Additive antifungal activity of anidulafungin and human neutrophils against *Candida parapsilosis* biofilms

Aspasia Katragkou, Athanasios Chatzimoschou, Maria Simitisopoulou, Elpiniki Georgiadou and Emmanuel Roilides*

Laboratory of Infectious Diseases, 3rd Department of Pediatrics, Aristotle University, Hippokration Hospital, 54642 Thessaloniki, Greece

*Corresponding author. Tel: +30-2310-892444; Fax: +30-2310-992981; E-mail: roilides@med.auth.gr

Received 30 July 2010; returned 27 August 2010; revised 21 October 2010; accepted 5 November 2010

**Objectives:** To investigate the activities of two newer triazoles and two echinocandins combined with human phagocytes against *Candida parapsilosis* biofilms.

**Methods:** An in vitro model of *C. parapsilosis* biofilms was used. Biofilms were grown on silicone elastomer discs in 96-well plates at 37°C for 72 h. Biofilms or planktonic cells were incubated with voriconazole, posaconazole, caspofungin or anidulafungin, at clinically relevant concentrations, and human phagocytes (neutrophils or monocytes) alone or in combination with each of the antifungal agents for a further 22 h. Fungal damage induced by antifungal agents and/or phagocytes was determined by XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] metabolic assay.

**Results:** Each of the antifungal agents alone and in combination with human phagocytes induced less damage against *C. parapsilosis* biofilms compared with planktonic cells. No antagonistic interactions between antifungal agents and phagocytes were found. Furthermore, anidulafungin, but not caspofungin, and neutrophils exerted additive activity against *C. parapsilosis* biofilms.

**Conclusions:** Besides a lack of antagonistic interactions between newer antifungal agents and phagocytes, anidulafungin exerts additive immunopharmacological activity against *C. parapsilosis* biofilms.

**Keywords:** antifungal agents, host defences, echinocandins, azoles, caspofungin

**Introduction**

*Candida parapsilosis* is the second or third most common cause of invasive candidiasis behind *Candida albicans*; however, on some occasions, it has outranked *C. albicans*.1 *C. parapsilosis* is a frequent cause of candidaemia, particularly among neonates, in patients with vascular catheters and in those who have received prior antifungal agents, have received parenteral nutrition or have undergone transplantation.1,2 Biofilm formation is a potent virulence factor for *Candida* species, as it confers significant resistance to antifungal agents and the innate immune response.3 While a vast body of research concerns the study of host defences against planktonic *C. albicans*,3 there is scant information on *C. parapsilosis* biofilms, as reviewed previously.1 Further, it is unknown how antifungal agents interact with human phagocytes against *C. parapsilosis* biofilms and whether the antifungal agents could influence the interactions between innate immune cells and biofilms.

In this study we set out to answer some fundamental questions regarding the immunopharmacological effects of the newer antifungal agents against *C. parapsilosis* biofilms. We investigated the damage of *C. parapsilosis* biofilms induced by human neutrophils (PMNs) and monocytes (MNCs) alone and in combination with voriconazole, posaconazole, caspofungin or anidulafungin. In addition, we compared interactions of antifungal agents and human phagocytes against biofilms with those against planktonic cells.

**Materials and methods**

The clinical isolate *C. parapsilosis* PA/71, from a sputum culture, was used in this study. *Candida* cells from fresh Sabouraud dextrose agar plates were grown overnight in yeast-nitrogen-base broth (Scharlau Chemie SA, Spain) supplemented with 50 mM glucose at 37°C. Before their use for biofilm formation, planktonic cells were suspended in 0.15 M PBS (pH 7.2, Ca²⁺ and Mg²⁺ free; Biochrom KG, Germany), standardized to 10⁶ blastoconidia/mL and used immediately.5 *C. parapsilosis* biofilms were grown on the surface of silicone elastomer discs (Bioplexus Corp., Saticoy, CA, USA), pre-treated with fetal bovine serum (FBS; Gibco, Paisley, Scotland), in 96-well plates at 37°C for 72 h under constant linear shaking for bloodstream flow simulation.5 PMNs were isolated from heparinized whole blood of healthy adult volunteers by dextran sedimentation and Ficoll centrifugation, as
Antifungals, phagocytes and C. parapsilosis biofilms

described previously. The THP-1 monocyctic cell line (ATCC TIB202; ATCC, Manassas, VA, USA) was used as the MNC source.

Mature biofilms were then incubated in the presence of a range of clinically relevant concentrations of voriconazole (Pfizer, Groton, CT, USA; 0.5, 2, 32 mg/L), posaconazole (Schering-Plough, Kenilworth, NJ, USA; 0.25, 1, 16 mg/L), caspofungin (Merck, Whitehouse Station, NJ, USA; 0.06, 0.12, 1 mg/L) or anidulafungin (Pfizer; 0.06, 0.12, 0.5 mg/L) alone or in combination with human phagocytes (PMNs or MNCs) at effector cell:target (E:T) ratios of 1:1 and 5:1 at 37°C in a humidified 5% CO₂ incubator for 22 h. Biofilms containing only RPMI-1640 served as controls. Four replicate biofilms were used for each condition.

After incubation, phagocytes were lysed hypotonically. Fungal damage induced by phagocytes and/or antifungal agents was assessed by modification of the XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; 0.25 mg/mL] metabolic assay using coenzyme Q₀ (2,3-dimethoxy-5-methyl-1,4-benzoquinone; 40 mg/L) as the final electron acceptor agent. Antifungal activities were expressed as percentages of damage and were calculated by the formula: \( \frac{X}{C} \times 100 \), where \( X \) is the average optical density of treated biofilms or planktonic cells and \( C \) is the average optical density of control biofilms or planktonic cells. Optical density was measured using a spectrophotometer (Anthos 2000, Austria) at 450 nm with a reference wavelength at 690 nm. Planktonic cells were treated and tested in the same way as biofilms, except that no silicone elastomer discs were used.

To evaluate the differences of three or more groups, statistical analysis included tests of homogeneity of variance, analysis of variance and post hoc analysis. The combined effect of phagocytes and antifungal agents was calculated as follows: the damage induced by the phagocytes alone and the antifungal drug alone was calculated and compared with the effect of treatment with the combination of phagocytes and drug. Synergism was defined as an antifungal effect (damage) caused by the combination that was significantly greater than the effect of phagocytes alone plus the effect of the drug alone. An additive effect was defined as an antifungal effect of the combination that was significantly greater than the effect produced by either phagocytes or drug alone but that did not reach synergism. Antagonism was defined as an antifungal effect of the combination that was less than the effect produced by either phagocytes or drug alone. Differences between biofilm and planktonic growth forms were analysed by Student’s t-test (SPSS Statistical Package, version 11.5; SPSS Inc., Chicago, IL, USA).

Results and discussion

The percentages of fungal damage induced by voriconazole, posaconazole, caspofungin or anidulafungin, tested over a range of concentrations, by PMNs or MNCs or by the combination of each antifungal agent with phagocytes are shown in Figures 1

![Figure 1](image_url)

Figure 1. Percentage damage induced by human PMNs and/or voriconazole (VRC; a), MNCs and/or VRC (b), PMNs and/or posaconazole (POS; c) or MNCs and/or PSC (d) after incubation at 37°C for 22 h against C. parapsilosis biofilms (white bars) or planktonic cells (grey bars) at different E:T ratios shown under the horizontal axis. The values are means ± SEM of six to eight experiments. Each experiment with PMNs was conducted with PMNs of one donor and by use of quadruplicate wells for each condition. The mean value of the replicate wells was considered as the value of that particular donor and experiment. The means of the replicate wells of each experiment were then used in the data analysis to calculate the mean ± SEM for all the experiments conducted under the same conditions. The results of the damage induced by the combination of human phagocytes (PMNs or MNCs) and each triazole were compared with the results of human phagocytes or each triazole alone by analysis of variance with Dunnett’s test. The brackets above the bars denote that the combined effect of PMNs (at a 1:1 or 5:1 E:T ratio) with each triazole (at the indicated concentration) was additive (P < 0.01 versus the two components) against C. parapsilosis planktonic cells, as indicated. The asterisks denote differences between biofilm and planktonic conditions (P < 0.05).
and 2. Overall, phagocyte-induced fungal damage against biofilms was E:T dependent, and in general lower (either statistically significant or with a trend) than that against their planktonic counterparts. Similarly, the damage induced by voriconazole, posaconazole, caspofungin or anidulafungin alone or by the combination of each of the antifungal agents with phagocytes against biofilms was concentration dependent and in general lower than that against planktonic cells.

The effect of combining voriconazole and phagocytes against *C. parapsilosis* planktonic cells was additive. In contrast, no significant collaboration between voriconazole and phagocytes occurred against biofilms. For example, the combined activity of voriconazole and PMNs or MNCs was similar to the activities of each of them alone against biofilms. This was also the case for posaconazole and phagocytes against biofilms (Figure 1).

Published data, thus far, have reported a collaboration of phagocytes with voriconazole for enhanced killing of *C. albicans* planktonic cells.8 Our study extends this finding, showing that an additive interaction exists between phagocytes and voriconazole against *C. parapsilosis* planktonic cells.

From all the conditions tested, caspofungin in combination with phagocytes consistently showed a lack of collaborative effect against both phenotypes of *C. parapsilosis* (Figure 2a and b). In contrast, the combination of anidulafungin at 0.5 mg/L with PMNs at a 5:1 E:T ratio induced significantly greater biofilm damage than PMNs alone (*P* = 0.004) or anidulafungin alone (*P* = 0.041) (Figure 2c). Therefore, this interaction, at the above conditions, was additive. The combined activity of anidulafungin with phagocytes, for the other conditions tested, showed a trend toward collaboration, as a statistically significant difference occurred only between the combination and phagocytes alone, not between the combination and anidulafungin alone.

Despite repeated attempts, the increased assay variability did not show statistically significant differences in fungal damage between biofilm and planktonic cells. Such an increased variability is not surprising for *C. parapsilosis*, as it has also been noted by other researchers.6 It could be attributed to the distinctive *C. parapsilosis* biofilm characteristics, which, unlike *C. albicans* biofilms, have a heterogeneous architecture with a patchy and easily disruptable appearance. Our confocal microscopy studies verified these observations (data not shown). Furthermore, another source of variability was the different host responses among the different donors used as PMN sources. Indeed, polymorphism in dectin-1 germline genes have been reported to affect the host responses against fungal pathogens.9

We recently reported that exposure of *C. albicans* biofilms to subinhibitory concentrations of anidulafungin produced phagocyte-mediated damage.7 Here, for the first time, we report that this immunopharmacological effect of anidulafungin is extended to *C. parapsilosis* biofilms. Moreover, we found that, among the antifungal agents tested, only anidulafungin exhibits such an effect against Candida spp. biofilms. Caspofungin, although an echinocandin-class compound like anidulafungin, does not demonstrate a similar collaborative effect against Candida biofilms. This discrepancy between anidulafungin and other echinocandins has also been raised by others.10 Some *in vitro* data also suggest that caspofungin treatment of *C. parapsilosis* has an immunosuppressive effect on host defences.11 Differences in echinocandin configuration may explain the relatively more extensive anidulafungin ‘unmasking’

**Figure 2.** Percentage damage induced by human PMNs and/or caspofungin (CAS; a), MNCs and/or CAS (b), PMNs and/or anidulafungin (AND; c) or MNCs and/or AND (d) after incubation at 37 °C for 22 h against *C. parapsilosis* biofilms (white bars) or planktonic cells (grey bars) at different E:T ratios. For remaining information, see the legend for Figure 1.
effect to the underlying β-glucan in the cell wall of C. parapsilosis biofilms. This augmented exposure of β-glucan could initiate an enhanced immune response mediated by innate immune receptors, like dectin-1. 12

Conclusions
In our study, within the limits of in vitro experimental conditions, we examined the immunopharmacological effects of voriconazole, posaconazole, caspofungin and anidulafungin against C. parapsilosis biofilms. Our results showed that C. parapsilosis biofilms exhibit significantly reduced susceptibility to host immune cells compared with their planktonic counterparts. We also found that while there is no antagonistic antifungal interaction between antifungal agents and phagocytes, exposure of C. parapsilosis biofilms to subinhibitory concentrations of anidulafungin (0.5 mg/L) is associated with a significant increase in phagocyte-mediated damage. Further, this immunopharmacological effect of anidulafungin does not appear to be echinocandin class dependent, as caspofungin did not demonstrate such a collaborative effect against C. parapsilosis biofilms.

Acknowledgements
This work was presented in part at the Forty-seventh Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, USA, 2007 (Abstract M-1853).

We thank Professor Mahmoud Ghannoum for kindly providing us with the isolate used in this study.

Funding
This study was partially supported by Aristotle University, Thessaloniki, Greece, by a grant from the European Society of Pediatric Infectious Diseases (2006–2007 Small Grant Awards) and by a grant from Pfizer (GA900095).

Transparency declarations
None to declare.

References