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Short Communication

Tricyclic antidepressants inhibit *Candida albicans* growth and biofilm formation

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ABSTRACT

Candida albicans is a commensal yeast of the human body, able to form biofilms on solid surfaces such as implanted medical devices, and contributes to nosocomial infections. Biofilms have the capacity to resist higher levels of antifungals compared with planktonic cells, and can develop tolerance to commonly used treatments. The necessity to overcome acquired drug resistance and identify new active molecules with low toxicity is a significant problem. It has been reported that some antidepressants have antibacterial properties, but little is known regarding the effect of these drugs on fungi. This study demonstrated the capacity of three tricyclic antidepressants (doxepin, imipramine and nortriptyline) to inhibit the growth and biofilm formation of *Candida* spp. The antimicrobial potential of the drugs was assessed by studying gene expression, hyphae formation, biofilm growth and maturation. Their negative impact on the growth of *C. albicans* and other *Candida* spp. is shown in vitro and with the hepatic S9 system, which is preliminary to any in-vivo test. This study found that the antidepressants considered can inhibit not only hyphae and biofilm formation, but also kill cells in a mature biofilm. Moreover, cell lysis by nortriptyline was observed, along with its synergistic activity with amphotericin B. These findings suggest that tricyclic antidepressants, particularly nortriptyline, should be studied further in drug repositioning programmes to assess their antimycotic capacity in full.

1. Introduction

Candida albicans is a commensal yeast of the human gastrointestinal and gastrourinary tracts. In immunocompromised individuals, *C. albicans* can cross the host's protective membranes and colonize internal organs, producing life-threatening infections. In addition, *C. albicans* may become an opportunistic pathogen for healthy individuals because of its ability to form biofilms on solid surfaces, such as implanted medical devices, where they contribute to nosocomial infections [1]. Biofilm formation is a systematic, highly controlled process involving different stages. First, yeast cells attach to a surface where they proliferate. Next, filamentous cells create the basal layer, where the biofilm can develop and accumulate extracellular matrix. Finally, cells are dispersed from the biofilm, ready to colonize a new environment. Key transcriptional factors (TFs) (i.e. Bcr1, Efg1, Ndt80) are necessary to establish this community of cells [2–4], and the *ALS* gene family, encoding for proteins implicated in surface attachment, are among their target genes [5]. Later phases of biofilm formation and production of the extracellular matrix are mainly regulated by the TF Zap1 [6]. From a clinical point of view, one of the most worrying characteristics of a biofilm is the capacity to resist higher levels of antifungal drugs compared with planktonic cells, and the possibility of developing tolerance to active compounds like fluconazole or amphotericin B (AmB) [7], posing serious problems for patients with candidiasis. The major cause of these infections remains *C. albicans*, but other *Candida* spp. such as *C. glabrata, C. parapsilosis, C. krusei* and *C. utilis* are isolated more and more frequently [8]. Therefore, treatments based on new pharmaceutical formulations and combinations of therapies are needed.

Previous studies have shown that the antidepressant sertraline, a serotonin-specific re-uptake inhibitor, can hinder both bacteria and yeast growth, while the tricyclic antidepressant (TCA) amitriptyline can block prokaryotic cell growth [9]. However, the antifungal activity of TCAs is largely unknown and was investigated in this study.

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2. Materials and methods

2.1. Strains, cultures and chemicals

C. albicans SC5314, *C. utilis* CBS 5609, *C. krusei* (Castellani) Berkhout, *C. glabrata* 8706 (Neqas collection), *Saccharomyces cerevisiae* BY4742, *Escherichia coli* K-12, *Staphylococcus aureus* ATCC 6538 and *Lactobacillus casei* were used in this study. Yeast strains were grown in Yeast extract-Peptone medium (YP) with 2% glucose at 37°C or RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) for biofilm formation and maturation. Bacteria strains were grown in Luria Bertani broth (LB) at 37°C (*E. coli* and *S. aureus*) or De Man, Rogosa and Sharpe medium (MRS) medium at 28°C (*L. casei*). AmB, doxepin, imipramine and nortriptyline were purchased from Sigma-Aldrich Corp. (St Louis, MI, USA). Plastic consumables were purchased from Sarstedt AG & Co. (Numbrecht, Germany).

2.2. RNA isolation and quantitative reverse transcription polymerase chain reaction

Exponentially growing *C. albicans* strain SC5314 was inoculated into medium containing the minimum inhibitory concentration of each drug required to inhibit the growth of 75% of organisms (MIC_{75}), and grown for 3 h at 37°C. Expression in biofilm was tested on cells grown for 48 h and treated as described above. Extractions of total RNA, retrotranscriptions and quantitative reverse transcription polymerase chain reaction (qRT-PCR) were performed as described previously [10]. The primers employed are listed in Table S1 (see online supplementary material). All qRT-PCR data were analysed using the $2^{-\Delta\Delta Ct}$ method, comparing the relative expression levels with that of *TAF145*, the reference gene. The expression of the latter is stable for the period considered, supporting the fact that cell death is not yet induced. Each qRT-PCR data point represented the mean of three technical replicates.

2.3. Assays on biofilm formation and maturation

Attachment of cells to a multi-well plate (flat bottom, polystyrene, Sarstedt AG & Co) was described previously [11]. After the attachment phase, cells were submerged in medium, with or without the addition of the drugs at MIC_{99} or MIC_{10}, and grown at 37°C for 48 h. For biofilm maturation**,** cells were grown in RPMI-1640 medium for 48 h and washed twice with phosphate buffered saline (PBS). Next, fresh medium alone or medium containing the specific MIC_{99} (or MIC_{10}) concentration of each molecule was added to the plate, which was then incubated for 48 h. Cells were scraped from the surface, diluted and plated in order to count the colony-forming units (CFUs). In parallel, biofilm formation was monitored with an assay based on XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay [11]. Percentage survival was calculated using the following formula: (number of survivors in the treated sample)/(number of cells in the untreated control) × 100.

2.4. Visualization of hyphae

C. albicans cells were grown overnight and freshly diluted in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 2% glucose, with or without the addition of TCAs at different concentrations. After 16 h of incubation, cells were stained with Calcofluor white (0.1%) and propidium iodide (0.05%), and visualized using an AXIO Image Z2 (Zeiss, Jena, Germany).

2.5. Evaluation of cellular lysis, measurement of protein concentration and flow cytometry

Exponentially growing cells were diluted in 2 mL of PBS at an optical density of 1 measured at a wavelength of 600 nm (OD_{600}) with or without the supplement of the drugs at MIC_{99}. Samples were incubated for 24 h at 37°C at 150 rpm. Next, the OD_{600} of each sample was measured, and 1.5 mL was transferred to a new tube and centrifuged. The supernatant was concentrated with a SpeedVac (Savant Instruments Pvt Ltd, Maharashtra, India). The protein concentration was evaluated with Bradford Protein Assay according to the manufacturer (Bio-Rad Laboratories Inc., Hercules, CA, USA). Fifty microlitres of the same samples were also analysed with a flow cytometer (NovoCyte; ACEA Biosciences, Inc., San Diego, CA, USA), where forward scatter and side scatter channels were evaluated.

2.6. S9 mix digestion

Cells were prepared as described for the biofilm formation inhibition assay. In addition, S9 mix was added to wells containing the drugs (present at MIC_{99}), and survival after 48 h of incubation was tested. Comparison with samples (prepared in parallel) that contained the drug, but not the S9 enzymatic mix, was performed. S9 mix was used at the final concentration of 0.02 10^{-3} mg/L, as no negative effect was observed on the control samples containing only the media and the cells at this concentration.

2.7. Statistical analysis

Data analysis was performed using SPSS (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Tricyclic antidepressants inhibit yeast growth

The TCAs doxepin, imipramine and nortriptyline inhibited cell growth for all bacterial and yeast species tested, but with different efficacy. MIC_{99} data (see Table S2, online supplementary material) indicated that bacterial cells were four to 40 times less sensitive to these molecules than yeast cells, with nortriptyline and imipramine being the most effective in yeast cells. Subsequent experiments focused on the effect of the TCAs on the opportunistic pathogen *C. albicans.*

3.2. Transcription of key biofilm regulatory genes is modified by tricyclic antidepressants

In order to test whether incubation with TCAs could affect the expression of key biofilm regulators, the effect of TCAs at MIC_{75} was studied. This concentration, for a short incubation time of 3 h, modifies gene expression without affecting cell viability. AmB, a known antifungal, was used as a control. For planktonic cells, the presence of TCAs downregulated the transcriptions of *BCR1* and *EFG1* (Fig. 1A) and the cell surface encoding gene *ALS1,* while in a treated biofilm, the same genes displayed increased gene expression with respect to the untreated control (Fig. 1A). In both growing conditions, expression of *ZAP1* or *NDT80* was barely affected by TCAs.

3.3. Hyphae formation is inhibited by the presence of TCAs

To analyse the effect of TCAs on hyphal growth, cells were grown in medium containing a hyphal inducer (10% FBS) along with different amounts of TCAs. Control samples without TCA

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