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Arachidonic acid increases antifungal susceptibility of *Candida albicans* and *Candida dubliniensis*

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***Objectives*: During *Candida albicans* infection, arachidonic acid (AA) is released from phospholipids of infected host cell membranes and used by *C. albicans* as the sole carbon source and for production of eicosanoids. AA can be incorporated into the phospholipids of yeasts, influencing the saturation level and fluidity of yeast cell membranes. It is suggested that the effectiveness of polyene (e.g. amphotericin B) and imidazole (e.g. clotrimazole) antifungals may depend upon the level of unsaturation and ergosterol in the membrane. Therefore, the aim of this study was to evaluate the effect of AA on the cell membrane and susceptibility of *C. albicans* and *Candida dubliniensis* biofilms towards amphotericin B and clotrimazole.**

***Methods*: Both yeasts were grown in the presence and absence of AA and the effect of amphotericin B and clotrimazole was examined by confocal laser scanning microscopy, determination of mitochondrial metabolism, unsaturation index of the phospholipid fractions and ergosterol content of the membranes.**

***Results*: AA had no effect on the viability of the cells in the biofilm; however, there was an increase in ergosterol levels as well as antifungal susceptibility of biofilms grown in the presence of AA.**

***Conclusions*: AA influences phospholipid unsaturation and ergosterol content of both yeasts *C. albicans* and *C. dublininensis*, increasing susceptibility towards the antifungals. Pretreatment of biofilms with polyunsaturated fatty acids may result in the reduction in antifungal dose needed to inhibit biofilms.**

Keywords: amphotericin B, clotrimazole, phospholipids, ergosterol

Introduction

During *Candida albicans* infection, arachidonic acid (AA) is released from phospholipids of infected host cells by fungal phospholipases.[1] *C. albicans* utilizes the released AA as the sole carbon source for cell growth and morphogenesis. In addition, AA is a precursor for production of eicosanoids, known virulence factors, stimulating germ tube formation and inflammation during infection[2] and can be incorporated into the phospholipids of yeasts, influencing the level of unsaturation in yeast membranes.[3]

Two classes of membrane-active antifungals are commonly used to treat *Candida* infections: the polyenes, e.g. amphotericin B, which binds to ergosterol in fungal cell membranes, causing membrane disruption, and the azoles, e.g. clotrimazole, an ergosterol synthesis inhibitor, which increases cellular permeability.[4] Hąc-Wydro *et al.*[5] suggested that nystatin, a polyene drug, may bind more strongly to phospholipid monolayers containing unsaturated fatty acids than to monolayers of saturated phospholipids with similar acyl chain lengths. Similarly, Yamaguchi[6] found that imidazole antifungals interact with unsaturated phospholipids extracted from *C. albicans* protoplast membranes as well as polyunsaturated free fatty acids (PUFAs) (including AA) and that the presence of unsaturated phospholipids influenced the sensitivity of liposomes towards imidazole antifungals.[7]

Since AA is present in the natural habitat of *C. albicans* and plays an important role during infection, the aim of this

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study was to investigate the effect of AA on the susceptibility of biofilms of both yeasts towards amphotericin B and clotrimazole.

Materials and methods

Strains used

C. albicans CBS 562T and *Candida dubliniensis* NRRL Y-17841T were used in this study and maintained on yeast–malt extract agar at room temperature.

Effect of arachidonic acid on biofilm viability

Biofilms of each strain were formed in chamber slides (Lab-Tek® Chamber Slide™ System, Naperville, IL, USA) containing 3 mL of RPMI-1640 medium (Sigma Aldrich, USA) for 48 h at 37°C in the presence and absence of 1 mM AA (Sigma Aldrich). Biofilms were stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen, Molecular Probes, USA)[8] and viewed using a confocal laser scanning microscope (Nikon TE 2000, Japan).

Antifungal susceptibility assay

Biofilms were formed in the presence and absence of 1 mM AA for 48 h at 37°C in microtitre plates (Corning Incorporated, Costar®, USA). Stock solutions (50 g/L) of amphotericin B (Sigma Aldrich) and clotrimazole (Sigma Aldrich) were prepared and added as described previously.[9] The mitochondrial metabolism of the biofilms were studied using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H tetrazolium hydroxide (XTT) (Sigma Chemicals, USA) as described previously.[10]

Visualization of antifungal susceptibility

Amphotericin B (12.5 mg/L) and clotrimazole (1.25 mg/L), diluted in buffered (pH 7.4) growth medium, were added to biofilms formed in chamber slides and incubated for another 5 h at 37°C with appropriate controls. Biofilms were stained and viewed as described above.[8]

Phospholipid composition and unsaturation index

Biofilms were formed in polystyrene Petri dishes containing RPMI-1640 medium for 48 h at 37°C. Washed biofilms were scraped off and the cells placed into 2 mL plastic tubes, resuspended in hexane (Lasec, SA), vortexed (1 min) and extracted (6 h) to remove neutral lipids. After filtration, cells were resuspended in chloroform/methanol (2:1) (Lasec, SA) and the remaining polar lipids (including glyco- and phospholipids) extracted and separated into glyco- and phospholipids by column chromatography.[3] The phospholipids were separated into phosphatidylethanolamine, phosphatidylcholine (PC), phosphatidylinositol and phosphatidylserine, visualized by iodine vapour, scraped off, extracted with chloroform/methanol (2:1) and the fatty acids transesterified with trimethyl sulphonium hydroxide. The fatty acid methyl esters were analysed on a Shimadzu GC-2010 gas chromatograph (Japan) with a flame ionization detector equipped with a SGE-BPX-70 column (60 m × 0.32 mm inner diameter) with nitrogen as carrier gas (flow rate: 0.5 mL/min) and determined by reference to authentic standards. The unsaturation index for each phospholipid fraction was calculated as described by Mishra and Prasad.[11]

Ergosterol content

Biofilms were formed in Petri dishes as described. Washed biofilms were scraped off and placed into glass borosilicate tubes (Schott, USA) and the wet weight of the cells determined. Extraction of ergosterol was done according to Arthington-Skaggs *et al.*[12] with modifications. Potassium hydroxide (Merck, SA), dissolved in methanol/ethanol/water (700:315:15), was added to the cells. A further 2 mL of ethanol was added and the tubes were gassed with nitrogen gas. The tubes were incubated in a water bath at 80°C for 90 min and shaken every 10 min. Sterols were extracted with *n*-heptane (Burdick & Jackson, USA) and stored at 4°C. The heptane fraction was dried under nitrogen gas and dissolved in 2 mL of ethanol. Any solids were precipitated overnight at 4°C. The samples were filtered (0.45 μm) and the absorbance measured at 282 nm on a SpectraMax M2 Microplate Reader (Molecular devices, USA). This was done in duplicate.

Statistical analysis

Unless stated otherwise, all experiments were performed in triplicate and the *t*-test was used to determine significance.

Results and discussion

Effect of arachidonic acid on biofilm viability

Results indicate that 1 mM AA has no influence on the viability of the biofilms of both yeast strains studied.

Antifungal susceptibility

The XTT assay indicates an increase in susceptibility of biofilms of both yeast strains towards both amphotericin B (Figure 1a and c) and clotrimazole (Figure 1b and d) after growth in the presence of AA.

The effect of growth in the presence of AA on antifungal susceptibility was also examined microscopically. An increase in dead cells was observed when biofilms, grown in the presence of AA, were treated with amphotericin B (Figure 2a and d) and clotrimazole (Figure 2e and h), confirming that growth in the presence of 1 mM AA renders the *Candida* strains more susceptible to amphotericin B and clotrimazole.

Phospholipid composition and unsaturation index

The fatty acid profile of the major phospholipid fraction (PC) of *C. albicans* biofilms grown in the presence of AA showed an increase in the percentage of polyunsaturated linoleic acid [from 5.68 (±1.60) to 10.99 (±1.54)] and a decrease in the percentage of saturated palmitic acid [from 42.65 (±2.69) to 34.33 (±4.22)] and stearic acid [from 17.92 (±1.92) to 9.73 (±1.72)]. The PC fraction of *C. dubliniensis* biofilms grown in the presence of AA showed an increase in the percentage of linoleic acid [2.87 (±4.77) to 11.35 (±1.75)] and a decrease in the percentage of palmitic acid [from 50.47 (±1.94) to 38.79 (±1.32)]. This, together with an increase in AA in the PC fraction [*C. albicans*: 1.87 (±0.50); *C. dubliniensis*: 5.07 (±1.10)], contributed to the increase in unsaturation index of this fraction from 0.42 (±0.04) to 0.70 (±0.05) for *C. albicans* and from 0.30 (±0.05) to 0.67 (±0.11) for *C. dubliniensis*.

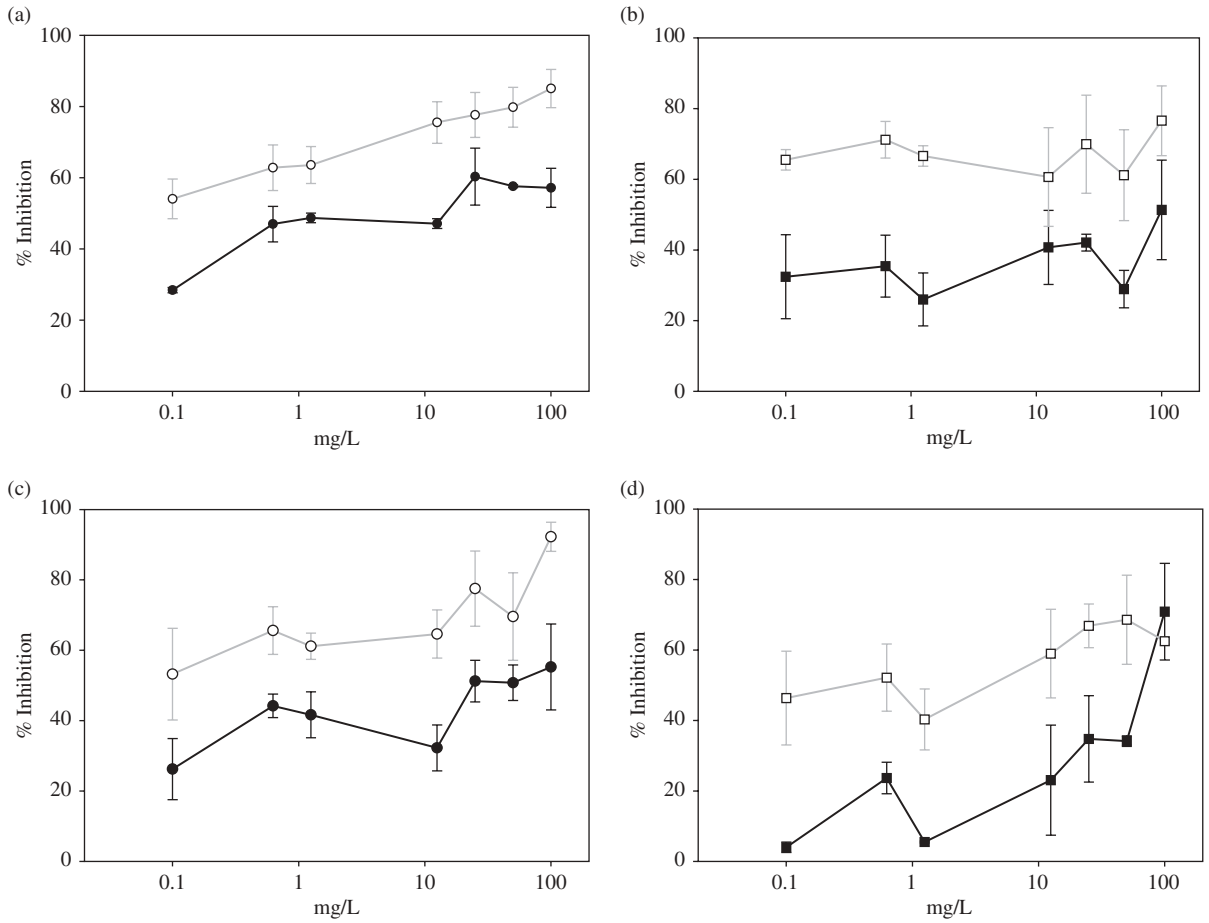

Figure 1. Percentage inhibition of antifungals on the mitochondrial metabolism of biofilms formed by *Candida* species grown in the presence and absence of AA. (a and b) *C. albicans* CBS 562T. (c and d) *C. dubliniensis* NRRL Y-17841T. Open circles, biofilms grown in the presence of AA, treated with amphotericin B; filled circles, biofilms grown in the absence of AA, treated with amphotericin B; open squares, biofilms grown in the presence of AA, treated with clotrimazole; filled squares, biofilms grown in the absence of AA, treated with clotrimazole. Results correspond to the mean and SD of three measurements.

Ergosterol content

The ergosterol content of both organisms increased in biofilms grown in the presence of AA, i.e. for *C. albicans* from 629.13 mg/g (±24.56) to 680.09 mg/g (±4.13) and for *C. dubliniensis* from 302.96 mg/g (±4.85) to 340.83 mg/g (±27.36).

It is known that yeasts (e.g. *Saccharomyces cerevisiae*) can react to the uptake of exogenous fatty acids by altering their phospholipids and, in some strains, the ergosterol content.[13] Similar results were obtained in this study. The increase in ergosterol content of the *Candida* strains grown in the presence of AA may explain the increase in amphotericin B susceptibility of these strains; however, the mechanism behind increased clotrimazole susceptibility is unclear.

Membrane saturation may play an important role in the effectiveness of antifungal therapy.[5–7] The unsaturation index indicates an increase in unsaturation of the phospholipids of biofilms grown in the presence of AA. This may affect membrane fluidity and membrane protein function,[13] possibly allowing increased uptake of antifungals, such as clotrimazole.

An alternative/complementary mechanism may be increased oxidative stress when PUFAs are incorporated into cellular lipids,[14] which may change the susceptibility of both yeasts towards antifungals, including amphotericin B.[15]

Since there is a need to develop new drug therapies for the treatment of mycoses, pre-treatment of biofilms with AA and/or other long-chain PUFAs may result in the reduction in antifungal doses needed to inhibit *Candida* biofilms; however, the conserved status of this phenomenon within these species will have to be determined.

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Transparency declarations

None to declare.

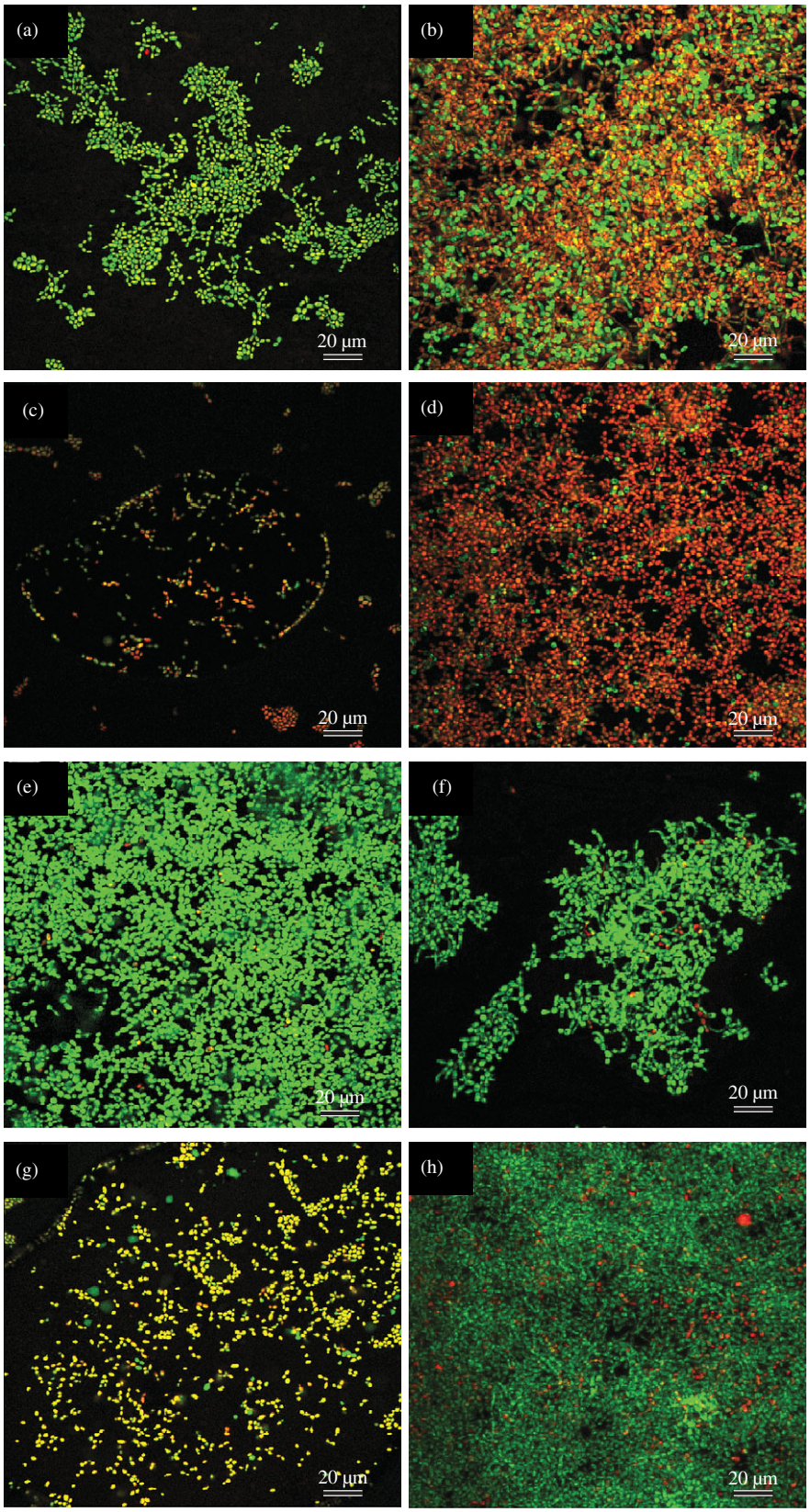

Figure 2. Confocal laser scanning micrographs of biofilms grown in the absence of AA treated with amphotericin B: (a) *C. albicans* CBS 562T and (b) *C. dubliniensis* NRRL Y-17841T. Biofilms grown in the presence of AA, treated with amphotericin B: (c) *C. albicans* CBS 562T and (d) *C. dubliniensis* NRRL Y-17841T. Biofilms grown in the absence of AA treated with clotrimazole: (e) *C. albicans* CBS 562T and (f) *C. dubliniensis* NRRL Y-17841T. Biofilms grown in the presence of AA treated with clotrimazole: (g) *C. albicans* CBS 562T and (h) *C. dubliniensis* NRRL Y-17841T. Green fluorescence indicates live cells and red fluorescence indicates dead cells.

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