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ORIGINAL ARTICLE/ARTICLE ORIGINAL

Histatin-5 induces the reversal of Pdr5p mediated fluconazole resistance in *Saccharomyces cerevisae*

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Fluconazole;
Multidrug resistance;
Histatin-5;
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Summary
Background. — Candidiasis is a major opportunistic fungal infection in humans. The low number of antifungal drugs available to treat *Candida* infections and the increasing incidence of multidrug resistant (MDR) strains point to an urgent need of identifying new therapeutic options. The role of salivary components can provide insights for the development of new methodologies of control.
Objective. — The aim of this study was to evaluate the ability of histatin-5, a constitutive immunological peptide present in saliva, in reversing fungal MDR phenotype, using a resistant *Saccharomyces cerevisiae* strain as model of study.
Results. — A total of 2.5 μg and 5 μg of histatin-5 revealed to be able to chemosensitize (to revert antifungal resistance) a MDR strain to fluconazole impairing its intrinsic resistance. The presence of histatin-5 decreased the strain growth when associated to fluconazole, and also assisted in the retention of rhodamine 6G within cell cytoplasm. The ATPase activity of Pdr5p, an ABC efflux transporter, was significantly reduced up to 65% within physiological concentration of the peptide.
Conclusion. — Results revealed that histatin-5 is able to revert MDR phenotype and may be considered a potential alternative MDR inhibitor. Since Pdr5p is homologous to *Candida albicans* CaCdr1p and CaCdr2p, data obtained might be extrapolated to these transporters, inferring that associating fluconazole and histatin-5 may be a useful tool to circumvent failure treatments of infections caused by *Candida* MDR strains.

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Introduction

Among all the opportunistic infections associated with HIV, lesions due to *Candida* spp. are the most studied up to date. Indeed, the growing number of immunocompromised patients in the population led to significant changes in the epidemiology of human fungal infections [1,2]. The overuse of fluconazole and other azole drugs as therapeutic and prophylactic agents for candidiasis in immunocompromised patients resulted in the increasing resistance of the organism and also changed the conventional etiology of this mycosis to species inherently less susceptible to azoles [3].

The appearance of multiresistant strains may occur due to an increase in the expression of transporters related to the multiple drug resistance (MDR). This MDR phenotype can be defined as the cell resistance to several structurally and functionally distinct compounds and is characterized by the ability to extrude drugs of the cell, thereby decreasing the drug concentration in the intracellular environment [4].

Overexpression of efflux pumps belonging to ATP-Binding Cassette (ABC) superfamily is the main responsible for the development of resistance to antifungal agents, herbicides and cytotoxic drugs [5]. CaCdr1p and CaCdr2p transporters present in *Candida* spp. membranes are functionally homologous to Pdr5p, Snq2p and Yor1p transporters. These transporters are members of the pleiotropic drug resistance (PDR) subfamily of *Saccharomyces cerevisiae* and frequently described as promoters of the MDR phenotype, especially to azole antifungals [6,7].

The increasing incidence of multidrug resistant (MDR) strains and the low number of antifungal drugs available to control the infection caused by this species highlight the urgent need to discover new molecular mechanisms that could be exploited to overcome this situation [8]. The search for substances able to inhibit the multidrug resistance transporters is extremely relevant, since several studies have already suggested the use of alternative drugs as potential adjuvants to treat multidrug resistant infections [9,10]. However, the ability of constitutive molecules such as salivary proteins and peptides in reversing this phenotype still remains unclarified.

Salivary antimicrobial peptides such as histatins, defensins and cathelicidins are essential for the microbial balance in the oral cavity. Due to their highly cationic nature, they are able to bind strongly to the cell wall of many bacterial and fungal species, inducing the formation of pores in the cytoplasmic membrane and consequent cell lysis [11].

Histatin-5 is a constitutive peptide produced and secreted by all major salivary glands, and therefore reaching high concentrations in saliva, maintaining its physiological range between 10.5 to 300 μg/mL [12]. This peptide possesses great antifungal activity, causing structural changes in the cell wall that induce the release of potassium and ATP [13].

The evaluation of the antifungal properties of salivary immune components has a great potential in the discovery of new methodologies for the treatment of candidiasis, including the reversion of antifungal resistance. Since Pdr5p shares a high similarity with CaCdr1p [14] and CaCdr2p [15], the major *Candida albicans* efflux proteins related to MDR phenotype, *S. cerevisae* mutants overexpressing Pdr5p transporter are considered a suitable model for the study of antifungal resistance related to ABC transporters in *C. albicans*. Thus, the aim of this study was to evaluate the ability of histatin-5 in reversing the MDR phenotype using a *S. cerevisiae* strain that overexpresses the Pdr5p transporter in vitro.

Materials and methods

Strains and culture conditions

In this study, two mutant strains of *S. cerevisiae* were used. The first strain, namely AD124567 (Pdr5p+), overexpresses Pdr5p, while the genes encoding the Pdr3p regulator and the other five ABC transporters (Yor1p, Snq2p, Pdr10p, Pdr11p and Ycf1p) have been deleted. The second one, namely AD1234567 (Pdr5p−), had all the six genes related to ABC transporters deleted, and also the gene that encodes the Pdr5p transporter. Consequently, Pdr5p+ strain shows fluconazole resistance, while Pdr5p− strain is sensitive to this antifungal agent [16,17]. Both strains were grown in YPD medium (2% glucose, 1% yeast extract, 2% peptone) at 30 °C with agitation and were harvested in the exponential phase of growth whenever experiments were about to be performed.

Chemicals

Fluconazole was obtained commercially from Farmacopa Pharmaceuticals (Rio de Janeiro, Brazil) and had a potency rate of 98.5—101.5%. Fluconazole stock solutions were prepared in distilled water, sterilized by filtration (0.22 μm) and maintained at −20 °C. Histatin-5 was acquired commercially from GenScript (Piscataway, NJ, USA) and a stock solution was prepared in phosphate buffer solution (PBS) 1 mM pH 7.2 to a final concentration of 1.0 mg/mL. Rhodamine 6G (R6G) was purchased from Sigma-Aldrich® (St Louis, USA) and a stock solution was prepared in distilled water and stored at room temperature.

Agarose diffusion chemosensitization assay

To evaluate the ability of histatin-5 in reversing the MDR phenotype of Pdr5p+ *S. cerevisiae* strain, the chemosensitization assay was performed with slight modifications [9]. Briefly, YPD medium containing 120 μg/mL of fluconazole was prepared with 1.5% agarose (Sigma-Aldrich®, St. Louis, MO, EUA). Cell suspensions containing 2.4×10^6 yeasts/mL were incorporated into molten agarose (45 °C), several amounts of histatin-5 (0.31; 0.62; 1.25; 2.5 and 5 μg) were applied to Whatman 3MM® blotting paper disks (Sigma-Aldrich®, St. Louis, MO, EUA), dried at 30 °C for 30 min and placed on the solidified medium surface. The plate was incubated at 30 °C for 48 h. A fluconazole-free medium was also used to evaluate the effect of histatin-5 alone on the growth of yeast cells.

Synergistic activity between histatin-5 and fluconazole

To assess the possible combined effect between the peptide and fluconazole, Pdr5p+ yeast cells were collected on the

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