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Exposure of the yeast *Candida albicans* to the anti-neoplastic agent adriamycin increases the tolerance to amphotericin B

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Abstract

Cancer patients experience a high incidence of fungal infections due to their immuno-suppressed condition. This work has investigated the interaction of an anti-neoplastic agent, adriamycin (doxorubicin), with the yeast *Candida albicans* and examined whether this drug altered the susceptibility of the yeast to amphotericin B – an anti-fungal agent used for the treatment of systemic fungal infections in cancer patients. Exposure to adriamycin for 24 h increased the growth of *C. albicans* and increased the tolerance to amphotericin B by a small, but statistically significant, extent. Growth in adriamycin-supplemented medium suppressed the respiration rate of *C. albicans*, which resulted in a decrease in the ergosterol content of the fungal cell membrane. The tolerance to amphotericin B was lost after exposure to adriamycin for 48 h, which coincided with a restoration in the respiration rate and the ergosterol content of the fungal cell membrane. This work demonstrated that short-term exposure (24 h) to adriamycin increased the tolerance of *C. albicans* for amphotericin B, which may be mediated by a decrease in the ergosterol content as a result of an adriamycin-induced disruption of oxidative phosphorylation.

Introduction

Cancer is a debilitating, multifactorial disease that attacks indiscriminately across all demographic sectors of society. There are several families of anti–neoplastic agents used to treat cancer and these are sub-divided on the basis of their mode of action. One class of anti-neoplastic agent, the anthracyclines, are non-covalent DNA binding agents and one of these, adriamycin, was isolated in 1969 from *Streptomyces peucetius* (Pratt & Ruddon 1979). Adriamycin (also known as doxorubicin) is used in the treatment of Hodgkin's disease and non-Hodgkin's lymphoma and in cancers of the breast, ovaries and lymph system (Lowenthal & Eaton 1996). Adriamycin is a topoisomerase II inhibitor (Cummings et al 1991) and its structure allows it to intercalate between base pairs of DNA causing subsequent template disorders, which lead to strand breakage. Adriamycin also affects mitochondrial function by selectively binding to cardiolipin in the mitochondrial membrane and disrupting the action of complex I (Parker et al 2001).

Cancer patients are at an increased risk of a range of opportunistic fungal infections by virtue of their debilitated state and the immuno-suppressive nature of anti-neoplastic regimes (Saral 1991; De Pauw 1997). The yeast *Candida albicans* is an important opportunistic fungal pathogen, affecting individuals whose immune systems become immuno-compromised due to diseases such as leukaemia or anti-neoplastic therapy (Schuler & Haag 1997). The incidence of infections caused by *C. albicans* has increased in recent years among immuno-compromised patients due, in part, to inefficient or incomplete therapies and by advances in medicine allowing the survival of immuno-compromised patients for prolonged periods. *C. albicans* accounts for between 52 and 63% of all nosocomial fungal infections, and yeast of the genus *Candida* are the fourth most common organisms isolated from blood (Pfaller et al 1998; Verduyn Lunel et al 1999).

Treatment of fungal infections is generally achieved by using azole or polyene anti-fungal agents such as amphotericin B (Schuler & Haag 1997; Prentice et al 1999; Laverdiere et al 2000). Amphotericin B is employed to treat advanced systemic fungal infections, but its efficacy can be low (Saral 1991; Walsh et al 1999). In addition,

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amphotericin B can have severe side effects, which may limit its use in certain patient groups. However, amphotericin B still remains an important anti-fungal agent for the treatment of recalcitrant systemic fungal infections (Hartsel & Bolard 1996). The anti-fungal activity of amphotericin B lies in its ability to bind to ergosterol in the fungal cell membrane and create pores through which cytoplasmic contents leak and protons enter, leading to cytoplasmic acidification and subsequent cell death (Abu Salah 1996).

The aim of this work was to determine whether exposure of *C. albicans* to the anti-neoplastic agent adriamycin altered the susceptibility of the yeast to amphotericin B. We postulated that in certain instances anti-neoplastic therapy may inadvertently alter the tolerance of *C. albicans* to selected anti-fungal drugs employed to arrest the development of infections in cancer patients.

Materials and Methods

Growth conditions

C. albicans ATCC 10231 was originally obtained from the American Type Culture Collection (Manasas, VA) and was grown to the stationary phase (approximately 1.5×10^8 cells mL^{-1}) at 30 °C overnight in yeast extract-peptone-D-glucose broth (YEPD; 1% (w/v) yeast extract (Sigma Aldrich Chemical Co., Dorset, UK), 2% (w/v) bacteriological peptone (Sigma Aldrich) and 2% (w/v) glucose (Sigma Aldrich)) in an orbital incubator at 200 rev min^{-1}. Where appropriate, media were solidified by the addition of 2% (w/v) agar (Oxoid). Yeast cultures were maintained on YEPD agar at 4 °C and sub-cultured every four to six weeks.

Antifungal and anti-neoplastic agents

Adriamycin was purchased as Doxorubicin (Ebewe Arzneimittel Gmbh., Unterach, Austria). Amphotericin B (Sigma Aldrich) was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich) and diluted to working concentrations in sterile phosphate-buffered saline (PBS, pH 7.2) (Life Technologies, Paisley, UK) before use. The maximum DMSO concentration employed with cells was 2% (v/v).

Amphotericin B susceptibility testing

Yeast cultures were grown to the stationary phase in antibiotic medium 3 (AB 3, Oxoid) supplemented with 2% (w/v) glucose overnight at 30 °C and 200 rev min^{-1}. Cultures were harvested by centrifugation (2220 *g* for 5 min in a Beckmann GS-6 centrifuge) and diluted to 1×10^6 cells mL^{-1}. Cells (1×10^5 in 100 μL) were added to each well of a 96-well plate (Sarsdtedt, Wexford, Ireland) containing amphotericin B (Sigma Aldrich) dissolved in antibiotic medium 3 (AB3) in serial dilutions from 2.5 to 0.0048 $\mu g\,mL^{-1}$. The plates were incubated at 37 °C for 24 h and the optical density was read at 450 nm using a MRX spectrometer (Dynax Technology). The MIC90 was determined to be the lowest concentration of amphotericin B required to reduce growth by 90%, relative to the control (Moran et al 1997).

Effect of pre-growth in adriamycin on the growth of *C. albicans* in amphotericin B

AB3 medium (supplemented with 2% (w/v) glucose and adriamycin (20 $\mu g\,mL^{-1}$)) was inoculated with *C. albicans* at a density of $5 \times 10^5\,mL^{-1}$ and grown at 37 °C for 24 h. Cells were harvested by centrifugation and resuspended in AB3 containing amphotericin B (0.625 $\mu g\,mL^{-1}$) at a density of 5×10^5 cells mL^{-1}. A drug free control consisted of cells that had not been exposed to adriamycin. Cell density was determined after growth for 24 h using a PAMAS SVSS-C Particle Counter (Rutesheim, Germany).

Oxygen consumption

Cells were grown in YEPD supplemented with adriamycin (20 $\mu g\,mL^{-1}$) at 30 °C for 24 or 48 h, harvested by centrifugation and resuspended at a density of $1 \times 10^8\,mL^{-1}$ in PBS (pH 7.2). A Clark Type oxygen electrode (Rank Brothers Ltd, Cambridge, UK) was employed to determine the respiration rate of cells and the rate of oxygen consumption was expressed as the number of μmoles of oxygen consumed per minute per 1×10^8 cells.

Sterol extraction and gas chromatography analysis

Sterol extraction was performed as described by Arthington-Skaggs et al (1999). Equivalent wet weight samples (2.28 g) of stationary phase cells were resuspended in 2 mL denaturing solution (20% w/v potassium hydroxide (Sigma Aldrich), 60% v/v ethanol) and placed in a 90 °C shaking water bath (190 rev min^{-1}) for 1.5 h. Heptane (600 μL) was added to each of the samples. Samples were left in the dark until a distinct heptane layer formed. Sterols were identified using a Varian Series II Gas Chromatograph (Varian Ltd, Victoria, Australia). An ergosterol standard curve was constructed over the range 100–0.25 $\mu g\,mL^{-1}$ and the lowest level of detection of ergosterol was 0.1 $\mu g\,mL^{-1}$.

Statistical analysis

Using either the Kruskal–Wallis test or Mann-Whitney U test (Sigma Stat Statistical Analysis Package Version 1.00; SPSS Inc., Chicago, IL), P values less than 0.05 denoted significance (Wardlaw 2000). All experiments were performed on three independent occasions and the means were expressed ± s.e.

Results

Effect of adriamycin exposure on *C. albicans* growth and susceptibility to amphotericin B

Adriamycin did not display a significant fungistatic or fungicidal effect on *C. albicans* over the concentration range

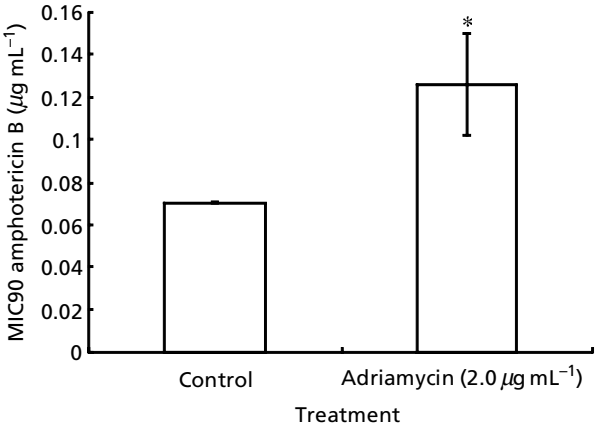

Figure 1 Amphotericin B MIC90 of *C. albicans* grown in AB3 supplemented with adriamycin B ($2.0\,\mu g\,mL^{-1}$). $^*P = 0.05$ compared with control.

$0.1–40\,\mu g\,mL^{-1}$ (data not presented). Experiments were performed to establish the response of *C. albicans* to amphotericin B, and to amphotericin B when co-cultured in the presence of adriamycin ($2.0\,\mu g\,mL^{-1}$). Exposure of *C. albicans* in AB3 supplemented with adriamycin to amphotericin B yielded an amphotericin B MIC90 of $0.13\,\mu g\,mL^{-1}$ compared with the control which gave an amphotericin B MIC90 value of $0.07\,\mu g\,mL^{-1}$ (Figure 1). These data were significant at $P = 0.05$ and indicated that 24-h exposure of *C. albicans* to adriamycin altered the susceptibility of the yeast to amphotericin B. No alteration in tolerance to the azoles, ketoconazole and miconazole, or the polyene, nystatin, was observed following pre-growth of *C. albicans* in adriamycin (data not presented).

Experiments were performed to determine whether a correlation existed between the enhanced tolerance of *C. albicans* to amphotericin B and cell growth. The cell density attained in the culture that had been pre-grown in adriamycin was $1.55 \times 10^7\,mL^{-1}$ whereas the cell number attained in the control was $3.57 \times 10^6\,mL^{-1}$ (Figure 2). Cultures that had been exposed to adriamycin for 48 h showed a lower final cell density ($1.28 \times 10^6\,mL^{-1}$) than the control.

Investigation of the effect of adriamycin on respiration of *C. albicans*

The oxygen consumption of *C. albicans* pre-grown in adriamycin ($20\,\mu g\,mL^{-1}$) for 24 or 48 h was determined. Oxygen consumption of control cells was 9.6 μmol oxygen/10^8 cells min^{-1} while cells pre-grown in adriamycin for 24 h displayed a respiration rate of 5.4 μmol oxygen/10^8 cells min^{-1}, which was significantly less (56%) than the control at $P = 0.05$ (Figure 3). Cells pre-grown in adriamycin for 48 h demonstrated a 23% reduction in oxygen consumption (7.4 μmol oxygen/10^8 cells min^{-1}) relative to the control.

Effect of adriamycin on the ergosterol content of *C. albicans*

Ergosterol is a major component of the fungal cell membrane which helps maintain structure and regulates the influx and efflux of extra/intracellular components. Amphotericin B functions by binding to ergosterol leading to the creation of pores through which intracellular constituents leak and protons enter the cell (Abu Salah 1996). The combined effect of these two processes is the acidification of the cytoplasm and the death of the cell. Resistance of fungal cells to amphotericin B is rare but, where encountered, is characterized by a reduction in the amount of ergosterol in the cell membrane (Kelly et al 1997; White et al 1998).

Ergosterol quantification by GC analysis demonstrated (Figure 4) that following growth for 24 h in adriamycin-supplemented medium the ergosterol content of *C. albicans* was reduced from 58.5 to $29.6\,\mu g\,mL^{-1}$ $(g\ cells)^{-1}$ (wet wt),

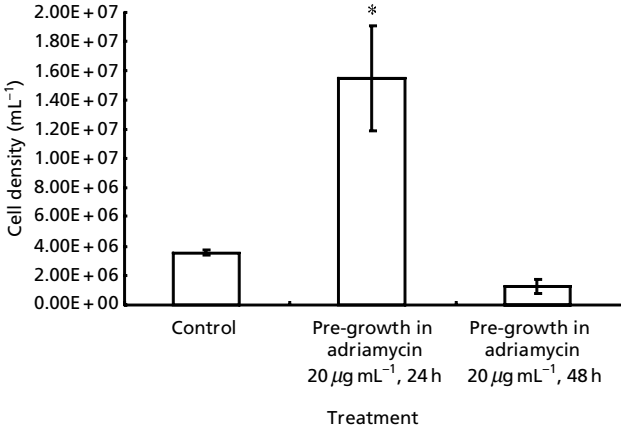

Figure 2 Growth of *C. albicans* in AB3 supplemented with amphotericin B ($0.625\,\mu g\,mL^{-1}$) following pre-growth in adriamycin ($20\,\mu g\,mL^{-1}$) for 24 or 48 h. $^*P = 0.05$ compared with control.

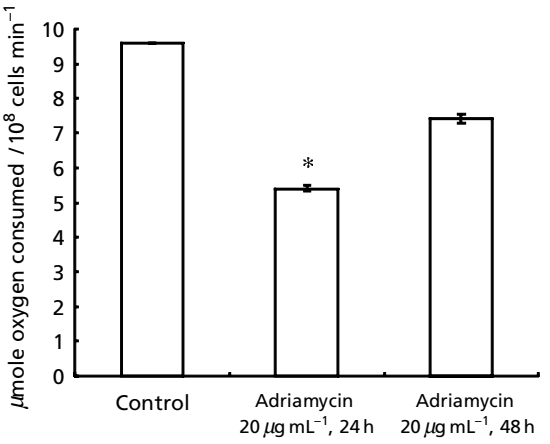

Figure 3 Oxygen consumption of *C. albicans* following growth in adriamycin ($20\,\mu g\,mL^{-1}$) for 24 or 48 h. $^*P = 0.05$.

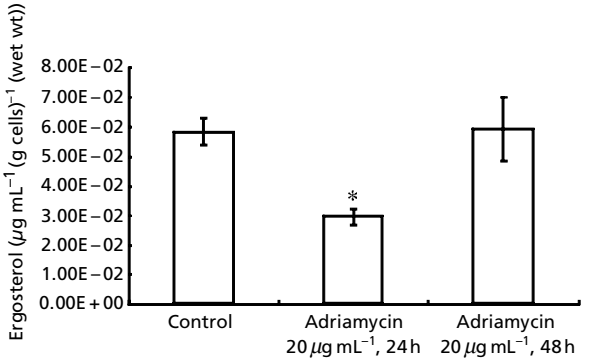

Figure 4 Ergosterol content of *C. albicans* following growth in adriamycin ($20\,\mu g\,mL^{-1}$) for 24 or 48 h. $*P = 0.05$.

a decrease of 49% (significant at $P = 0.05$). Growth of *C. albicans* for 48 h in adriamycin resulted in a cellular ergosterol content similar to the control.

Discussion

The anthracycline antibiotics are one of the most widely used groups of anti-neoplastic agents for the treatment of solid and non-solid malignancies (Pratt & Ruddon 1979; Lowenthal & Eaton 1996). Adriamycin is an anthracycline which functions by inhibiting the action of topoisomerase II (Cummings et al 1991), inducing DNA strand breakage, and kills mammalian cells by inducing apoptosis (programmed cell death) (Skladanowski & Konopa 1993; Huschtscha et al 1995). In addition, it interacts with a phospholipid (cardiolipin) of the mitochondrial membrane leading to an alteration in the permeability of the mitochondrion with a concomitant adverse effect on oxidative phosphorylation (Parker et al 2001).

Fungal infections in cancer patients are a serious, and in some cases life-threatening, problem which arise due to the debilitated state of the patient or as a consequence of immuno-suppression induced by anti-neoplastic therapy (De Pauw 1997). Amphotericn B is one of the anti-fungal agents used for the treatment of systemic fungal infections frequently seen in leukaemic and solid tumour patients (Hartsel & Bolard 1996) and acts by binding to ergosterol in the fungal cell membrane and creating pores through which intracellular constituents escape (Abu Salah 1996).

This study demonstrated that exposure of *C. albicans* to the anti-neoplastic agent adriamycin led to an increase in tolerance to amphotericin B (Figure 1). Pre-exposure to adriamycin resulted in higher cell growth in the presence of amphotericin B (Figure 2). While adriamycin did not affect the viability of *C. albicans* to a significant extent it did reduce oxygen consumption (Figure 3). Cells exposed to adriamycin for 24 h showed a 56% reduction in respiration and demonstrated a reduced amount of ergosterol (Figure 4). Ergosterol biosynthesis requires a functional mitochondrion for the provision of NADPH which is necessary for one of the intermediate stages in its biosynthesis (Daum et al 1998). The depleted ergosterol content of adriamycin-treated cells might have been a consequence of their depressed respiration rate. In contrast, the ergosterol content of cells grown in the presence of adriamycin for 48 h was similar to the control, which reflected the higher respiration rate in these cells relative to that in cells exposed to adriamycin for 24 h.

Exposure of *C. albicans* to adriamycin for 24 h caused a small, but statistically significant, increase in tolerance to amphotericin B, which might have been due to the depleted ergosterol levels as a result of the reduced respiration rate. Adriamycin affects respiration by binding to the phospholipid cardiolipin (diphosphatidylglycerol) – a component of the mitochondrial membrane (Cummings et al 1991; Parker et al 2001). This has the effect of disrupting membrane permeability and inhibiting the action of complex I (Prebble 1981; Das & Mazumdar 2000), which is a critical component of the electron transport chain of oxidative phosphorylation in *C. albicans* (Helmerhorst et al 2002). It is postulated that adriamycin binds to cardiolipin which disrupts the action of complex I leading to a depression in the respiration rate. This leads to depleted levels of NADPH (which is required for ergosterol biosynthesis), reduced levels of ergosterol and an increase in tolerance of amphotericin B. While tolerance of amphotericin B is clinically rare (Van den Bossche et al 1998) it has been observed in cases where ergosterol levels are depleted leaving fewer binding sites for amphotericin B (Kelly et al 1997; White et al 1998; Kontoyiannis & Lewis 2002). Reduced levels of ergosterol have been implicated in fluconazole tolerance in *C. albicans* (Loffler et al 2002). In this case it was postulated that ergosterol deficient mutants had an altered membrane permeability that retarded the entry of fluconazole.

The reappearance of susceptibility to amphotericin B after growth of *C. albicans* in adriamycin for 48 h was accompanied by a simultaneous restoration in the respiration rate and ergosterol content of the cell. The resumption of respiration might have been due to the cells replacing cardiolipin damaged by adriamycin in the initial 24 h. As a consequence, respiration resumed at near normal levels with a concomitant increase in the ergosterol level leading to a restoration in the susceptibility to amphotericin B.

This work demonstrated that adriamycin anti-neoplastic therapy had the potential to increase the tolerance of *C. albicans* for amphotericin B by disrupting respiration, which had an adverse effect on the ergosterol content of the fungal cell membrane. This may have deleterious consequences for cancer patients and could lead to the appearance of amphotericin B-tolerant *C. albicans* infections in patients receiving adriamycin anti-neoplastic therapy.

Conclusion

Short-term exposure (24 h) to adriamycin increased the tolerance of *C. albicans* for amphotericin B, which may be mediated by a decrease in the ergosterol content as a result of an adriamycin-induced disruption of oxidative phosphorylation.

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