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In vitro anti-*Candida* activity of selective serotonin reuptake inhibitors against fluconazole-resistant strains and their activity against biofilm-forming isolates

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ABSTRACT

Recent research has shown broad antifungal activity of the classic antidepressants selective serotonin reuptake inhibitors (SSRIs). This fact, combined with the increased cross-resistance frequency of the genre *Candida* regarding the main treatment today, fluconazole, requires the development of novel therapeutic strategies. In that context, this study aimed to assess the antifungal potential of fluoxetine, sertraline, and paroxetine against fluconazole-resistant *Candida* spp. planktonic cells, as well as to assess the mechanism of action and the viability of biofilms treated with fluoxetine. After 24 h, the fluconazole-resistant *Candida* spp. strains showed minimum inhibitory concentration (MIC) in the ranges of 20 −160 μg/mL for fluoxetine, 10−20 μg/mL for sertraline, and 10−100.8 μg/mL for paroxetine by the broth microdilution method (M27-A3). According to our data by flow cytometry, each of the SSRIs cause fungal death after damaging the plasma and mitochondrial membrane, which activates apoptotic signaling pathways and leads to dose-dependant cell viability loss. Regarding biofilm-forming isolates, the fluoxetine reduce mature biofilm of all the species tested. Therefore, it is concluded that SSRIs are capable of inhibit the growth in vitro of *Candida* spp., both in planktonic form, as biofilm, inducing cellular death by apoptosis.

1. Introduction

In recent years, hospital-acquired fungal infections have received attention due to their greater prevalence and high mortality rates, ranging between 40 and 70% depending on the risk factor involved [1]. Among clinically relevant yeasts, those in the genus *Candida* are important opportunistic pathogens isolated from these hospitalized patients, mainly immunocompromised [2].

Some hospital-acquired fungal infections, such as in the blood stream or in the urinary tract, are directly linked to implanted medical devices, which may serve as support for biofilm growth, one of the main virulence factors of the genus *Candida* [3]. Mature biofilms are much more resistant against both conventional antimicrobials and host defense mechanisms compared to free (planktonic) cells in the medium [3,4].

The high incidence of fungal infections caused by *Candida* spp. and the increase in strains resistant against the main treatment

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(fluconazole), associated with these species' ability to form biofilms, have led to a growing number of treatment failures, which requires new, effective anti-*Candida* agents [4].

The literature carries reports of selective serotonin reuptake inhibitors (SSRIs) impacting antimicrobial activity, such as against gram-positive and some gram-negative bacteria, against fungi, and in the reversal of *Plasmodium falciparum* resistance [5–7].

Lass-Flörl et al. [8] reported in vitro antifungal activity of SSRIs against *Aspergillus* spp. and *Candida parapsilosis*. Among the five SSRIs tested, fluoxetine and sertraline showed high activity against these fungi.

The anti-*Candida* activity of three SSRIs was analyzed in the present study: fluoxetine, paroxetine, and sertraline. Given the antifungal potential of SSRIs, this study aimed to verify the antifungal effect of the candidates against fluconazole-resistant *Candida* spp. strains while elucidating the possible mechanisms involved in the cytotoxic action by employing procedures such as flow cytometry and comet assay. The study also assessed the activity of SSRIs against biofilms of different *Candida* species.

2. Materials and methods

2.1. Yeast strains

Fluconazole-resistant clinical strains of *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata* (four strains from each species) were used. These strains are from the yeast collection of the Laboratory of Bioprospection in Antimicrobial Molecules (LABIMAN/FF/UFC) [9,10].

2.2. Molecular identification

Genomic DNA was purified from the yeast strains using a cetyltrimethylammoniumbromide (CTAB)-based protocol as previously described [11]. The nuclear DNA region comprising the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) as suggested by White et al. [12]. Amplification reactions were performed in a final volume of 25 μL, which contained genomic DNA (300–400 ng), 1× GoTaq reaction buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 200 μM each dNTP (GE Healthcare Life Sciences, Piscataway, NJ, USA), 0.5 μM each primer and 1.25 U of GoTaq DNA Polymerase (Promega). Reactions were carried out in a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) programmed for an initial denaturation step (2 min at 95 °C) followed by 35 cycles of 1 min at 95 °C, 1 min at 60 °C, and 3 min at 72 °C. The last cycle was followed by a final incubation of 10 min at 72 °C. Control samples containing all reaction components, except DNA, were used to test that no DNA contamination occurred. The amplifications specificity was determined by 1.0% agarose gel electrophoresis [13]. The remaining amplified products were purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) and the concentrations of the purified amplicons were determined by measuring the absorbance at 260 nm of ten-fold dilutions. DNA sequencing was performed at the Macrogen Inc. (Seoul, South Korea) using the Sanger's dideoxy chain termination method. Both strands of each amplicon were sequenced using the ITS4 and ITS5 primers. Sequences were then assembled using the Phred/Phrap/Consed package [14–16]. The start and end boundaries of the ITS1 and ITS2 were identified by comparisons to annotated sequences from the ITSoneDB [17] and ITS2 database [18], respectively. The ITS/5.8S sequences were deposited in the GenBank database (accession numbers: AB861478, AB861479, KJ740174, KJ740175, KJ740184, AB861491, AB861493, AB861490, AB861486, AB861487, AB861488, AB861485, AB861484) and compared to those available on public DNA sequence databases using the BLAST program [19].

2.3. L929 cell proliferation inhibition using the MTT test

L929 cells were cultivated under standard conditions in MEM with Earle's salts. All culture media were supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/mL penicillin/streptomycin at 37 °C with 5% CO_2. L929 cells were plated in 96-well plates (0.3×10^6 cells/well) and test compounds (0.156–100 μg/mL) dissolved in ethanol were added to each well, followed by incubation for 24 h, under standard cultivation conditions. Cells were treated with MTT assay reagents (0.5 mg/mL). Three hours later, MTT formazan product was dissolved in 150 μL DMSO and absorbance was measured using a multiplate reader (Packard Spectra Count, Canada). Test substances effect was quantified as reduced dye control absorbance percentage at 595 nm. Experiments were carried out in duplicate and repeated at least three times [9].

2.4. In vitro antifungal activity

The broth microdilution (BMD) susceptibility test was performed according to the document M27-A3 using RPMI broth (pH 7.0) buffered with 0.165 M MOPS (Sigma Chemical, St. Louis, MO). Fluconazole (FLC; Merck Sharp & Dohme, São Paulo, Brazil) was dissolved in distilled water and tested at concentrations ranging from 0.125 to 64 μg/mL. The selective serotonin reuptake inhibitors (SSRIs): fluoxetine, sertraline and paroxetine (FLX, SER, PAR; Galena Química e Farmacêutica, Brasil) were dissolved in 95% ethanol and their effects on yeast cells were evaluated at concentrations ranging from 4.68 to 600 μg/mL. The yeasts and compounds were incubated in 96-well culture plates at 35 °C for 24 h and the results were examined visually, as recommended by CLSI [20]. The minimum inhibitory concentration (MIC) of each compound was determined as the concentration that inhibited 50% of fungal growth. The strains were classified as susceptible (S) or resistant (R) to FLC according to the following cutoff points, as recommended by the CLSI document M27-S4: MIC $\leq$ 2 μg/mL (S), MIC $\geq$ 8 μg/mL (R). The strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as controls [21].

2.5. Cell treatments

To assess cell density, membrane integrity, mitochondrial transmembrane potential, and DNA damage, one representative FLC-resistant strain of *C. albicans* (strain 2) was exposed for 24 h to various concentrations (MIC, 2 × MIC, and 4 × MIC) of fluoxetine, sertraline or paroxetine. Cells treated with FLC (64 μg/mL) or amphotericin B (4 μg/mL) were included for comparison. All the tests were performed in triplicate in three independent experiments [9,10].

2.6. Preparation of yeast suspensions

Cell suspensions were prepared from cultures in the exponential growth phase. The cells were collected, washed, re-suspended and adjusted to 10^6 cells/mL in HEPES buffer (pH 7.2) supplemented with 2% glucose [9,10].

2.7. Determination of cell density and membrane integrity

Cell density and membrane integrity of the fungal strain were evaluated by the exclusion of 2 mg/L propidium iodide (PI). Aliquots