The calcineurin inhibitor cyclosporin A exhibits synergism with antifungals against Candida parapsilosis species complex

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Candida parapsilosis complex comprises three closely related species, C. parapsilosis sensu stricto, Candida metapsilosis and Candida orthopsilosis. In the last decade, antifungal resistance to azoles and caspofungin among C. parapsilosis sensu lato strains has been considered a matter of concern worldwide. In the present study, we evaluated the synergistic potential of antifungals and the calcineurin inhibitor cyclosporin A (Cys) against planktonic and biofilms of C. parapsilosis complex from clinical sources. Susceptibility assays with amphotericin, fluconazole, voriconazole, caspofungin and Cys were performed by microdilution in accordance with Clinical and Laboratory Standards Institute guidelines. Synergy testing against planktonic cells of C. parapsilosis sensu lato strains was assessed by the chequerboard method. Combinations formed by antifungals with Cys were evaluated against mature biofilms in microtitre plates. No differences in the antifungal susceptibility pattern among species were observed, but C. parapsilosis sensu stricto strains were more susceptible to Cys than C. orthopsilosis and C. metapsilosis. Synergism between antifungals and Cys was observed in C. parapsilosis sensu lato strains. Combinations formed by antifungals and Cys were able to prevent biofilm formation and showed an inhibitory effect against mature biofilms of C. parapsilosis sensu stricto, C. metapsilosis and C. orthopsilosis. These results strengthen the potential of calcineurin inhibition as a promising approach to enhance the efficiency of antifungal drugs.

INTRODUCTION

Candida parapsilosis complex comprises three closely related species, C. parapsilosis sensu stricto, Candida metapsilosis and Candida orthopsilosis. Although phenotypically similar, these micro-organisms show differences regarding virulence, which in turn correlates with their epidemiological characteristics. C. metapsilosis – the least virulent species – also has a low prevalence in human infections, followed by C. orthopsilosis and C. parapsilosis sensu stricto, the last considered the most virulent, and consequently the most frequent pathogen of the group (Gács et al., 2007; Lockhart et al., 2008; Orsi et al., 2010; Cantón et al., 2011).

Abbreviations: AMB, amphotericin B; CAS, caspofungin; Cys, cyclosporin A; FICI, fractional inhibitory concentration index; FLC, fluconazole; VRZ, voriconazole.
Apart from these questions, the C. parapsilosis complex is considered the second most important agent of candidemia in Latin America and Asia, and is also very frequent in surveys conducted in Europe (Costa et al., 2000; Godoy et al., 2003; Almirante et al., 2006; Colombo et al., 2006; Medrano et al., 2006; Falagas et al., 2010; Pereira et al., 2010).

Members of the C. parapsilosis complex cause disease mainly in severely ill patients from intensive care units, low-birth-weight neonates and those receiving parenteral nutrition (Colombo et al., 2006; Nosek et al., 2009; van Asbeck et al., 2009). These micro-organisms have been considered important opportunistic and nosocomial pathogens (Pfäler et al., 2008; van Asbeck et al., 2009; Nucci et al., 2010), causing invasive diseases such as fungaemia, endocarditis, osteomyelitis and meningitis. C. parapsilosis sensu lato candidaemia has a mortality rate up to 45% (Trofa et al., 2008; Sidrim et al., 2011; Miranda et al., 2013).

Worldwide, susceptibility studies have shown that antifungal resistance in C. parapsilosis sensu lato strains is uncommon. However, in the last decade, many studies have described the isolation of fluconazole-resistant strains (Moudgal et al., 2005; Sarvikivi et al., 2005; Legout et al., 2006; Pfäler et al., 2008; Bonfietti et al., 2012b). Resistance has also been detected among C. orthopsilosis and C. metapsilosis clinical strains (Chen et al., 2010; Bonfietti et al., 2012a). In addition, reduced echinocandin susceptibility has been described among C. parapsilosis sensu stricto (Bonfietti et al., 2012b; Spreghini et al., 2012), C. orthopsilosis and C. metapsilosis (García-Effron et al., 2008). These findings are a matter of concern among clinicians (Moudgal et al., 2005; Sarvikivi et al., 2005; Legout et al., 2006; Pammi et al., 2013).

Calcineurin is a calcium/calmodulin-dependent protein phosphatase that takes part in several metabolic processes in the fungal cell, including morphogenesis and virulence. Experimental studies have shown that calcineurin inhibitors are able to increase the sensitivity to azoles in Canädia albicans (Uppuluri et al., 2008; Chen et al., 2011; Zhang et al., 2012). Additionally, synergistic combinations formed by azoles and calcineurin inhibitors are able to inhibit both planktonic and biofilms of C. albicans (Uppuluri et al., 2008). In the present study, we evaluated the synergistic potential of the calcineurin inhibitor cyclosporin A (Cys) with amphotericin B (AMB), azoles and caspofungin against planktonic and biofilms of C. parapsilosis complex from clinical sources.

METHODS

Micro-organisms. Strains of C. parapsilosis sensu stricto (n=12), C. orthopsilosis (n=12) and C. metapsilosis (n=12) from clinical sources were included in this study. Reference strains were kindly provided by Dr Elisa Borghi from the Department of Health Sciences, Università degli Studi di Milano, Italy. Upon arrival at our laboratory, strains were plated onto chromogenic medium (HiCrome Candida Differential Agar; HI Media Laboratories) to ensure purity. Phenotypic tests included examination of micromorphological features on cornmeal/Tween 80 agar, carbohydrate/nitrogen assimilation and urease production (De Hoog et al., 2000).

Molecular identification. Isolates were plated onto potato dextrose agar (HiMedia) and incubated at 35 °C for 48 h. Individual colonies were picked with a micropipette tip, transferred to microtubes containing 6 µl of 0.02 M NaOH and heated at 99 °C for 10 min. C. parapsilosis species complex identification was conducted as suggested by Tavanti et al. (2005), with the primers S1F (5’-GGTTAGGCTTGTTAGTTGTTG-3’) and S1R (5’-CAATGCAAATCTCCGCAAAA-3’), which amplified a fragment of 716 bp. Reactions were performed in a total volume of 25 µl containing 2 µl yeast supernatant. The amplification conditions were as follows: a first cycle of 7 min at 94 °C, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 74 °C for 1 min, with a final extension step of 10 min at 74 °C. Enzymic digestion of PCR amplicons was performed with BanI (Fermentas Life Sciences) at 37 °C for 16 h. Products were separated by electrophoresis on 2% agarose gel and visualized under UV light. Identification was confirmed based on the restriction pattern: one, zero and three restriction sites for C. parapsilosis sensu stricto, C. orthopsilosis and C. metapsilosis, respectively.

Antimicrobial drugs. Stock solutions of AMB (Sigma Chemical Corp.) and voriconazole (VRZ; Pfizer) were prepared in DMSO. Fluconazole (FLC; Pfizer) and caspofungin (CAS; Merck Sharp & Dohme) were diluted in sterile water (CLSI, 2008). Cys (Merck) was diluted in RPMI 1640 with l-glutamine and without sodium bicarbonate (Sigma Chemical Co.), buffered to pH 7.0 with 0.165 M MOPS (Sigma Chemical Co.). Serial twofold dilutions of each drug were performed in RPMI 1640.

Inoculum preparation for susceptibility testing. Inocula of all tested isolates were prepared from 48 h cultures grown on potato dextrose agar at 35 °C. The colonies were suspended in 5 ml sterile 0.9% saline and the turbidity was adjusted to 0.5 on the McFarland scale. Afterwards, the suspension was diluted 1:100 and then 1:20 with RPMI 1640 to obtain an inoculum of planktonic cells containing 5 × 10²–2.5 × 10³ cells ml⁻¹ (CLSI, 2008).

Antifungal susceptibility of C. parapsilosis complex planktonic cells. Broth microdilution testing was performed according to the Clinical and Laboratory Standards Institute (CLSI, 2008). The final concentrations of each antimicrobial combination ranged as follows: 0.03125–16 µg ml⁻¹ for AMB, 0.125–64 µg ml⁻¹ for FLC, 0.03125–16 µg ml⁻¹ for VRZ, 0.03125–16 µg ml⁻¹ for CAS and 6.25–100 µg ml⁻¹ for Cys. Microlution plates were incubated at 35 °C and read after 24 h for FLC and CAS or 48 h for AMB and Cys. MICs were defined as the lowest drug concentration that caused complete inhibition (AMB) or a significant diminution of growth (50% inhibition; VRZ, FLC and CAS) when compared with the control well (CLSI, 2008). For Cys, MICs were arbitrarily defined as 50% inhibition.

Synergistic potential of Cys and antifungals by the chequerboard method. The in vitro synergism between Cys and antifungals was evaluated in a chequerboard assay (Odds, 2003). Chequerboard synergy testing was performed in duplicate in microdilution assays for each fungal strain. Combinations included Cys+AMB, Cys+FLC, Cys+VRZ and Cys+CAS. Combinations were formed with each drug at the following concentrations: Cys, 0.078–5 µg ml⁻¹; AMB, 0.0078–1.0 µg ml⁻¹; FLC, 0.03125–2 µg ml⁻¹; VRZ, 0.0039–0.0625 µg ml⁻¹; and CAS, 0.0039–0.0625 µg ml⁻¹. Positive growth controls were performed in RPMI 1640 without antifungicals. The MIC of each drug in combination (MICsyn) was defined as the lowest concentration that caused complete inhibition of visible fungal growth. Drug interactions were classified as synergistic, indifferent or
antagonistic according to the fractional inhibitory concentration index (FICI). The interaction was defined as synergistic if the FICI was ≤0.5, indifferent if it was >0.5–4.0 and antagonistic if it was >4.0 (Odds, 2003).

**Biofilm formation.** Biofilms of *C. parapsilosis sensu lato* strains were prepared as described by Ruiz et al. (2013) with slight modifications. In brief, strains of *C. parapsilosis sensu stricto* (n=7), *C. orthopsilosis* (n=7) and *C. metapsilosis* (n=7), chosen randomly from the set of clinical isolates, were grown in Sabouraud dextrose agar for 24 h at 35 °C. After this period, the colonies were suspended in sterile 0.9 % saline, the turbidity was adjusted to 4 on the McFarland scale and 20 μl inoculum aliquots were transferred to flat wells of 96-well polystyrene plates containing 180 μl Sabouraud broth supplemented with 8 % glucose. The plates were incubated at 35 °C for 24 h and the wells were then washed twice with sterile 0.9 % saline to remove non-adhered cells. Following incubation, the supernatant was aspirated and an aliquot of 100 μl 0.4 % crystal violet was added to each well. After 45 min at 35 °C, the dye solution was aspirated and the wells were washed twice with sterile distilled water. The wells were filled with 200 μl 100 % ethanol, and after 45 min at 25 °C, the mixture was aspirated and the absorbance read in a spectrophotometer at 595 nm.

**Effect of Cys against mature biofilms.** The inhibitory activity of Cys against mature biofilms of *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis* was evaluated according to Ruiz et al. (2013), with slight modifications. The following solutions were tested: Cys, AMB, FLC, VRZ, CAS, Cys+AMB, Cys+FLC, Cys+VRZ and Cys+CAS. Aliquots of 200 μl each test solution at two different concentrations (10 × MICCyan, 50 × MICCyan) were added to viable 48 h biofilms. Controls were grown in medium without antimicrobials. Measurement of *C. parapsilosis* complex biofilms was obtained by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium hydroxide (XTT; Sigma) reduction assay. For each strain, 75 μl XTT salt solution (1 mg ml⁻¹ in PBS), 6 μl menadione solution (1 mM in acetone; Sigma) and 50 μl sterile PBS were added to each well. The microtitre plates were incubated at 36 °C for 5 h. The metabolic activity of the yeast cells within the biofilm was evaluated by the enzymic reduction in XTT tetrazolium salt to XTT formazan, resulting in a colorimetric change, which was measured at 492 nm. Experiments were performed in duplicate and repeated three times independently.

**Effect of Cys in preventing biofilm formation.** The ability of Cys to inhibit biofilm formation of *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis* was evaluated as suggested by Ruiz et al. (2013). Biofilm formation was conducted as described previously except for the addition of the following test solutions to the culture medium: Cys+AMB, Cys+FLC, Cys+VRZ and Cys+CAS. At the same time, Cys and antifungals were also tested alone. Drugs were tested at 10 × MICCyan and 50 × MICCyan. Controls were grown in culture medium without antimicrobials. The metabolic activity of the biofilms was measured by an XTT reduction assay, as described above. Experiments were performed in duplicate and repeated three times independently.

**Statistical analysis.** The antimicrobial susceptibilities were compared using one-way ANOVA and Tukey’s multiple comparisons post-test. Differences between treatments were evaluated for significance using the Wilcoxon signed-rank test. A P value of <0.05 was considered to be significant. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software).

**RESULTS**

**Antimicrobial susceptibility of planktonic cells**

The susceptibility profile of *C. parapsilosis* complex strains is shown in Table 1. All of the studied strains were susceptible to AMB, FLC, VRZ and CAS (CLSI, 2008). No differences in the antifungal susceptibility pattern among species were observed, but *C. parapsilosis sensu stricto* strains were more susceptible to Cys than *C. orthopsilosis* and *C. metapsilosis* (P<0.05).

Synergism between Cys and antifungals was observed (Table 2). Cys significantly reduced (P<0.05) the MICs of antifungal drugs in a similar pattern among the three species: approximately five times for AMB, about four times for FLC and approximately seven times for VRZ. For CAS, MICs were reduced approximately 11 times for *C. parapsilosis sensu stricto* and nearly five times for *C. orthopsilosis* and *C. metapsilosis*. Synergism was observed for all tested strains, except for one *C. parapsilosis* (Cys+FLC) and one *C. orthopsilosis* (Cys+AMB) strain, which were classified as indifferent.

**Effect of antifungals and Cys against *C. parapsilosis* complex biofilms**

In the present study, biofilms quantified by the crystal violet staining method showed similar results among *C. parapsilosis sensu stricto* strains and *C. orthopsilosis* strains (mean absorbance value). However, *C. metapsilosis* strains

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**Table 1. Susceptibility pattern of *C. parapsilosis* complex strains**

<table>
<thead>
<tr>
<th>Drug</th>
<th><em>C. parapsilosis sensu stricto</em> (n=12)</th>
<th><em>C. orthopsilosis</em> (n=12)</th>
<th><em>C. metapsilosis</em> (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC range</td>
<td>GM</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>AMB</td>
<td>0.0625–0.5</td>
<td>0.176</td>
<td>0.125</td>
</tr>
<tr>
<td>FLC</td>
<td>0.125–2.0</td>
<td>0.471</td>
<td>0.375</td>
</tr>
<tr>
<td>VRZ</td>
<td>0.0312–0.25</td>
<td>0.049</td>
<td>0.0312</td>
</tr>
<tr>
<td>CAS</td>
<td>0.0312–0.25</td>
<td>0.132</td>
<td>0.125</td>
</tr>
<tr>
<td>Cys</td>
<td>6.25</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
showed significantly lower amounts of biofilm ($P<0.05$), as shown in Fig. 1.

Antifungal drugs alone were effective only when tested at $50 \times \text{MIC}$ ($P<0.05$) for AMB, VRZ and CAS. Metabolic activities were reduced by up to 40, 31, 29 or 33% when mature biofilms were treated with AMB, FLC, VRZ or CAS, respectively (Fig. 2a). No significant differences in susceptibility of mature biofilms to antifungals among species were detected. Biofilm formation was inhibited by up to 37, 22, 45 or 41% by AMB, FLC, VRZ or CAS, respectively (Fig. 2b). C. metapsilosis biofilms were more susceptible to VRZ and CAS ($P<0.05$). Cys alone at $10 \times$ or $50 \times$ MIC was not able to inhibit mature biofilm formation or prevent biofilm formation of any species.

Cys significantly reduced the formation of mature biofilms ($P<0.05$) when combined with antifungals at both $10 \times$ and $50 \times$ MICsyn (Fig. 3). Although no differences in the susceptibility of C. parapsilosis sensu stricto strains were observed, C. orthopsilosis strains were less susceptible to Cys+FLC ($P<0.05$), whereas C. metapsilosis strains were less susceptible to Cys+AMB or Cys+FLC at $50 \times$ MICsyn ($P<0.05$). Combinations formed by Cys and antifungals at $50 \times$ MICsyn were significantly more efficient at inhibiting biofilm formation in C. parapsilosis complex strains than at $10 \times$ MICsyn ($P<0.05$), as shown in Fig. 4. In comparison with controls, the reductions achieved were approximately 50% for every combination at $50 \times$ MICsyn, except for C. parapsilosis sensu stricto and C. orthopsilosis for which biofilms were inhibited approximately 70% by Cys+AMB ($P<0.05$).

**DISCUSSION**

In recent decades, the rising frequency of *Candida* infections among immunocompromised patients has been followed by reports of antifungal resistance (Chong et al., 2007; Nucci et al., 2010). In addition, the epidemiology of candidiasis has changed, and non-*C. albicans* species have gained attention worldwide (Bassetti et al., 2011; Cantón et al., 2011). Among such species, *C. parapsilosis sensu lato* has been recognized as an important pathogen, especially among neonates and severely ill patients (Pfaller & Diekema, 2007).

Since the description of the *C. parapsilosis* complex (Tavanti et al., 2005), many studies have been conducted to describe its antifungal susceptibility pattern. Although resistance is not a common phenotype, the results of these studies have indicated the emergence ofazole resistance (Silva et al., 2009) and reduced echinocandin susceptibility (Spreghini et al., 2012) in the *C. parapsilosis* complex. The search for novel strategies to solve susceptibility issues is therefore of great importance.

In the present study, it was shown that Cys – a calcineurin inhibitor – is able to interfere with *C. parapsilosis sensu stricto* growth in the planktonic form (MIC 6.25 μg ml$^{-1}$),
but has little effect on cellular growth of *C. orthopsilosis* and *C. metapsilosis*. Li et al. (2008) demonstrated previously that Cys alone has weak antifungal activity against *C. albicans*, with an MIC of 512 µg ml⁻¹. The susceptibility to Cys could be a phenotypic trait exclusive to *C. parapsilosis sensu stricto* and not shared by the sibling species *C. orthopsilosis* and *C. metapsilosis*.

The results presented here confirm that Cys enhanced the *in vitro* activity of antifungal drugs against *C. parapsilosis sensu lato* strains. Synergism was observed between Cys and AMB, FLC, VRZ or CAS, with FICI values as low as 0.025 for Cys+VRZ. The increased susceptibility of *C. parapsilosis sensu stricto* to Cys combinations may be of practical importance, as epidemiological studies have shown that it is the most prevalent species of the complex (Cantón et al., 2011; García-Effron et al., 2012) and it is also prone to developing resistance to antifungals (Silva et al., 2009).

The synergistic potential of calcineurin inhibitors and antifungals has been demonstrated previously. One of the first such studies was performed by Marchetti et al. (2000), which showed the antifungal power of the combination of FLC and Cys against *C. albicans*. Since then, several studies have attested that calcineurin inhibitors show synergism with antifungals against *C. albicans* (Uppuluri et al., 2008), *Cryptococcus neoformans* (Del Poeta et al., 2000), *Aspergillus fumigatus* (Steinbach et al., 2004) and Mucorales (Shirazi & Kontoyiannis, 2013). To the best of our knowledge, the results of the present study show for the first time the inhibitory potential of combinations formed by Cys and antifungals against the species of the *C. parapsilosis* complex. Although all of the tested strains were susceptible to

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**Fig. 1.** Biofilm quantification of *C. parapsilosis sensu stricto* (open bars), *C. orthopsilosis* (shaded bars) and *C. metapsilosis* strains (filled bars) using the crystal violet staining method.

**Fig. 2.** Effect of antifungals against biofilms of *C. parapsilosis sensu stricto* (Cp), *C. orthopsilosis* (Co) and *C. metapsilosis* (Cm). (a) Biofilms were grown in RPMI 1640 without antimicrobials as controls and after 48 h were tested against AMB, FLC, VRZ or CAS at 50× MIC. (b) Biofilms were grown in RPMI 1640 without antimicrobials as controls or supplemented with antifungals at 50× MIC. The metabolic activities of the biofilms were measured by an XTT reduction assay. The results are shown as the percentage reduction in comparison with controls. Experiments were conducted in duplicate and the data are expressed as means ± SEM (n=7). An asterisk indicates a statistically significant difference from the control (P<0.05).
antifungals, the extent of MIC reduction caused by synergistic association with Cys led us to suppose that calcineurin inhibition may also have an effect against resistant *C. parapsilosis sensu lato* isolates, as shown previously for *C. albicans* (Uppuluri et al., 2008).

After establishing inhibitory concentrations in synergism (MICsyn) against planktonic cells, we sought to evaluate the effect of such combinations against biofilms. Previous studies have described *C. orthopsilosis* and *C. metapsilosis* as non-biofilm producers (Song et al., 2005; de Toro et al., 2011), although divergent results have been described by several authors (Melo et al., 2011; Pires et al., 2011; Ruiz et al., 2013). In the present study, *C. parapsilosis sensu stricto* strains, *C. orthopsilosis* and *C. metapsilosis* strains were able to produce biofilms in vitro. Similar results were obtained by Ruiz et al. (2013) in a set of clinical strains. Differences in biofilm methodology and heterogeneity among strains probably explain the variations observed in these studies.

In the present study, a significant reduction in mature biofilms, as well as inhibition of biofilm formation, was
achieved by Cys combined with antifungals. When tested alone at 10 × MIC, neither antifungals nor Cys were able to reduce or prevent biofilm formation.

C. parapsilosis sensu lato has frequently been related to biofilms on indwelling medical devices (Sardi et al., 2013). Fungal biofilm-associated infections are considered a therapeutic challenge mainly in immunocompromised individuals (Seneviratne et al., 2008; Chandra et al., 2012), as they are refractory to the antifungal agents currently available (Cantón et al., 2010). Although Cys alone has no effect against fungal biofilms, synergistic combinations with antifungals have proven effective against these structures (Uppuluri et al., 2008; Chen et al., 2011; Shinde et al., 2012). The results of the present study highlight the pharmacological potential of calcineurin inhibition as an anti-biofilm approach.

Although calcineurin inhibition might be considered a promising antifungal approach, at the present time no fungal-specific calcineurin inhibitors are available. Therefore, pharmacological inhibition of fungal calcineurin by either Cys or FK506 is the most common strategy adopted by scientists to eliminate calcineurin activity (Del Poeta et al., 2000; Marchetti et al., 2000; Li et al., 2008; Chen et al., 2011; Shinde et al., 2012; Shirazi & Kontoyiannis, 2013). Many studies have demonstrated the potent synergism between these immunosuppressive agents and antifungal drugs, even though they are not suitable for the treatment of fungal infections, as they also target human calcineurin. However, because of the importance of the calcineurin pathway for growth, morphogenesis and antifungal resistance in pathogenic fungi, calcineurin inhibition has been suggested as a promising target for the future development of novel antifungal agents.

Our results allowed us to conclude that the combinations formed by Cys and antifungals are able to inhibit the growth of C. parapsilosis species complex in the planktonic form, to reduce the survival of cells in mature biofilms and to prevent biofilm formation. These results strengthen the potential of calcineurin inhibition as a promising approach to enhance the efficiency of antifungal drugs.

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