Deng Z, Zhong JJ (2009). Effect of fermentation temperature on validamycin A production by *Streptomyces hygroscopicus* 5008. *J Biotechnol* 142:271-274
- López M, Tejera NA, Lluch C (2009) Validamycin A improves the response of *Medicago truncatula* plants to salt stress by inducing trehalose accumulation in the root nodules. *J Plant Physiol* 166:1218-1222
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-275
- Martínez-Esparza M, Martínez-Vicente E, González-Párraga P, Ros JM, García-Peñarribia P, Argüelles JC (2009) Role of trehalose-6P phosphatase (*TPS2*) in stress tolerance and resistance to macrophage killing in *Candida albicans*. *Int J Med Microbiol* 299:453-464
- Martínez-Esparza M, Tapia-Abellán A, Vitse-Standaert A, García-Peñarribia P, Argüelles JC, Poulain D, Jouault T (2011) Glycoconjugate expression on the cell wall of *tps1/tps1* trehalose-deficient *Candida albicans* strain and implications for its interaction with macrophages. *Glycobiology* 21:796-805
- Müller J, Boller T, Wiemken A (1995) Effects of validamycin A, a potent trehalase inhibitor, and phytohormones on trehalose metabolism in roots and root nodules of soybean and cowpea. *Planta* 197:362-368
- Odds FC, Brown AJ, Gow NA (2003) Antifungal agents: mechanisms of action. *Trends Microbiol* 11:272-279
- 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
- Pedreño Y, Maicas S, Argüelles JC, Sentandreu R, Valentin E (2004) The *ATC1* gene encodes a cell wall-linked acid trehalase required for growth on trehalose in *Candida albicans*. *J Biol Chem* 279:40852-40860
- Pedreño Y, González-Párraga P, Martínez-Esparza M, Sentandreu R, Valentin 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
- Pfaller MA, Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* 20:133-164
- Pfaller MA, Diekema DJ (2010) Epidemiology of invasive mycoses in North America. *Crit Rev Microbiol* 36:1-53
- Robson GD, Kuhn PJ, Trinci AP (1988) Effects of validamycin A on the morphology, growth and sporulation of *Rhizoctonia cerealis*, *Fusarium culmorum* and other fungi. *J Gen Microbiol* 134:3187-3194
- Sánchez-Fresneda R, González-Párraga P, Esteban O, Laforet L, Valentin E, Argüelles JC (2009) On the biochemical classification of yeast trehalases: *Candida albicans* contains two enzymes with mixed features of neutral and acid trehalase activities. *Biochem Biophys Res Commun* 383:98-102
- Serneels J, Tourmu H, Van Dijk P (2012) Tight control of trehalose content is required for efficient heat-induced cell elongation in *Candida albicans*. *J Biol Chem* 287:36873-36872
- Singer MA, Lindquist S (1998) Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. *Trends Biotechnol* 16:460-468