Published in final edited form as:

Mycoses. 2012 January ; 55(1): 80–85. doi:10.1111/j.1439-0507.2011.02047.x.

Addition of DNase Improves the In Vitro Activity of Antifungal Drugs against *Candida albicans* Biofilms

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SUMMARY

Background—Cells within *Candida albicans* biofilms display decreased susceptibility to most clinically used antifungal agents. We recently demonstrated that extracellular DNA (eDNA) plays an important role in biofilm integrity, as a component of the biofilm matrix.

Objective—To gain insight into the contributions of eDNA to *C. albicans* biofilms antifungal susceptibility by the investigation of the impact of the combined use of deoxyribonuclease I (DNase) and antifungals to treat biofilms.

Methods—*C. albicans* biofilms were formed using a simple and reproducible 96-well plate-based method. The activity of the combined use of 0.13 mg l⁻¹ DNase and antifungals was estimated by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay, and total viable counts.

Results and Conclusions—Here we report the improved efficacy of amphotericin B when in combination with DNase against *C. albicans* biofilms, as assessed by XTT readings and viable counts. Furthermore, although DNase increased the efficacy of caspofungin in the reduction of mitochondrial activity, no changes were observed in terms of culturable cells. DNase did not affect biofilm cells susceptibility to fluconazole. This work suggests that agents that target processes affecting the biofilm structural integrity may have potential use as adjuvants of a catheter-lock therapy.

Keywords

amphotericin B; antifungal adjuvant; *C. albicans* biofilm; caspofungin; fluconazole

Introduction

Candida albicans is an opportunistic fungal organism that is present as part of the normal microbiota of healthy individuals. However, when the host immune system is impaired or the competing bacterial microbiota is altered, *C. albicans* can overwhelm the host defences and gain access to the bloodstream, invading tissues, or contaminating medical devices, causing life threatening infections [1]. These infections are often associated with the formation of biofilms, and one of the major concerns with disease management is the fact that *C. albicans* biofilm cells display reduced susceptibility against azoles and polyenes in comparison to planktonic cells. This intrinsic property of biofilms is likely to be

multifactorial and has been associated with factors such as cells physiological state, activation of drug efflux pumps [2], and a protective effect of the extracellular matrix (ECM) namely β -glucans that have been shown to bind fluconazole [3] and amphotericin B [4]. Recently our group showed that extracellular DNA (eDNA) is a critical component of *C. albicans* biofilm ECM contributing to the biofilm structural integrity [5]. In order to gain further insight into the contributions of eDNA to *C. albicans* biofilms antifungal susceptibility, in the present study we have investigated the impact of the combined use of deoxyribonuclease I (DNase) (which degrades eDNA) and antifungals to treat *C. albicans* biofilms.

Materials and methods

Chemicals

A polyene (amphotericin B), an echinocandin (caspofungin), and an azole (fluconazole) were chosen for their different mechanisms of action. Drug solutions were prepared according to the Clinical and Laboratory Standards Institute (CLSI) recommendations [6], and the range of concentrations were as follows: amphotericin B (Sigma, St. Louis, MO), 0.06 to 16 mg l⁻¹, caspofungin (Merck, NJ, USA and Lisbon, Portugal), 0.008 to 2 mg l⁻¹, and fluconazole (Pfizer, CT, USA), 4 to 1,024 mg l⁻¹ for biofilm assays; and amphotericin B and caspofungin, 0.004 to 1 mg l⁻¹, and fluconazole, 0.13 to 32 mg l⁻¹ for planktonic cells assays. DNase I from bovine pancreas (Sigma) stock solutions were prepared in 0.15 mmol l⁻¹ NaCl supplemented with 5 mmol l⁻¹ of MgCl₂. The enzyme, which retains exonuclease activity in nutrient media for 24 h [7], was used at 0.13 mg ml⁻¹, concentration previously shown to decrease *C. albicans* biofilm biomass [5].

Effect of the combination of DNase and antifungal agents against *C. albicans* biofilms—*C. albicans* SC5314 biofilms were formed in 96-well microtiter plates as previously described, using RPMI 1640 medium (Mediatech Inc., Herdon, VA, USA) buffered with MOPS (Research Products International Corp Mount Prospect, IL, USA) to pH 7 [8]. Biofilms (24-h) were washed with PBS and challenged with RPMI containing the different concentrations of antifungals alone or in combination with DNase. Control biofilms were challenged with RPMI, RPMI MgCl₂, and RPMI DNase.

Following 24-h incubation at 37°C, biofilm cells were washed with PBS and mitochondrial activity estimated by the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay [8]. In addition, to determine the number of colony forming units (CFU), biofilm cells were scraped, resuspended in a final volume of 500 μ l, and vortexed as previously described [9]. After serial dilutions, cell suspensions were plated on Sabouraud dextrose agar medium and the number of CFU enumerated. Under the conditions used in this study the quantification limit was of 2.7 log₁₀ CFU cm⁻². The efficacy of biomass removal was assessed by crystal violet staining assay performed in each well [9].

Sessile minimum inhibitory concentrations (SMIC) were determined at 50% (SMIC50) and 80% (SMIC80) decrease in absorbance at 490 nm, in comparison with the corresponding control biofilms challenged with RPMI, for antifungals tested alone, or challenged with RPMI DNase, for antifungals tested in combination with DNase.

Effect of the combination of DNase and antifungal agents against *C. albicans* planktonic cells—*C. albicans* SC5314 planktonic cells were cultivated according to the CLSI microdilution method [6] in polystyrene, round-bottomed, 96-well microtitre plates. Cells were treated with antifungals alone or in combination with DNase 0.13 mg ml⁻¹. After 24 h incubation at 37°C under static conditions, spectrophotometric absorbance at 650 nm

(Abs650nm) was determined. Plate counts were also performed using standard procedures. The quantification limit was of $1.5 \log_{10}$ CFU ml⁻¹.

Planktonic minimal inhibitory concentrations (MIC) were determined as the lowest concentration of antifungal drug that prevented fungal growth relative to the corresponding controls challenged with RPMI, for antifungals tested alone, or challenged with RPMI DNase, for antifungals tested in combination with DNase.

Reproducibility and statistical analyses

All the experiments were repeated on three separated occasions, with three to eight dependent replicates. Data is presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using GraphPad statistical software (GraphPad Software, San Diego, CA, USA). The normality of the data was examined using D'Agostino and Pearson omnibus normality test. For statistical analysis of viable counts, cultures containing 0 CFU ml⁻¹ were ascribed one half of the quantification limit. To determine if the DNase improves the in vitro efficacy of antifungal drugs against *C. albicans*, the percentages of XTT colorimetric readings and Abs650nm readings, and the number of $\log_{10}$ CFU cm⁻² and $\log_{10}$ CFU ml⁻¹ were analysed using two-way ANOVA followed by Bonferroni post-hoc tests. Statistical significance was defined as $P < 0.05$.

Results and Discussion

In the present study, the combined effect of DNase with antifungal agents against *C. albicans* biofilm cells was characterized in terms of XTT reduction colorimetric readings, as an indication of the entire population mitochondrial activity, and CFU counts, to assess the ability of individual cells to proliferate when plated in solid medium.

Consistent with previous investigations [10–12], results revealed that the amphotericin B SMIC80 (1 mg l^{-1} , Fig. 1-Ia) is within the resistance range of this drug ($> 1 \text{ mg l}^{-1}$), although a complete eradication of the viable cells was not achieved with amphotericin B at 16 mg l^{-1} (Fig. 1-IIa). However, based on the % of XTT readings, addition of DNase to amphotericin B resulted in SMIC50 (0.06 mg l^{-1}) and SMIC80 (0.25 mg l^{-1}) values that were two dilutions lower than when this polyene agent was used alone (Fig. 1-Ia). Accordingly, between the amphotericin range of 0.06 and 0.25 mg l^{-1} the % of XTT colorimetric readings were lower when the antifungal drug was used in combination with DNase than when it was used alone ($P < 0.05$) (Fig. 1-Ia).

Furthermore, DNase increased the efficacy of amphotericin B, inducing a significant reduction in the $\log_{10}$ CFU cm⁻² in the amphotericin range between 1 and 8 mg l^{-1} , in comparison with biofilm cells treated with this polyene alone (Fig. 1-IIa). Notably, in the presence of DNase no survival cells were detected for amphotericin B levels above 2 mg l^{-1} (Fig. 1-IIa). These results suggest that the combined use of DNase and amphotericin B increases *C. albicans* biofilm cells susceptibility to this polyene drug.

As reported before [13–15], *C. albicans* biofilm cells were susceptible to caspofungin, with SMIC80 value (1 mg l^{-1} , Fig. 1-Ib) within the susceptible range ($\leq 2 \text{ mg l}^{-1}$) in terms of biofilm cells mitochondrial activity reduction. However, this echinocandin did not eliminate *C. albicans* culturable biofilm cells (Fig. 1-IIb), as previously shown by independent research groups [16–18], although it efficiently killed *C. albicans* planktonic cells (Fig. 2-IIb). Our results indicate that the in vitro activity of caspofungin against *C. albicans* biofilm cells mitochondrial activity was increased when used in combination with DNase (Fig. 1-Ib). In fact, SMIC50 and SMIC80 values for caspofungin plus DNase were three dilutions (0.13 mg l^{-1}) and two dilutions (0.25 mg l^{-1}) lower, respectively, when compared with

biofilms exposed to this antifungal alone ($P < 0.05$) (Fig. 1-Ib). Biofilm cells treated with DNase and caspofungin ranging from 0.13 to 0.5 mg l⁻¹ exhibited lower % of XTT readings in comparison with those treated with caspofungin only ($P < 0.05$). However, the combination of DNase and caspofungin did not induce major changes in terms of biofilm cells viable counts when compared with caspofungin treatment (Fig. 1-IIb). Of note, trailing growth with caspofungin is usually observed [19], and it was not abolished with the addition of DNase (Fig. 1-b). However, to confirm the trailing growth effect, higher caspofungin concentrations should be evaluated.

It is well established in the field that cells within biofilms are intrinsically less susceptible to azole derivatives [10, 14, 15]. Results for *C. albicans* biofilm cells reduced susceptibility to fluconazole are shown, with both SMIC80 and SMIC50 values higher than 1,024 mg l⁻¹ (Fig. 1-Ic) and reduction of viable counts $< 1\text{-log}_{10}$ unit (Fig. 1-IIc). In addition, the susceptibility of biofilm cells against fluconazole was not changed by the addition of DNase in terms of mitochondrial cells activity (Fig. 1-Ic) and viable counts (Fig. 1-IIc). Even though, other azole drugs need to be tested.

Several literature reports have shown that eDNA, a component of bacterial biofilms ECM [20], contributes to biofilms decreased susceptibility to some antibiotics [21, 22], antiseptics, and disinfectants agents [23], and may protect bacterial biofilms from sodium dodecyl sulphate treatment [24]. It has been suggested that this effect may be due to eDNA induction of genes involved in the modification of lipopolysaccharides [22] and/or poor penetration of drugs within biofilms [21]. However, this is probably not a biofilm specific trait, as there is some controversy on the effect of DNase on the antimicrobial susceptibility of bacterial planktonic cells [7, 25]. In addition, exogenous DNA was shown to decrease bacterial planktonic cells susceptibility to antibiotics [22]. We observed that DNase increases *C. albicans* planktonic cells susceptibility to amphotericin B (by one dilution) (Fig. 2-Ia) and caspofungin (by three dilutions) (Fig. 2-Ib), but not to fluconazole (Fig. 2-Ic), in comparison with the % of Abs650 readings of cells challenged only with the drugs (Fig. 2-I). Similar results were obtained for viable count assays (Fig. 2-II), showing that DNase treatment increases the efficacy of amphotericin B (Fig. 2-IIa) and caspofungin (Fig. 2-IIb) against *C. albicans* planktonic cells. In contrast, in *C. albicans* biofilm and planktonic cultures treated with antifungals and DNA at 320 ng ml⁻¹, concentration previously shown to increase *C. albicans* biofilm biomass [5], the drug susceptibility was not affected, when compared to antifungals tested alone (data not shown). Overall, these results suggest that eDNA, as a component of biofilm ECM, may not impede antifungals diffusion through biofilms, in accordance to previous reports showing a fast drug diffusion through *C. albicans* biofilms [26]. Nevertheless, products of the enzymatic digestion of eDNA or DNase might interact with cell component(s) facilitating the action of antifungal agents. In fact, it was shown that ECM β -glucans are involved in biofilm antifungal resistance by hampering the access of the drugs to their targets inside the cells [27].

In conclusion, our results show that *C. albicans* biofilm cells susceptibility, particularly to amphotericin B, is enhanced by the addition of DNase. Although additional studies might include a much higher number of strains and *Candida* species, as well as the evaluation of the mechanism(s) and in vivo efficacy of DNase, this work suggests that agents that target processes affecting the biofilm structural integrity may have potential use as a therapeutic adjuvant in the treatment of *Candida* biofilms, at least as part of antifungal lock-like therapy of devices.

Acknowledgments

This work was supported by the National Institute of Dental & Craniofacial Research (NIDCR) (grant number 5R21DE017294 to J. L.-R.); and National Institute of Allergy and Infectious Diseases (NIAID) (grant number R21AI080930). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIDCR, the NIAID or the National Institutes of Health. The authors would like to thank Merck Sharp & Dohme Portugal for providing part of the caspofungin used in this study. M. M. was financially supported by a fellowship from Fundação para a Ciência e Tecnologia, Portugal (contract SFRH/BD/28222/2006), co-financed by the Programa Operacional Potencial Humano (POPH)/Fundo Social Europeu (FSE).

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Fig. 1.

Activity of amphotericin B (a), caspofungin (b), and fluconazole (c) alone (white bars) or in combination with DNase (black bars) against *C. albicans* SC5314 24-h biofilm cells. DNase at 0.13 mg ml^{-1} was added to all serial dilutions of antifungal agents. The effects were assessed in terms of the percentage of colorimetric readings for XTT as compared with drug-free controls (mean % of XTT colorimetric readings $\pm$ SEM) as compared with drug-free controls (I) and in terms of viable counts (mean CFU $\text{cm}^{-2} \pm$ SEM) (II). Biofilm cells recovery was $\geq 90 \%$. Statistically significant differences are indicated with an asterisk (*, $P < 0.05$).

Fig. 2.

Activity of amphotericin B (a), caspofungin (b), and fluconazole (c) alone (white bars) or in combination with DNase (black bars) against *C. albicans* SC5314 24-h planktonic cells. DNase at 0.13 mg ml^{-1} was added to all serial dilutions of antifungal agents. The effects were assessed in terms of the percentage of Absorbance 650 nm readings (mean % of Abs650nm readings $\pm$ SEM), as compared with drug-free controls (I) and in terms of viable counts (mean CFU $\text{ml}^{-1} \pm$ SEM) (II). Statistically significant differences are indicated with an asterisk (*, $P < 0.05$).