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β-lapachone and α-nor-lapachone modulate *Candida albicans* viability and virulence factors

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ABSTRACT

Background. – *Candida albicans* is the most important fungal pathogen that causes infections in humans, and the search for new therapeutic strategies for its treatment is essential.
Objective. – The aim of this study was to evaluate the activity of seven naphthoquinones (β-lapachone, β-nor-lapachone, bromide-β-lapachone, hydroxy-β-lapachone, α-lapachone, α-nor-lapachone and α-xyloidone) on the growth of a fluconazole-resistant *C. albicans* oral clinical isolate and the effects of these compounds on the viability of mammalian cells, on yeast's morphogenesis, biofilm formation and cell wall mannoproteins availability.
Results. – All the compounds were able to completely inhibit the yeast growth. β-lapachone and α-nor-lapachone were the less cytotoxic compounds against L929 and RAW 264.7 cells. At IC_{50}, β-lapachone inhibited morphogenesis in 92%, while the treatment of yeast cells with α-nor-lapachone decreased yeast-to-hyphae transition in 42%. At 50 μg/ml, β-lapachone inhibited biofilm formation by 84%, whereas α-nor-lapachone reduced biofilm formation by 64%. The treatment of yeast cells with β-lapachone decreased cell wall mannoproteins availability in 28.5%, while α-nor-lapachone was not able to interfere on this virulence factor. Taken together, data show that β-lapachone and α-nor-lapachone exhibited in vitro cytotoxicity against a fluconazole-resistant *C. albicans* strain, thus demonstrating to be promising candidates to be used in the treatment of infections caused by this fungus.
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1. Introduction

Candida albicans is the most important fungal pathogen that causes infections in humans [1]. Nevertheless, the research for new therapeutic strategies for its treatment continues to be essential due to the high mortality associated with candidiasis, especially in immunocompromised individuals, as well as the small number of drugs available for treatment, the adverse effects associated with the usage of certain compounds and the increasing incidence of antifungal resistance [2].

Since fungal and animal cells share a conserved eukaryotic biology, the discovery of new antifungal medicines has been considered an outstanding challenge to modern research [3]. An alternative strategy for the development of anti-*C. albicans* drugs may be the use of virulence factors of yeast as pharmacological targets. These virulence factors can be defined as features that allow the establishment of infection, and hence, of the disease itself [4]. *C. albicans* has developed several of these virulence factors throughout its evolution, and three of the most relevant of them are the cell wall mannoprotein production [5], the yeast-to-hyphae transition, also called morphogenesis [6] and the biofilm formation [7].

Natural products have been traditionally used as therapeutic agents. In the last two decades, several studies have found that quinone compounds exhibit valuable pharmacological properties such as antitumor [8] and trypanocidal activities [9]. The development of synthetic organic chemistry has allowed structural modifications of compounds obtained naturally, in order to improve pharmacological and/or toxicological properties. Two examples are β-lapachone and α-nor-lapachone, quinones that possess a variety of pharmacological activities and can be synthesized from lapachol [10,11].

Due to the current epidemiological situation of candidiasis and the difficulty in obtaining effective therapeutic strategies, mainly against infections caused by resistant microorganisms, the aim of

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this study was to evaluate the activity of seven naphthoquinones on the growth of a fluconazole-resistant *C. albicans* oral clinical isolate, and the effects of these compounds on the viability of mammalian cells, on yeast's morphogenesis, biofilm formation and cell wall mannoproteins availability.

2. Materials and methods

2.1. Yeast strain and growth conditions

A *C. albicans* strain (namely PRI) isolated from the oral mucosa of a pediatric HIV patient known to be resistant to fluconazole (MIC $>$ 256 μg/ml) was used in this study [12]. Yeasts were maintained at low temperature (liquid nitrogen) and before any experimental procedures, cells were subcultured into brain heart infusion (BHI) broth and incubated at 37 °C for 48 h to reach exponential growing phase.

2.2. Compounds

β-lapachone (β-lap), β-nor-lapachone (β-nor-lap), bromide-β-lapachone (Br-β-lap), hydroxy-β-lapachone (OH-β-lap), α-lapachone (α-lap), α-nor-lapachone (α-nor-lap) and α-xyloidone (α-xyl) (Fig. 1) were synthesized by Laboratory of Heterocyclic Chemistry from Institute of Natural Products Research (IPPN/UFRJ). Compounds were dissolved in dimethyl sulfoxyde (Sigma Aldrich®, St. Louis, USA) to a final concentration of 10 mg/ml.

2.3. Antifungal susceptibility testing

The minimal inhibitory concentration (MIC) was determined according to the M27-A3 methodology for microdilution from CLSI [13]. Briefly, cells were inoculated into RPMI-1640 medium at a concentration of 5×10^3 cells/ml and incubated at 37 °C for 48 hours with agitation (75 rpm), in the presence of serial concentrations (100–0.2 μg/ml) of the compounds. Cell growth was measured using a microplate reader at 600 nm (Fluostar Optima, BMG Labtech, Offenburg, Germany). The minimum concentration able to inhibit 100% and 50% of yeast growth were defined as MIC and IC_{50}, respectively.

2.4. Cytotoxicity

Cytotoxicity of the compounds was determined using the "dye-uptake" technique [14] with slight modifications. Two cell lines were used in this study, L929 fibroblast cells and RAW 264.7 macrophage cells obtained from mice. Serial dilutions of the compounds were made, using Minimum Eagle Medium (MEM) (Cultilab®, São Paulo, Brazil) without serum as diluent. Then, the substances were placed in contact with confluent cell monolayers. The cells were incubated at 37 °C for 72 h in 5% CO_2. Then, neutral red dye was added to each well of a 96-well flat-bottomed microtitre plate, followed by incubation at 37 °C for 3 h. After this period, the dye was discarded; the cell monolayers were washed three times with PBS 10 mM (KH_2PO_4 0.43 g, NaCl 7.2 g, Na_2HPO_4 2.54 g, final volume 1000 ml, pH adjusted to 7.2) and added 4% formaldehyde during 5 minutes for fixation. After this procedure, a solution containing 1% acetic acid and 50% methanol was added to the system. Cells were then incubated at room temperature and the cell viability was evaluated in a spectrophotometer (FLUOstar OPTIMA, BMG Labtech®, Offenburg, Germany) at 492 nm. The results are expressed as the concentration of compounds able of inhibiting mitochondrial metabolism by 50%, also referred as CC_{50}.

2.5. Morphogenesis

The effect of the naphtoquinones on yeast-to-hyphae transition was evaluated as described by Braga-Silva and coworkers (2007) with slight modifications. Briefly, 5×10^6 cells were incubated in 1.0 ml fetal bovine serum (FCS) (Cultilab®, São Paulo, Brazil) in the presence or absence of the compounds at IC_{50} for 3 h at 37 °C with agitation (75 rpm). Cell viability was tested by the trypan blue exclusion method. Then, cells were suspended in 1 M NaOH containing 10 mM EDTA and 1% (v/v) β-mercaptoethanol to eliminate clusters that could have hampered counting accuracy. A haemocytometer Neubauer chamber was used for differential counting, and the percentage of germ tubes was determined. This procedure was performed four times, and a minimum of 500 cells were counted at each experiment.

2.6. Biofilm formation inhibition

The effect of the naphthoquinones on biofilm formation was evaluated as described by Thein and coworkers (2007) with slight modifications [15]. Briefly, 10^6 cells were added to a 96-well flat-bottomed microtitre plate and incubated at 37 °C for 90 min with gentle agitation (75 rpm). Supernatant was removed and wells were gently washed twice with PBS to remove non-adherent cells. YNB medium BD® (Franklin NJ, USA) supplemented with 100 mM of glucose containing serial concentrations of the compounds (range: 100–0.2 μg/ml) was added to the plates containing the

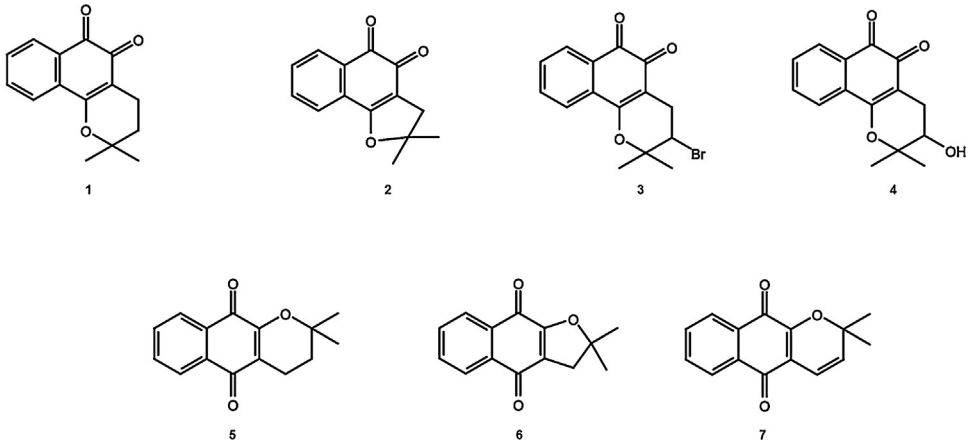

Fig. 1. Structure of the compounds tested in this study. 1–β-lapachone; 2–β-nor-lapachone; 3–bromide-β-lapachone; 4–hydroxy-β-lapachone; 5–α-lapachone; 6–α-nor-lapachone; 7–α-xyloidone.

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