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ROS formation is a differential contributory factor to the fungicidal action of Amphotericin B and Micafungin in *Candida albicans*

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ABSTRACT

The hypothetical role played by the intracellular formation of reactive oxygen species (ROS) in the fungicidal action carried out by Amphotericin B (AmB) and Micafungin (MF) was examined in *Candida albicans*, which remains the most prevalent fungal pathogen. The clinical MICs for MF and AmB were 0.016 and 0.12 μg/ml, respectively. Whereas AmB (0.5–1.0 × MIC) induced a marked production of intracellular ROS accompanied by a high degree of cell killing in the *C. albicans* SC5314 strain, the fungicidal effect of MF was still operative, but ROS generation was slight. Preincubation with thiourea suppressed the formation of ROS and caused a marked increase in cell viability, regardless of the antifungal used. Simultaneous measurement of several well established antioxidant enzymes (catalase, glutathione reductase and superoxide dismutase) revealed strong AmB-induced activation of the three enzymatic activities, whereas MF only had a weak stimulating effect. Likewise, AmB but not MF promoted a conspicuous rise in the mitochondrial membrane potential together with the intracellular synthesis of trehalose, the non-reducing disaccharide which acts as a specific protector against oxidative stress in *C. albicans*. Optical and electronic microscopy analysis revealed a significant damage to cell integrity and structural alterations caused by both antifungals. Taken together, our results strongly suggest that the induction of an internal oxidative stress in *C. albicans* through the accumulation of ROS is a preferential contributory factor to the antifungal action of a widely used polyene (AmB) but not of MF (echinocandin).

1. Introduction

The clinical problem of fungal resistance to chemotherapeutics is not so worrying as the dramatic scenario of bacterial resistance to antibiotics, exemplified by the growing isolation of multiresistant highly virulent bacterial strains (Shor and Perlin, 2015). In recent years, however, a dangerous rise in resistant fungal strains has been recorded, which, together with the limited arsenal of true fungicidal compounds available and their low selective toxicity, plus the spread of nosocomial bloodstream candidiasis and aspergillosis, complicates the efficacy of antifungal chemotherapy (Pfaller and Diekema, 2010, 2007).

The molecular mechanisms of resistance have been elucidated in the three main groups of clinical antifungals: polyenes, azoles and echinocandins. Azoles interfere with ergosterol biosynthesis, the main sterol of the plasma membrane, by inhibiting the key cytochrome P-450-dependent enzyme lanosterol 14α-demethylase (Erg11p). Resistance to azole compounds is achieved through mutations in the gene target (*ERG11*) or genes involved in the drug efflux (*CDR1*;*CDR2* and *MDR1*) (Liu et al., 2005). In turn, echinocandins are non-competitive inhibitors of the β-(1,3)-D-glucan synthase that catalyzes the formation of β-glucan polymers, essential components of the cell wall. In some pathogenic yeasts, mutations in the FKS1 gene, which codes for a subunit of the glucan synthase complex, confer resistance to echinocandins (Akins, 2005; Chandrasekar and Sobel, 2006; Liu et al., 2005). Of note is the fact that clinical resistance to polyenes (AmB) remains extremely rare 50 years after its first application as monotherapy (Vincent et al., 2013). Polyenes specifically bind to membrane ergosterol, triggering pore formation and loss of selective permeability through ion leakage (Ellis, 2002).

Nevertheless, our knowledge on the action mechanisms of the main types of antifungals used in medical practice is still incomplete. A convenient strategy to prevent the appearance of resistance would involve identifying the whole sets of fungicidal activities, which, for some established compounds, is currently far from being reached. In the

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case of AmB it is still a matter of dispute whether the postulated lytic mechanism, closely depends on the instability triggered in the plasma membrane followed by subsequent pore aperture and potassium loss (Chen et al., 1978; Palacios et al., 2007), or whether full fungicidal activity can be achieved by simple and specific binding of AmB to the ergosterol, in which case pore formation would be irrelevant (Cereghetti and Carreira, 2006; Gray et al., 2012; Palacios et al., 2007).

On the other hand, compelling evidence supports the generation of intracellular oxidative stress through the production of ROS as an important contributory mechanism of the lethal effect caused by AmB (Mesa-Arango et al., 2014; Sokol-Anderson et al., 1986). In fact, the addition of enzymatic scavengers of free radicals, *e.g.* catalase or superoxide dismutase, protects *Candida albicans* protoplasts against AmB (Sokol-Anderson et al., 1986). In turn, *C. albicans* mounts an AmB-induced antioxidant response that encompasses trehalose synthesis (González-Párraga et al., 2011). Both the Krebs cycle and the electron transport chain play critical roles in oxidative-mediated cell death (Belenky et al., 2013). In the present work we analyze the effect of AmB and MF on the intracellular production of ROS and the generation of an antioxidant response in the *C. albicans* SC5314 standard strain. Our results point to a clearly distinct effect of both antifungals.

2. Materials and methods

2.1. Yeast strains and culture conditions

The *C. albicans* SC5314 wild type strain was used throughout this study. Yeast cell cultures (blastoconidia) were grown at 37 °C by shaking in YPD medium consisting of 2% peptone, 1% yeast extract and 2% glucose.

2.2. Preparation of cell-free extracts

After exposure to different stresses, samples from the cultures were harvested and resuspended at known densities (10–15 mg/ml, wet weight) in the extraction buffer, 100 mM 4-morpholine-ethanesulfonic acid (MES) pH 6.0, containing 5 mM cysteine and 0.1 mM phenyl methyl sulphonyl fluoride (PMSF). The cellular suspensions were transferred into small pre-cooled tubes (1.0 cm diameter) with 1.5 g Ballotini glass beads (0.45 mm diameter). Cells were broken by vigorously vibrating the tubes in a vortex mixer. The tubes were quickly cooled in ice. The crude extract was then centrifuged at 10,000 × *g* for 5 min and the pellet was resuspended in the same buffer at the initial density. For antioxidant assays, the supernatant fraction obtained was filtered through Sephadex G-25 NAP columns (Amersham Pharmacia Biotech AB) previously equilibrated with 50 mM K-phosphate buffer, pH 7.8, to remove low-molecular-weight compounds.

2.3. Enzymatic assays

Catalase activity was determined at 240 nm by monitoring the removal of H_2O_2, as described elsewhere (González-Párraga et al., 2003). Glutathione reductase (GR) activity was assayed by measuring the GSSG-dependent oxidation of NADPH as described in (González-Párraga et al., 2003). Measurements of superoxide dismutase (SOD) were made spectrophotochemically by the ferricytochrome C method using xanthine/xanthine oxidase as the source of $O_2\cdot^-$ radicals (González-Párraga et al., 2003). Fumarase activity was analyzed at 240 nm following the transformation of malate to fumarate according to (Walk and Hock, 1977). Data of enzymatic activity were normalized in relation to a control measurement (100%).

2.4. ROS and membrane potential determinations by cytometric analysis

Intracellular ROS formation by flow cytometry with dihydrofluorescein diacetate (DHF), was measured following the procedure described in (Sangalli-Leite et al., 2011) with the additional modifications indicated elsewhere (Sánchez-Fresneda et al., 2015). Mitochondrial membrane potential was also determined by flow cytometry using Rhodamine 123 as fluorochrome. DHF and Rhodamine 123 were added to the samples at a final concentration of 40 μM and 20 μM respectively, and incubated at 37 °C for 30 min. After treatment with Rhodamine 123, the cells were washed twice with PBS to remove the excess of fluorochrome. Data acquisition and analysis were performed using WINMDI software (available from http://facs.scripps.edu).

2.5. Morphological analysis.

The cellular morphology after exposure to the different antifungals was recorded with a Leica DMRB microscope using Nomarski interference contrast technique. The microscope is equipped with a Leica DC500 camera connected to a PC containing the Leica Application Suite V 2.5.0 R1 software.

2.6. Transmission electron microscopy

The cells were harvested by centrifugation at 4 °C, and the pellet was immediately fixed in 2.5% glutaraldehyde (Serva) in 0.1 M cacodylate buffer, pH 7.2–7.4 for 1 h at 4 °C. Then, the pellet was postfixed in 1% OsO_4 and embedded in Epon prior to sectioning on a Reichert Jung ultramicrotome. Ultrathin sections were stained with uranyl acetate plus lead citrate and examined with a Philips Tecnai 12 electron microscope with a digital camera incorporated.

2.7. Other measurements

Intracellular trehalose was measured following the procedure described elsewhere (González-Párraga et al., 2011). Briefly, cell samples (20–50 mg, wet weight) were washed, resuspended in 2 ml water and boiled for 30 min with occasional shaking. The concentration of trehalose released into the supernatant was determined with commercial trehalase (Sigma). The assay contained 90 μl 25 mM sodium acetate buffer pH 5.6, 100 μl cell-free supernatant and 10 μl trehalase (2 units ml^{-1}). After incubation overnight at 37 °C, the glucose produced was estimated by the glucose oxidase-peroxidase procedure. Parallel controls were run to correct the basal glucose levels.

Growth was monitored by measuring the optical density of cultures at 600 nm in a Shimadzu U/V spectrophotometer. Protein was estimated by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

2.8. Statistical analysis

The statistical analysis was performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, California, USA). The statistical significance of differences between sets of data was determined by Mann Whitney-test. Every experiment was performed in triplicate and repeated at least three times.

3. Results

3.1. Cell-killing and intracellular ROS formation in SC5314 cells following treatment with Micafungin (MF) and Amphotericin B (AmB)

Following the CLSI (Clinical & Laboratory Standards Institute) protocol, the corresponding clinical MICs values for the *C. albicans* laboratory strain SC5314 were determined as 0.016 μg/ml for MF and 0.12 μg/ml for AmB. The quality control *Candida parapsilosis* ATCC®