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Evaluation of four calcium channel blockers as fluconazole resistance inhibitors in *Candida glabrata*

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ABSTRACT

Objectives: The aim of this study was to evaluate the ability of four calcium channel blockers (CCBs), namely verapamil, diltiazem, nicardipine (NIC) and nifedipine (NIF), to enhance the susceptibility of *Candida glabrata* strains to fluconazole (FLC).
Methods: Synergistic antifungal effects of the CCBs with FLC were examined by the chequerboard method, and fractional inhibitory concentration indices (FICIs) were determined. The time–kill curve method was used for the most promising combination to further evaluate the synergetic effects.
Results: NIC showed an additive effect with FLC against FLC-resistant and FLC-susceptible-dose-dependent strains (DSY 565 and CBS 138) known to express efflux pumps, but not against FLC-susceptible strains. NIF exhibited an additive effect with FLC both by the chequerboard method ($0.5 < FICI < 1$) and time–kill curves (<2 $\log_{10}$ CFU/mL decrease in viable count). In addition, NIF had its own antifungal effect consistently against most of the strains used in this study, with minimum inhibitory concentrations (MICs) of 8 μg/mL.
Conclusions: NIC showed an additive effect with FLC against FLC-resistant *C. glabrata* strains, most probably via efflux pump inhibition as demonstrated selectively in FLC-resistant strains with known efflux pumps. NIF displayed a promising antifungal effect alone as well as an additive effect with FLC against most of the strains.

1. Introduction

Candida glabrata has emerged as the second most common cause of mucosal and invasive fungal infections after *Candida albicans* [1]. According to a US Centers for Disease Control and Prevention (CDC) report, *C. glabrata* is considered to be an upcoming threat due to its increasing frequency of resistance to antifungal drugs such as fluconazole (FLC) [2]. It has intrinsic low susceptibility to azole antifungals that are usually effective in treating infections caused by other *Candida* spp., thus it requires specific consideration in treatment approaches [3]. In general, there is an increasing rate of fungal infections and resistance with a limited number of available antimycotics compared with antibacterial agents [4,5]. This has led to a continuous search for new antifungals, alone or in combination, able to reverse resistance to these agents.

Antimicrobial resistance can be primary (intrinsic) or secondary (acquired) [4]. One of the major mechanisms of resistance in *Candida* spp. involves reduced drug accumulation via the development of active efflux pumps. These pumps are encoded by two gene families of transporters: *CDR1* and *CDR2* of the ATP-binding cassette (ABC) superfamily; and the *MDR* genes of the major facilitator superfamily (MFS) [5].

A wide range of compounds have been studied for the purpose of reversing multidrug resistance (MDR) in cancer cells, with calcium channel blockers (CCBs) probably being the most extensively investigated [6]. As fungal and human cells are both eukaryotic, using the same principle of MDR reversal, some CCBs were also studied for synergistic effect with FLC in *C. albicans*. Bulatova and Darwish studied 15 chemosensitisers and demonstrated that some of them, including CCBs, caused a shift in FLC susceptibility in *C. albicans* [7]. However, to the best of our knowledge, none of the previous studies investigated the ability of CCBs to reverse FLC resistance in *C. glabrata*.

The aim of the study was to evaluate the ability of four CCBs to reverse resistance to FLC in FLC-resistant strains of *C. glabrata*.

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2. Materials and methods

2.1. Candida glabrata strains

Candida glabrata ATCC 22553 was used as a control strain. *Candida glabrata* strains CBS 138 and CBS 850821 were a kind gift from Prof. Richard Cannon (University of Otago, Dunedin, New Zealand), whilst *C. glabrata* DSY 565 and DSY 562 were kindly gifted by Prof. Dominique Sanglard (Lausanne University Hospital, Lausanne, Switzerland). Two *C. glabrata* clinical strains from The University of Jordan Hospital (Amman, Jordan) were also used after being identified using CHROMagar™ differential medium. Each strain was cultured twice on Sabouraud dextrose agar (SDA) at 35 °C for 24–48 h.

2.2. Chemicals, materials and media used

Verapamil (VER), diltiazem (DLT), nifedipine (NIF), nicardipine (NIC) and FLC were purchased from Sigma-Aldrich. RPMI broth (with L-glutamine and phenol red but without sodium bicarbonate) was buffered with 0.165 M MOPS (morpholinepropanesulfonic acid) (Sigma-Aldrich) and supplemented with 2% glucose. Stock solutions of antifungals were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). To detect the cut-off point at which DMSO produces an antifungal effect, preliminary experiments were conducted with a starting DMSO concentration of 25%; the lowest concentration of DMSO found to produce an antifungal effect was 12.5%. Therefore, a starting DMSO concentration of 0.25% was selected for use in the experiments, which was further diluted according to the particular experiment.

2.3. Antifungal susceptibility testing

Broth microdilution susceptibility testing was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines [8] with some variations. The concentration range was 2–256 μg/mL for FLC, DLT, VER and NIC and 1–64 μg/mL for NIF. A 100 μL yeast inoculum suspension (ca. 0.5–2.5 × 10^3 cells/mL) was added to each well of the microdilution trays. Drug-free and yeast-free controls were included. The plates were incubated at 35 °C and minimum inhibitory concentrations (MICs) were assessed visually and by spectrophotometer reading at 530 nm after 24 h and 48 h. An MIC of ≤8 μg/mL was interpreted as susceptible, ≥64 μg/mL as resistant and 16–32 μg/mL as susceptible-dose-dependent (SSD) [8].

2.4. Chequerboard microdilution assay for combinations studies

The chequerboard method was applied for combinations of FLC with each CCB for each strain of *C. glabrata*, including CBS 138, CBS 850821, DSY 565, DSY 562, clinical strains 1 and 2, and reference strain ATCC 22553. Each experiment was conducted in triplicate.

Modes of interaction between drugs were classified as synergistic, additive or antagonistic based on the fractional inhibitory concentration index (FICI), which was calculated by the following formula [9]:

$$(MIC_{A\text{ in combination}}/MIC_{A\text{ alone}}) + (MIC_{B\text{ in combination}}/MIC_{B\text{ alone}})$$

where A is FLC and B is the CCB in each chequerboard experiment.

The interaction was defined as synergistic when the FICI was ≤0.5, indifferent when the FICI was >0.5 but <4.0, and antagonistic when the FICI was ≥4.0 [10]. However, when there was no antifungal effect of the CCB, the individual fractional inhibitory concentration (FIC) for FLC and not the sum of the FICs was used, and the results were interpreted as follows: FIC ≤0.25, synergism; FIC >0.25 but ≤0.5, additive; FIC >0.5 but <2, indifference; and FIC ≥2, antagonism [11]. In other words, an individual FIC of ≤0.25, indicative of a four-fold reduction, was assumed as synergy [10,11].

2.5. Time–kill curve studies

The time–kill curve study was performed using the combination of FLC and NIF on four different strains of *C. glabrata* (CBS 138, CBS 850821, DSY 565 and DSY 562).

Each experiment was performed using the microdilution method under experimental conditions identical to those described for the chequerboard microdilution method as follows: FLC alone; NIF alone; four wells for the combination with different concentrations chosen; one well for growth control; and another for sterility control. These steps of the time–kill curve method were done under the same experimental conditions as the chequerboard assay.

These procedures resulted in a starting inoculum of ca. 0.5–2.5 × 10^3 CFU/mL. Antifungal concentrations equal to 0.5×, 1× and 2× MIC for FLC alone and 1× and 2× MIC for NIF alone were used. Each experiment was conducted in duplicate.

Wells containing RPMI 2% G medium were added with either FLC, NIF or their combination. The combinations were studied at different concentrations to determine the most suitable concentration for each drug in the combination. Four different combinations for each strain were studied: MIC_{FLU}/0.5× MIC_{NIF}; 0.5× MIC_{FLU}/MIC_{NIF}; 0.5× MIC_{FLU}/0.5× MIC_{NIF}; and 0.5× MIC_{FLU}/0.25× MIC_{NIF}. For sterility control testing, a 10 μL sample was plated on SDA one time for each experiment. Test solutions were placed in an incubator at 35 °C. At pre-determined times (0, 4, 6, 24 and 48 h), 10 μL samples were obtained from each solution, were ten-fold serially diluted if necessary, and a 10 μL sample from each dilution was plated on SDA for colony counting. Colony counts were recorded following incubation at 35 °C for 24–48 h.

The following criteria were used to interpret time–kill results: synergism, ≥2 $\log_{10}$ decrease in CFU/mL compared with the most active agent; antagonism, ≥2 $\log_{10}$ increase in CFU/mL compared with the least active agent; additivity, <2 but >1 $\log_{10}$ decrease in CFU/mL compared with the most active agent; and indifference, <2 but >1 $\log_{10}$ increase in CFU/mL compared with the least active agent [12].

3. Results

3.1. Antifungal susceptibility testing

The strains used in this study ranged from susceptible and SDD to resistant to FLC (Table 1).

NIC, VER and DLT showed no antifungal effects. In contrast, NIF showed an antifungal effect consistently against most of the strains used in this study (MIC of 8 μg/mL) (Table 1).

3.2. Chequerboard combination assay

3.2.1. Fluconazole/verapamil and fluconazole/diltiazem combinations

Neither VER nor DLT demonstrated the ability to reverse FLC resistance in any *C. glabrata* strains. The mean FICIs ranged from 0.5–1 for all strains, indicating indifference.

3.2.2. Fluconazole/nicardipine combination

The FLC + NIC combination was either additive or indifferent (Table 2). FICIs for the combination in resistant and SDD isolates (DSY 565 and CBS 138, respectively) were additive (>0.25 and ≤0.5). In contrast, for susceptible strains (DSY 562 and clinical strains 1 and 2), the MIC of the FLC + NIC combination showed almost no change compared with FLC alone (FICIs of >0.5 and ≤1, indicating indifference).