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HEALTH SCIENCES

Antifungal and anti-biofilm effect of the calcium channel blocker verapamil on non-*albicans* Candida species

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Abstract: Candida is a human fungal pathogen that causes a wide range of diseases. *Candida albicans* is the main etiologic agent in these diseases; however, infections can be caused by non-*albicans Candida* species. Virulence factors such as biofilm production, which protect the fungus from host immunity and anti-fungal drugs, are important for the infection. Therefore, available antifungal drugs for candidiasis treatment are limited and the investigation of new and effective drugs is needed. Verapamil is a calcium channel blocker with an inhibitory effect on hyphae development, adhesion, and colonization of C. *albicans*. In this study, we investigated the effect of verapamil on cell viability and its antifungal and anti-biofilm activity in non-*albicans Candida* species. Verapamil was not toxic to keratinocyte cells; moreover, *C. krusei, C. parapsilosis*, and *C. glabrata* were susceptible to verapamil with a minimal inhibitory concentration (MIC) of 1250 µM; in addition, this drug displayed fungistatic effect at the evaluated concentrations. After treatment with verapamil, reduced viability, biomass, and mitochondrial activity were observed in biofilms of the non-*albicans Candida* species *C. krusei, C. glabrata*, and *C. parapsilosis*. These findings highlight the importance of the study of verapamil as an alternative treatment for infections caused by non-*albicans Candida* species.

Key words: Verapamil, antifungal activity, biofilm, *Candida* non-*albicans*, drug repurposing.

INTRODUCTION

In the last decade, an increase in the incidence of *Candida* spp. infections was observed; this was found to be associated with an increase in the number of immunocompromised patients (Quindós et al. 2018, Salehi et al. 2019, Walsh et al. 2019). *Candida* species are common human fungal pathogens that cause a wide range of clinical diseases, ranging from superficial infections to life-threatening systemic disease (Lin et al. 2018, Meletiadis et al. 2017, Pfaller & Diekema 2007). *Candida albicans* is the most common species responsible for causing infections worldwide; however, there has been an increase in the incidence of infections caused by non-*albicans Candida* species (Alim et al. 2018, Santolaya et al. 2019, Silva et al. 2012). *Candida albicans* represents 50-82% of the *Candida* spp. isolates, while among non-*albicans* species *C. glabrata*, *C. krusei*, and *C. parapsilosis* correspond to the 9.4-19%, 2-3%, and 1-2%, respectively (Dos Santos Abrantes et al. 2014, Thompson et al. 2010).

The use of steroid drugs or broad-spectrum antibiotics, as well as smoking, carrying

prostheses, and possessing a compromised immune system are risk factors for the production of a microenvironment that favors the overgrowth of *Candida* spp. This may trigger oral candidiasis, which is the most prevalent opportunistic fungal infection affecting the oral mucosa (Singh et al. 2014). Oral candidiasis is mainly caused by overgrowth of *C. albicans*; however, non-*albicans* species have been isolated as well in samples of oropharyngeal candidiasis (Patel et al. 2012, Singh et al. 2014). Oral candidiasis associated with *Candida* spp. can be classified as pseudomembranous, erythematous, nodular, or plaque-like. Depending on the host's immune system, oral candidiasis may become systemic by spreading into the bloodstream or gastrointestinal tract, leading to a serious infection that can cause the death of the individual (Akpan & Morgan 2002, Niimi et al. 2010).

One of the most studied *Candida* spp. virulence factors are biofilms, i.e., sessile communities of microbes found either attached to a surface or buried firmly in an extracellular matrix (ECM), which is a complex and highly polar mixture of biomolecules including proteins, polysaccharides, nucleic acids, and lipids (Overhage et al. 2008)

The biofilm matrices of *Candida* species have a strong network of exopolymers, providing protection against host and environmental factors, such as the immune system and antimicrobial drugs. Although biofilm matrices do not inhibit the diffusion of antibiotics, they restrict antibiotic entry into the biofilm. Matrix components like exopolymers hinder the diffusion of drugs into the cells of the biofilm by binding to them, making the fungi refractory to antifungals (Al-Fattani & Douglas 2004, Dominguez et al. 2018, Rodrigues et al. 2017). This property of biofilms leads to persistent biofilm infections, despite treatment with antibiotics, predisposing the organism to develop resistance against these drugs (genetic resistance) (Ciofu et al. 2017).

In addition, the biofilm formation allows microorganisms to resist hostile environmental conditions such as starvation and desiccation, and causes a wide range of chronic diseases. Consequently, biofilms are a major source of persistent nosocomial infections in immunosuppressed patients (Davies 2003, Singh et al. 2000) who come in contact with medical devices, such as catheters, cardiac pacemakers, joint prosthesis, dentures, prosthetic heart valves, and contact lenses (Piozzi et al. 2004, Wu et al. 2015), that are known to provide an ideal surface for the attachment of microorganisms. Saliva contains molecules that reduce the adhesion of microorganisms, but in contrast also produces salivary proteins that have been shown to act as microbial receptors, thus facilitating the formation of biofilms in the oral cavity (Junqueira 2012).

Consequently, the treatment and effective elimination of fungal biofilms has become a challenge (Roy et al. 2018). Earlier studies showed that elimination of biofilms requires higher concentrations of antimicrobials than the conventional dose (Bjarnsholt et al. 2009, Brandl et al. 2008, Hoyle & Costerton 1991, Parsek & Singh 2003, Rasmussen & Givskov 2006, Wu et al. 2015). The effect of antifungal drugs on biofilms depends on the class on the antifungal, as well as on the antifungal susceptibility of the species and the isolate (Taff et al. 2013). Recent studies demonstrated that fluconazole can reduce biomass and cell number in *Candida* spp. biofilms from fluconazole-susceptible and -resistant *C. albicans* and *C. glabrata* isolates, albeit in a high concentration (from 40 µg/mL to 1,280 µg/mL, depending on the isolate) corresponding to five times the previously reported Minimal Inhibitory Concentration (MIC) of Panariello et al. (2018).

Furthermore, amphotericin B in liposomal or deoxycholate formulation was active against *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* biofilms when administered in a concentration between four and eight times greater than the reported MIC concentration (2-8 mg/L). In addition, *C. glabrata* was the least susceptible yeast species to both evaluated formulations (Rodrigues & Henriques 2017). Finally, echinocandins represent the first choice in the treatment of biofilm-related *Candida* spp. infections (Almirante et al. 2017); however, lack of effectiveness of echinocandin drugs against *C. parapsilosis* biofilm has been reported (Thomaz et al. 2020). Therefore, the search for alternative drugs that could disperse and eliminate biofilms is warranted.

Drug repurposing consists of investigating new uses for already approved drugs to treat another disease (Ashburn & Thor 2004). This strategy could be used as an alternative to *de novo* drug development to treat fungal diseases. Verapamil is a calcium channel blocker that is widely used in the treatment of angina and hypertension (Fahie & Cassagnol 2020). This compound has an inhibitory effect on hyphae development, adhesion, and colonization of *C. albicans* (Yu et al. 2013, 2014a, b). Therefore, in this study, we evaluated the antifungal activity of verapamil against non-*albicans Candida* species as well as the effect of verapamil on biofilm formation.

MATERIALS AND METHODS

Compounds and antifungals

The calcium channel blocker verapamil (Sigma-Aldrich, St Louis, MO) and the antifungal amphotericin B (Sigma-Aldrich, St Louis, MO) were used in this study. Stock solutions were prepared in dimethyl sulfoxide (DMSO) and subsequently diluted in Roswell Park Memorial Institute 1640 (RPMI 1640) medium. The maximum DMSO concentration in the final medium was 5%.

Evaluation of cell viability (in vitro cytotoxicity) using resazurin

Evaluation of cell viability was performed using the resazurin method. For this assay, the HaCaT cell line (human keratinocytes) was purchased from the Rio de Janeiro Cell Bank (BCRJ) and was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 2% antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). HaCaT cells were seeded at a concentration of 10^6 cells/mL in a 96-well microplate and incubated for 24 h at 37 °C with 5% CO_2. Cells were treated with verapamil concentrations ranging from 2.4 to 1250 µM for 24 h. Resazurin (Sigma-Aldrich, St Louis, MO) was used to measure cell viability as described earlier (Pavan et al. 2010). According to ISO 10993-5 (2009), drug concentrations provoking a >30% reduction in cell viability are considered cytotoxic.

Microorganisms and culture conditions

Candida krusei ATCC 6258, *C. parapsilosis* ATCC 90018, and *C. glabrata* ATCC 9030 obtained from the Laboratory of Microbiology of the Institute of Science and Technology of São José dos Campos/UNESP were used for the experiments. Sabouraud dextrose (HiMedia, Mumbai, India) medium was used to culture the yeasts by shaking at 150 rpm at an incubation temperature of 37 °C for 24 h.

Minimum Inhibitory Concentration (MIC)

Susceptibility assays were performed according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2017). RPMI 1640 medium with L-glutamine and sodium bicarbonate (Sigma-Aldrich, St

Louis, MO), buffered to pH 7.0 with 0.165 M 4-Morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, St Louis, MO) and supplemented with 2% glucose was used for the assays. *Candida* spp. cells were suspended at a concentration of 0.5-2.5 × 10^5 cells/mL. Verapamil was applied at a concentration ranging between 2.4 and 1250 µM, while amphotericin B at a concentration between 0.016 and 8 µg/mL was used as a control. Plates were incubated at 37 °C for 24 h and readings were measured using spectrophotometry (530 nm).

Minimum Fungicidal Concentration (MFC)

After 48 h of incubation at 37 °C, an aliquot of the samples used for the susceptibility assay was removed from the 96-well plates and plated on Sabouraud dextrose agar (HiMedia, Mumbai, India). After 24 h of incubation at 37 °C, presence or absence of growth at each concentration was verified. The minimum fungicidal concentration is the lowest concentration at which no fungal growth is observed.

Verapamil effect on *Candida* spp. biofilm

Candida *spp. biofilm formation*

Biofilm formation was assessed according to the method of Thein et al (2006). In the assay, 100 µL aliquots of a 10^8 cells/mL suspension of *Candida* spp. in RPMI medium supplemented with 2% glucose were deposed in wells of 96-well plates. Plates were incubated at 37 °C with agitation at 75 rpm for 90 min for the pre-adhesion stage. Following this, wells were washed three times with phosphate-buffered saline (PBS) to remove non-adherent yeasts. Subsequently, 100 µL of RPMI medium was added to the wells, and the plate was incubated at 37 °C with agitation at 75 rpm for 24 h. The culture medium was changed after 24 h of incubation. Biofilms formed after 48 h were treated with the addition of verapamil at the MIC (1250 µM); moreover, because of the higher concentration of other compounds needed for biofilm eradication described in the literature, a five-times MIC (6250 µM) was used. Subsequently, these plates were incubated for 24 h and analyzed to determine total biomass, biofilm viability, and any reduction in the biofilm formed.

Biofilm viability by CFU assay

After 24 h of treatment, the wells containing biofilm were washed three times with PBS. Subsequently, the biofilm was disrupted in the wells using an ultrasonic homogenizer (Sonics Vibra Cell, Connecticut, USA) at 50 W for 20 s. Suspensions were serially diluted in PBS and diluted samples were plated on Sabouraud dextrose agar. The plates were incubated at 37 °C for 24 h and colonies were quantified as colony forming units per milliliter (CFU/mL).

Quantification of total biomass using crystal violet

The quantification of biofilm biomass was performed using a method described by Peeters et al (2008). Biofilms were washed with PBS and fixed with absolute ethanol for 15 min. Ethanol was then removed and the biofilm was dried at room temperature for 16 h. 100 µL of a 0.5% crystal violet solution was added to each well. After 20 min, excess dye was removed, and the wells containing biofilm were washed three times with PBS. Following this, 100 µL of absolute ethanol was added to dilute the dye. The absorbance of samples was read at 570 nm, and the results were expressed as percent decrease. *Candida* biofilm formed (after 48 h) without treatment with the drug, whose absorbance values represented 100% of the biomass, was used as the control for this experiment.

Quantification of biofilm metabolic activity (XTT assay)

Candida spp. biofilm viability was determined through a tetrazolium salt (XTT) assay. Biofilms were formed as described above. After biofilm formation and drug treatment, each of the wells was inoculated with 158 µL of PBS, 40 µL of XTT (Sigma-Aldrich, St Louis, MO), and 2 µL of menadione (Sigma-Aldrich, St Louis, MO). After 3 h of incubation in the dark at 37 °C, 100 µL of solution was transferred from each well to another plate and its absorbance was recorded at 490 nm.

Biofilm analysis using Scanning Electron Microscopy (SEM)

Biofilms formed on acrylic resin disks were fixed with 1 mL of 2.5% glutaraldehyde for 3 h. After this period, the specimens were washed with ethanol at concentrations of 10, 25, 50, 75, 90, and 100% alcohol. Acrylic resin disks were incubated at 37 °C for 24 h for complete drying. The acrylic resin disks were then removed from the bottom of the plate with the help of sterile forceps, transferred to aluminum stubs, and covered with gold for 160 s at 40 mA. After metallization, the biofilms were analyzed using an Inspect S50 scanning electron microscope 170 (FEI, Czech Republic) at the Institute of Science and Technology (ICT/Unesp), operating at 15 kV in increments of 2.000x.

Statistical analysis

Analysis of Variance (ANOVA) with Dunn's or Tukey's post-hoc test was used to analyze biofilm viability (CFU/mL counts), biomass (crystal violet), and metabolic activity (XTT assay), using the GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Susceptibility of non-*albicans Candida* species to verapamil

The evaluation of the antifungal activity of verapamil showed that this drug was active against *C. krusei*, C. *parapsilosis*, and *C. glabrata* with a MIC of 1250 µM. Moreover, an MCF assay demonstrated that verapamil displayed fungistatic activity at the evaluated concentration, able to growth in all evaluated concentrations (Table I).

Table I. Susceptibility of non-*albicans Candida* species to verapamil: Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of verapamil.

Minimum inhibitory concentration (MIC)			Minimum fungicidal concentration (MFC)
	Verapamil (µM)	Amphotericin B (µg/mL)	Verapamil (µM)
C. krusei	1250	1.0	fungistatic
C. parapsilosis	1250	0.5	fungistatic
C. glabrata	1250	0.5	fungistatic

In vitro cytotoxicity of verapamil on keratinocytes

Keratinocytes are cells present in the skin and oral mucosa (Turabelidze et al. 2014). Therefore, we evaluated verapamil cytotoxicity on HaCaT keratinocytes. We observed a 20% reduction in cell viability at higher concentrations (625 and 1250 µM, Figure 1). These results establish that verapamil is not toxic for keratinocytes, since according to ISO 10993-5 (2009) standards, only compounds provoking a reduction in cell viability of more than 30% are considered cytotoxic.

Evaluation of verapamil effect on non-*albicans Candida* spp. biofilms

Quantification of the effect of verapamil on biofilm viability

Since verapamil displayed antifungal activity against non-*albicans Candida* species, its activity against biofilms was further investigated. Firstly, the effect of verapamil on *Candida* spp. biofilm viability was assessed using CFU assay (Fig. 2). The treatment of *C. krusei* biofilm with verapamil at MIC (1250 µM) and five times the MIC (6250 µM) triggered a reduction in CFU/mL of 12% ($1.7 \times 10^8 \pm 1.8 \times 10^7$) and 28% ($5.8 \times 10^6 \pm 1.2 \times 10^6$), (Fig. 2a). respectively. The same treatments of *C. parapsilosis* biofilm induced a reduction in CFU/mL of 14% ($6.0 \times 10^6 \pm 1.5 \times 10^6$) and 26% (640000 ± 175879) respectively (Fig. 2b), while for *C. glabrata* biofilm the reduction amounted to 12% ($6.4 \times 10^6 \pm 1.2 \times 10^6$) and 20% ($1.3 \times 10^6 \pm$ 127366), respectively (Fig. 2b).

Evaluation of biomass reduction in the biofilm of non-albicans Candida spp. after treatment with verapamil

The effect of verapamil on biofilm biomass was evaluated using a violet crystal assay (Fig. 3). In this assay verapamil concentrations equivalent to the MIC (1250 µM) and five times the MIC (6250 µM) were also used. For *C. krusei,* the percent reduction in biomass was 53% (1.708 ± 0.2238) and 74% (0.9549 ± 0.1176) after treatment with the MIC and five times the MIC of verapamil, respectively (Fig. 3a). For *C. glabrata*, the percent reduction in biomass was 8% (5.747 ± 0.9715) and 47% (3.325 ± 0.6224) at the MIC and five times the MIC of verapamil, respectively (Fig. 3b). Finally, for *C. parapsilosis,* the biomass reduction at the MIC and five times the MIC of verapamil was 10% (4.373 ± 0.9002) and 72% (1.347 ± 0.2370), respectively (Fig. 3c). Statistically significant reductions in biomass were observed after treatment with both the MIC and five times the MIC of verapamil for *C. krusei* and with five times the MIC of verapamil for *C. glabrata* and *C. parapsilosis*.

Quantification of the effect of verapamil on the metabolic activity of Candida biofilm

Following the demonstration that verapamil treatment could reduce biofilm viability and biomass, its effect on the metabolic activity of biofilm was also investigated using XTT assay. *Candida glabrata* was the species that displayed the highest reduction of metabolic activity upon verapamil treatment, namely of 14% (2.308 ± 0.03517) and 89% (0.3001 ± 0.02388) when the MIC and five times the MIC of verapamil was used, respectively (Fig. 4c). Next, *C. parapsilosis* biofilm showed a percent reduction in metabolic activity of 39% (0.7888 ± 0.1135) and 43% (0.7434 ± 0.05242) upon the same treatments (Fig. 4b).; finally, verapamil-treated biofilm of *C. krusei* showed a 17% (0.8849 ± 0.05705) and 55% (0.4805 ± 0.04616) reduction in metabolic activity when compared to the control (Fig. 4a). These results corroborate our previous results relative to biofilm viability.

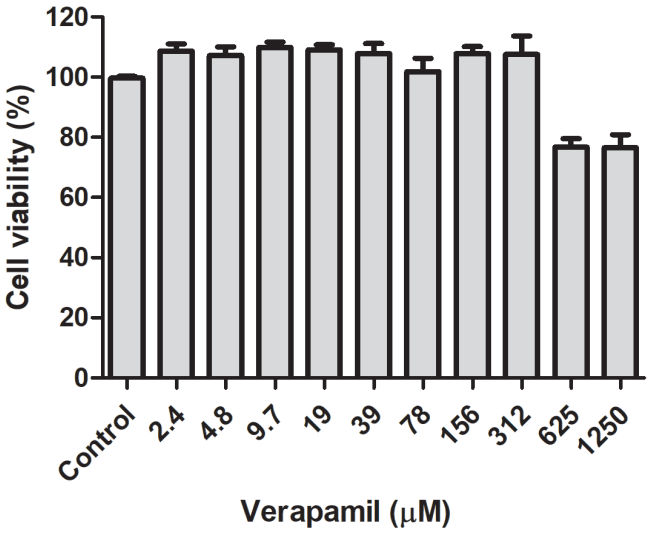

Figure 1. Verapamil cytotoxicity on a human keratinocyte (HaCaT) cell line was tested using the resazurin method. Viability is presented as percent control of untreated cells in the absence or presence of varying verapamil concentrations (μM). Error bars represent the standard deviation of five biological replicates.

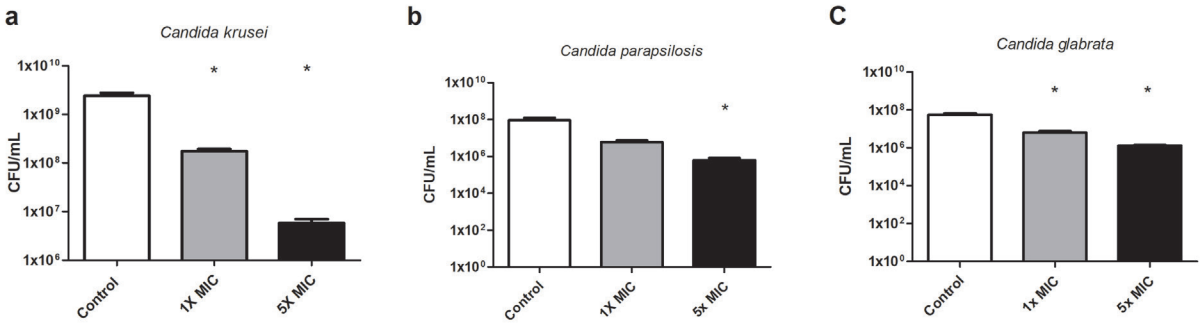

Figure 2. Biofilm viability of non-*albicans Candida* species after treatment with verapamil at the MIC and five times the MIC. (a) *C. krusei*; (b) *C. parapsilosis*; (c) *C. glabrata.* Asterisks represent statistically significant differences with untreated biofilm (control) as shown by ANOVA ($p < 0.05$). Error bars represent the SEM (standard error of the mean) of eight biological replicates.

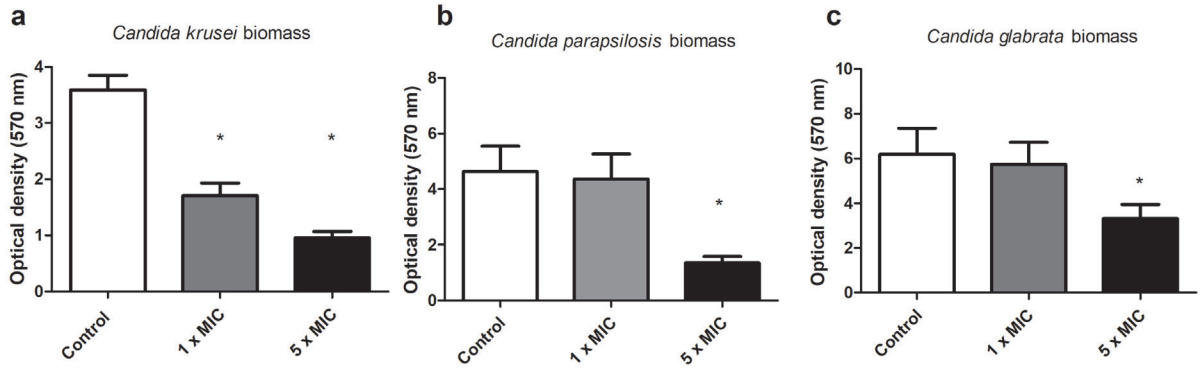

Figure 3. Evaluation of biomass reduction in biofilms of non-*albicans Candida* species after treatment with verapamil at the MIC and five times the MIC. (a) *C. krusei*; (b) *C. parapsilosis*; (c) *C. glabrata.* Asterisks represent statistically significant differences with untreated biofilm (control) as shown by ANOVA ($p < 0.05$). Error bars represent the SEM (standard error of the mean) of 10 biological replicates.

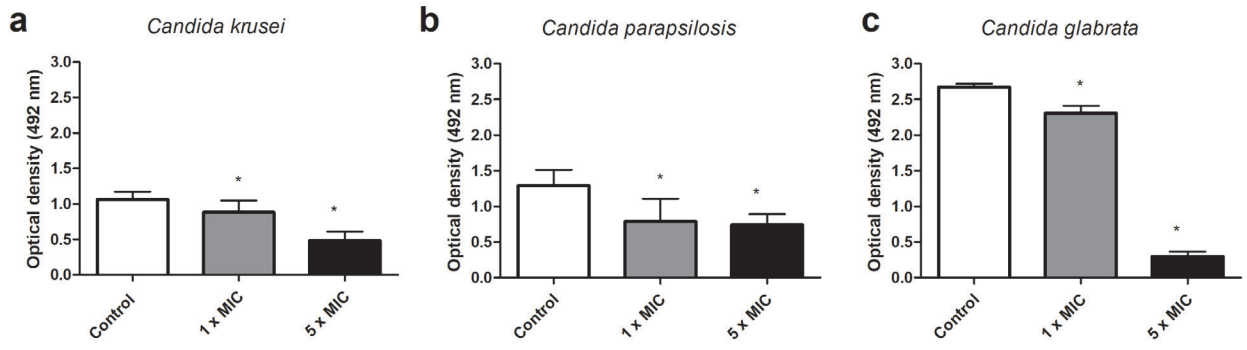

Figure 4. Metabolic activity of biofilms of non-*albicans Candida* species after treatment with verapamil at the MIC and five times the MIC. (a) *C. krusei*; (b) *C. parapsilosis*; (c) *C. glabrata.* Asterisks represent statistically significant differences with untreated biofilm (control) as shown by ANOVA ($p < 0.05$). Error bars represent the SEM (standard error of the mean) of 10 biological replicates.

Biofilm analysis using Scanning Electron Microscopy (SEM)

Once the activity of verapamil against biofilms of non-*albicans Candida* species was observed, the effect of treatment with this calcium channel blocker on biofilm constitution was visualized using SEM. A drastic reduction of biofilm was apparent when comparing untreated *C. krusei* (Fig. 5a), *C. parapsilosis* (Fig. 5d), and *C. glabrata* (Fig 5g) with their verapamil-treated counterparts at the MIC (Fig. 5b, e, and h) and five times the MIC (Fig. 5c, f, and i). These data corroborated our previous results on the effect of verapamil on biofilm.

DISCUSSION

Drug repurposing is considered a promising approach for the treatment of fungal diseases (Afeltra & Verweij 2003). This strategy allows cost reduction and accelerates the development of new drugs because the toxicology and pharmacology of the drug has been established (Afeltra & Verweij 2003, Katragkou et al. 2016, Krajewska-Kułak & Niczyporuk 1993, Yu et al. 2014a). Drug repurposing has been successfully used in the search for candidates to treat infections caused by *C. albicans*, *C. auris*, and *Cryptococcus neoformans* (Butts et al. 2013, de Oliveira et al. 2019, Siles et al. 2013, Wiederhold et al. 2017).

Calcium is an important factor for signal transduction mechanisms supporting the adaptation and survival of different fungi. Moreover, calcineurin plays an important role in the regulation of calcium homeostasis. In *Candida* spp. calcineurin is required for morphogenesis, azole tolerance, membrane stress, cell wall integrity, survival in serum, and virulence (Chen et al. 2012, Juvvadi et al. 2014, Liu et al. 2015, Sanglard et al. 2003). A recent report demonstrated that the calcium pump Spf1 participates in the development of *C. albicans* biofilm, indicating at the calcium homeostasis system as a potential target for biofilm eradication (Yu et al. 2012).

In this study, we first determined the susceptibility of non-*albicans Candida* species to verapamil as well as the MIC value of this drug. Verapamil was effective against *Candida krusei, C. glabrata*, and *C. parapsilosis* at the same MIC (1250 μM) and displayed fungistatic effect at the evaluated concentrations. Considering the

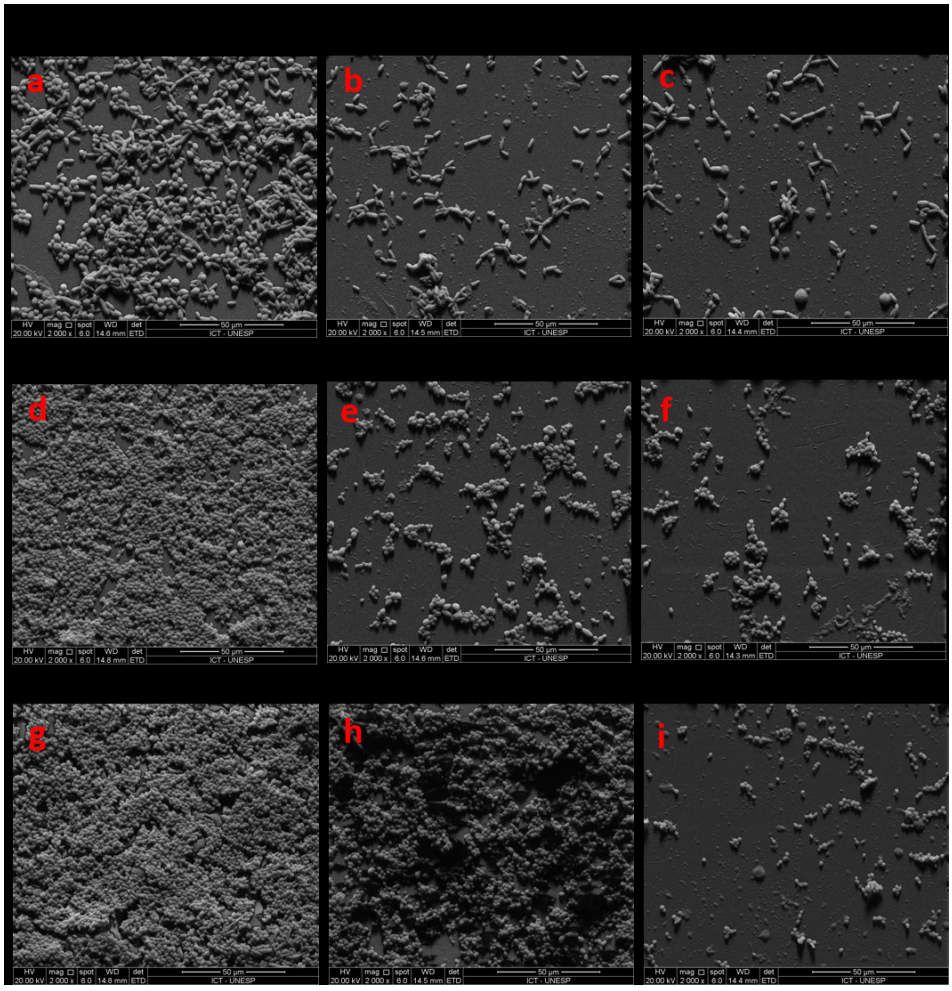

Figure 5. Analysis of non-*albicans Candida* biofilm by scanning electron microscopy. The figure sequence shows *C. krusei* biofilm without treatment (a); after treatment with verapamil at the MIC (b) and five times the MIC (c); *C. parapsilosis* biofilm without treatment (d); after treatment with verapamil at the MIC (e) and five times the MIC (f); and *C. glabrata* biofilm without treatment (g); after treatment with verapamil at the MIC (h) and five times the MIC (i).

development of a verapamil-based product with topical use (e.g. a mouthwash or cream), we then evaluated verapamil-induced cytotoxicity in the HaCaT cell line (human keratinocytes). We observed a 20% loss of cell viability at higher doses (625 and 1250 µM) of verapamil; however, a reduction in viability below 30% is acceptable in cytotoxicity testing according to ISO 10993 -5 (2009). A limitation of this study lies in the fact that the cytotoxicity of verapamil at five times the MIC (6250 µM or 2.841 mg/ml) was not evaluated. However, verapamil toxicity is well established, with the oral LD50 (median lethal dose) being 150 mg/kg in rats and 163 mg/kg in mice (National Center For Biotechnology Information 2020), which is much higher concentrations than the evaluated. Subsequently, the effect of verapamil on biofilm viability was evaluated. A concentration-dependent inhibitory effect of verapamil was observed. Next, verapamil was also found to effectively reduce biofilm biomass, as shown by crystal violet assay. Finally, SEM images confirmed the reduction of biofilms after treatment with verapamil at both concentrations.

Verapamil treatment of *C. albicans* results in increased sensitivity to oxidative stress, production of reactive oxygen species, and mitochondrial dysfunction, processes leading to toxicity for *C. albicans*. In addition, calcium channel blockage also triggers hyphae and biofilm inhibition (Brand et al. 2007, Yu et al. 2014a). Our results from the XTT assay,

that evaluated cellular metabolic activity by mitochondrial activity, are consistent with the literature. Indeed, after treatment of biofilms with verapamil, a reduction in metabolic activity of all studied species was observed, with *C. glabrata* being the species displaying the highest reduction of metabolic activity when treated with five times the MIC of verapamil (Yu et al. 2014a).

An important virulence mechanism in *Candida* spp. is filamentation, during which cells change their shape and pattern of growth to adhere to and penetrate the host tissues (Ribeiro et al. 2017, Vila et al. 2017, Yue et al. 2018). Filamentation occurs because of changes in environmental conditions, including high temperatures, nutrient limitation, and exposure to serum (Azadmanesh et al. 2017). Filamentous growth of *C. albicans* is tightly connected to biofilm formation because this process is necessary for adherence to surfaces (Woolford et al. 2016). The *C. albicans* gene *HWP1* encodes an adhesin associated with hyphal development and adhesion (de Barros et al. 2017). Verapamil treatment is reported to be most effective during these processes and to reduce yeast filamentation and adhesion to polystyrene probes and buccal epithelial cells (Yu et al. 2014a). The upregulation of *HWP1* in *C. albicans* was also observed during verapamil treatment of *C. albicans* biofilms; however, the expression of the *ALS3* gene, encoding a protein with an important role in adhesion and biofilm formation, did not change (Liu & Filler 2011, Yu et al. 2013). It is important to highlight that biofilm formation ability and its characteristics vary among laboratory reference *Candida* spp. strains; this could explain the reduced formation of hyphae observed in SEM images of *C. krusei* and *C. parapsilosis* (Alnuaimi et al. 2013).

In addition, verapamil has been reported to inhibit biofilm formation by *C. albicans* in synergy with fluconazole or tunicamycin, thus improving the effect of these antifungal drugs on biofilms (Yu et al. 2013). In contrast, a combination of verapamil and fluconazole (FLZ) did not display any inhibitory effect against FLZ-resistant *C. glabrata* isolates and could not enhance the effect of FLZ on *C. glabrata* isolates (Alnajjar et al. 2018). Different calcium channel blockers, such as verapamil hydrochloride, cinnarizine, nifedipine, and nimodipine, were effective against clinical isolates of *C. albicans,* with verapamil hydrochloride showing the strongest antifungal activity as compared to other compounds (Krajewska-Kułak & Niczyporuk 1993). However, only a few reports that study the effects of verapamil on non-*albicans Candida* species exist, which focus on the use of verapamil as an efflux blocker acting in synergy with other antifungals (Alnajjar et al. 2018, Pinto e Silva et al. 2009, Yu et al. 2013), and not on this drug alone.

In this study, we demonstrated that verapamil possesses antifungal and anti-biofilm activity against non-*albicans Candida* species, such as *C. krusei*, *C. glabrata,* and *C. parapsilosis*. This drug reduced biofilm growth, viability, and metabolic activity for all tested species. These findings are important for consideration of verapamil as a possible alternative treatment for infections caused by non-*albicans Candida* species. However, further studies are necessary to describe the activity and possible applications of this compound.

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