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Short Communication

In vivo and in vitro activity of a bis-arylidenecyclo-alkanone against fluconazole-susceptible and -resistant isolates of *Candida albicans*

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ABSTRACT

Objectives: Candida albicans is a commensal organism and opportunistic pathogen associated both with superficial (mucosal and cutaneous) and systemic infections. Extensive use of antifungal agents has led to reduced susceptibility to the few existing drugs, which has encouraged the search for novel antifungal agents. Therefore, the present study investigated the antifungal activity of 2,6-bis[(*E*)-(4-pyridyl) methylidene]cyclohexanone (PMC) against *C. albicans*.
Methods: The in vitro activity of PMC was evaluated against *C. albicans*. Additionally, an invertebrate infection model in *Caenorhabditis elegans* as well as two infected murine models of oral and systemic candidiasis were used to determine the antifungal efficacy of PMC in vivo.
Results: Minimum inhibitory concentrations (MICs) of PMC ranged from 4–32 μg/mL against nine clinical and two reference *C. albicans* isolates. Interestingly, PMC inhibited filamentation in vitro at subinhibitory concentrations similar to fluconazole. PMC also showed low toxicity against murine macrophages and human erythrocytes. In the invertebrate infection model, PMC was efficient in prolonging survival of *C. elegans* infected with *C. albicans* SC5314. Treatment with PMC was efficient both in murine models of systemic and oral candidiasis and was similar to that observed with conventional drug treatments (nystatin and fluconazole).
Conclusions: The results of this study indicate the therapeutic potential of PMC as it was able to inhibit filamentation of *C. albicans* in vitro. These alterations to the fungi by PMC resulted in a reduction of oral and systemic infection in mice. In conclusion, we present promising evidence of the anticandidal activity of PMC in vitro and in vivo.

1. Introduction

Candida albicans is part of the human microbiota and is an opportunistic pathogen. Candidiasis is the most prevalent of the human mycoses worldwide and, although more than 30 *Candida* spp. are recognised, *C. albicans* is the most common [1–4] The main classes of therapeutic agents used to treat such infections are polyenes, echinocandins and azoles [5–8]. The polyene amphotericin B is an effective antifungal agent, however its extended use can lead to nephrotoxicity. Echinocandins and azoles are less toxic, although there is an increasing incidence of fungal resistance to these agents [9].

2,6-Bis[(*E*)-(4-pyridyl)methylidene]cyclohexanone (PMC) belongs to a new class of antiparasitic compounds that were discovered by Braga et al. [10]. These authors demonstrated that PMC inhibited trypanothione reductase activity, a vital enzyme for antioxidant defences in *Trypanosoma cruzi*, and showed antileishmanial potency against the extracellular promastigote form of the parasite but exhibited almost no activity against the amastigote form. However, the antifungal activity of PMC is still being

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investigated and its target and action remain unknown. Therefore, we sought to determine the use of PMC against *C. albicans*, an important agent of human fungal disease, making this compound even more interesting from the new drug development perspective.

2. Materials and methods

2.1. Synthesis of 2,6-bis[(E)-(4-pyridyl)methylidene]cyclohexanone (PMC)

PMC was synthesised in one step by cross-aldol condensation as previously described by Braga et al. [10].

2.2. In vitro antifungal susceptibility testing

In vitro antifungal susceptibility was determined by the microbroth dilution method performed in sterile, flat-bottomed, 96-well microplates. Briefly, two reference isolates of *C. albicans* (SC5314 and ATCC 18804) as well as nine clinical *C. albicans* isolates from the Culture Collection of Microorganisms and Cells of the Federal University of Minas Gerais (UFMG) (Belo Horizonte, MG, Brazil) were cultured in Sabouraud dextrose agar (SDA) broth (HiMedia, Mumbai, India) at 37 °C for 24 h. Broth microdilution testing was performed in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [11]. PMC was tested at concentrations ranging from 0.125 μg/mL to 64 μg/mL. Fluconazole (Sigma-Aldrich, St Louis, MO) and nystatin (Squibb, New York, NY) were included as controls and were tested at concentrations of 0.125–64 μg/mL and 0.06–32 μg/mL, respectively. Plates were incubated at 35 °C for 48 h and culture growth was determined using a Benchmark Plus Microplate Spectrometer (Bio-Rad Laboratories, Hercules, CA) at 595 nm. The minimum inhibitory concentration (MIC) of fluconazole was considered as the concentration causing 80% inhibition [11], and the MIC of PMC and nystatin was the concentration causing 100% inhibition.

2.3. Inhibition of filamentation

To evaluate the effects on filamentation, *C. albicans* SC5314 in log growth phase was cultured in Lee's medium [12] with the modifications described by Lu et al. [13]. Fluconazole was included as a control using the same conditions as those for PMC at the MIC. Following 24 h of incubation at 37 °C, cell morphology was observed by optical microscopy (Axioplan; Carl Zeiss, Thornwood, NY). The degree of yeast–hypha inhibition was quantified by calculating the percentage of hyphal cells among 100 cells in a single sample. Counting was performed in triplicate.

2.4. Animal studies in mice

Female C57BL/6 mice were supplied by the Biological Center of the Federal University of Minas Gerais (CEBIO, UFMG, Belo Horizonte, MG, Brazil). All animal procedures were performed in accordance with the Ethics Committee for Animal Experimentation of UFMG.

2.5. Toxicity tests

2.5.1. Evaluation of cytotoxic effects against murine macrophages

Peritoneal macrophages were obtained from C57BL/6 mice for toxicity evaluation as described by Pereira Sá et al. [14]. Mice were injected intraperitoneally with 2 mL of 3% sodium thioglycollate (AMRESCO Inc., Solon, OH). Mice were euthanised after 72 h and were dipped in 70°GL alcohol for disinfection. The peritoneal membrane was exposed and 3 mL of ice-cold phosphate-buffered saline (PBS) was injected into the peritoneal cavity to collect macrophages. The obtained peritoneal lavage was centrifuged at 150 × *g* for 10 min at 4 °C. The cell pellet was suspended in RPMI (Sigma-Aldrich) with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) and the cell density was determined by counting in a Neubauer chamber. Macrophages from each mouse were seeded (1×10^5 cells/well) in 96-well plates and were incubated for 12 h at 37 °C in an incubator with 5% CO_2. Murine macrophages were then pre-incubated in 96-well plates for 24 h at 37 °C to allow cells to adapt prior to addition of the test compound. PMC and amphotericin B (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) prior to dilution, whereas fluconazole (Sigma-Aldrich) was dissolved in sterile deionised water. The half-maximal inhibitory concentration (IC_{50}) was determined over a range of concentrations (eight non-serial dilutions from 100 μM to 0.0128 μM). Cells were incubated with PMC or 0.5% DMSO as a control in a 5% CO_2/95% air-humidified atmosphere at 37 °C for 48 h. Cell viability was estimated by measuring the rate of mitochondrial reduction of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (AMRESCO Inc.). The selectivity index (SI) of PMC was determined by the ratio of the IC_{50} value in murine macrophages to the MIC (SI = IC_{50}/MIC).

2.5.2. Erythrocyte toxicity assay

An erythrocyte toxicity assay was performed as previously described by Sá et al. [15]. Human erythrocytes (Rockland Immunochemicals, Inc., Pottstown, PA) were diluted to 2% in PBS and then 190 μL aliquots were added to a 96-well plate. A 10 μL aliquot of PMC in PBS was added to the wells with the erythrocytes. The final concentration of PMC in the wells ranged from 0.125 μg/mL to 64 μg/mL. Triton X-100 was included as a positive control and PBS was used as a negative control; a control with DMSO was also performed. Plates were incubated at 37 °C for 1 h and were then centrifuged at 500 × *g* for 5 min to pellet the intact erythrocytes. A 100 μL supernatant sample from each well was transferred to a new 96-well plate. The percentage haemolysis was determined by measuring the absorbance at 540 nm using a Benchmark Plus Microplate Spectrometer (Bio-Rad Laboratories) and then normalising the mean absorbance value to the mean absorbance of the 1% Triton X-100-treated samples, which represented 100% haemolysis. Two independent experiments were performed in triplicate.

2.6. In vivo assays

2.6.1. Survival curve of Caenorhabditis elegans

For this experiment, the methodologies described by Breger et al. [16] and Scorzoni et al. [17] were used with some modifications. In brief, the *C. elegans* mutant strain *glp4–sek1* was used for this assay. Synchronised worms in the young adult stage were added to the centre of agar plates containing a lawn of *C. albicans* SC5314 and were incubated for 3 h at 25 °C. The worms were dislodged from the plate using M9 growth medium, were subsequently washed four times with M9 and were then collected by mild centrifugation. The fungus-infected worms were then transferred to liquid culture in a six-well plate containing 2 mL of 80% M9 buffer, 20% brain–heart infusion, 10 μg/mL cholesterol in ethanol and 90 μg/mL kanamycin.

To evaluate antifungal efficacy, PMC was added to the medium until a concentration of 8 μg/mL was reached. The plates were incubated overnight at 25 °C and were then examined at 24-h intervals for survival. In this assay, 45 worms per experimental group were used and worms were considered dead when they did not respond to touch by a platinum wire pick; dead worms were removed.

2.6.2. Murine model of oral candidiasis

The murine model of oral candidiasis was established as previously described by Wong et al. [18] with modifications. The