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Adriamycin Alters the Expression of Drug Efflux Pumps and Confers Amphotericin B Tolerance in *Candida albicans*

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Abstract. *The aim of the work presented here was to establish whether exposure of the yeast C. albicans to adriamycin altered the expression of the CDR1 drug efflux pump and consequently altered the susceptibility of the yeast to amphotericin B. Using a monoclonal antibody directed against human MDR 1 and polyclonal antibodies against CDR 1 (Candida drug resistance), we demonstrated that adriamycin induces an elevation in the expression of the CDR1 efflux pump which, together with previously recorded alterations in the composition of the fungal cell membrane, may confer tolerance to amphotericin B. This work highlights the fact that adriamycin therapy may inadvertently alter the susceptibility of C. albicans to amphotericin B, which may have deleterious consequences for anti-cancer chemotherapy regimes incorporating this anti-neoplastic agent.*

Cancer is a debilitating, multifactorial disease that attacks indiscriminately across all demographic sectors of society. There are several families of anti-neoplastic agents employed to treat cancer and these are sub-divided on the basis of their mode of action. Adriamycin is a member the anthracyclins, which are non-covalent DNA binding agents (1). 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 (2).

Successful treatment régimes with anti-neoplastic agents such as adriamycin are hampered by inefficient drug delivery to the target site or by resistance to this agent and other structurally and functionally unrelated agents. This has been termed multidrug resistance. Multidrug resistance is more prevalent today due to inadvertent selection for resistance strains and the maintenance of immuno-compromised individuals over prolonged periods of time (3). A common mode of action for the resistant cell in avoiding the effect of cytotoxic agents is the alteration of target sites, and / or, enhanced efflux of drug components from the cell (4). Several membrane pumps have been elucidated including MDR1 and MDR3 (Multidrug Resistance Pump) in humans (5) and CDR 1 (*Candida* Drug Resistance) in the pathogenic yeast *Candida albicans* (6). These membrane bound pumps can play a role in conferring tolerance to anti-neoplastic agents in cancer cells or anti-fungal agents in *C. albicans*.

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 treatments (7,8). The yeast *Candida albicans* is an important opportunistic fungal pathogen, affecting individuals whose immune systems become compromised due to disease, such as leukaemia or anti-neoplastic therapy (9).

In the work presented here, the role of CDR1 expression in increasing the tolerance of *C. albicans* to the anti-fungal agent amphotericin B, when *Candida* cells are pretreated with the anti-neoplastic agent adriamycin, was investigated. It is 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

Culture conditions. C. albicans ATCC (American Type Culture Collection, Virginia, USA) 10231 was grown at 30˚C and 200rpm in an orbital incubator overnight in antibiotic medium 3 (AB3, Oxoid, Ltd., Hampshire, UK) supplemented with 2% (w/v) glucose.

Antifungal and anti-neoplastic agents. Adriamycin was purchased as Doxorubicin (Ebewe Arzneimittel Gmbh., Unterach, Austria). Amphotericin B (Sigma Aldrich Chemical Co., Dublin, Ireland) was dissolved in DMSO and diluted to working concentrations in sterile phosphate-buffered saline (PBS, pH 7.2) (Life Technologies, Paisley, Scotland) prior to use.

Amphotericin B susceptibility testing. Yeast cultures were grown to the stationary phase. Cultures were harvested by centrifugation (2056 x g for 5 min in a Beckmann GS-6 centrifuge). AB3

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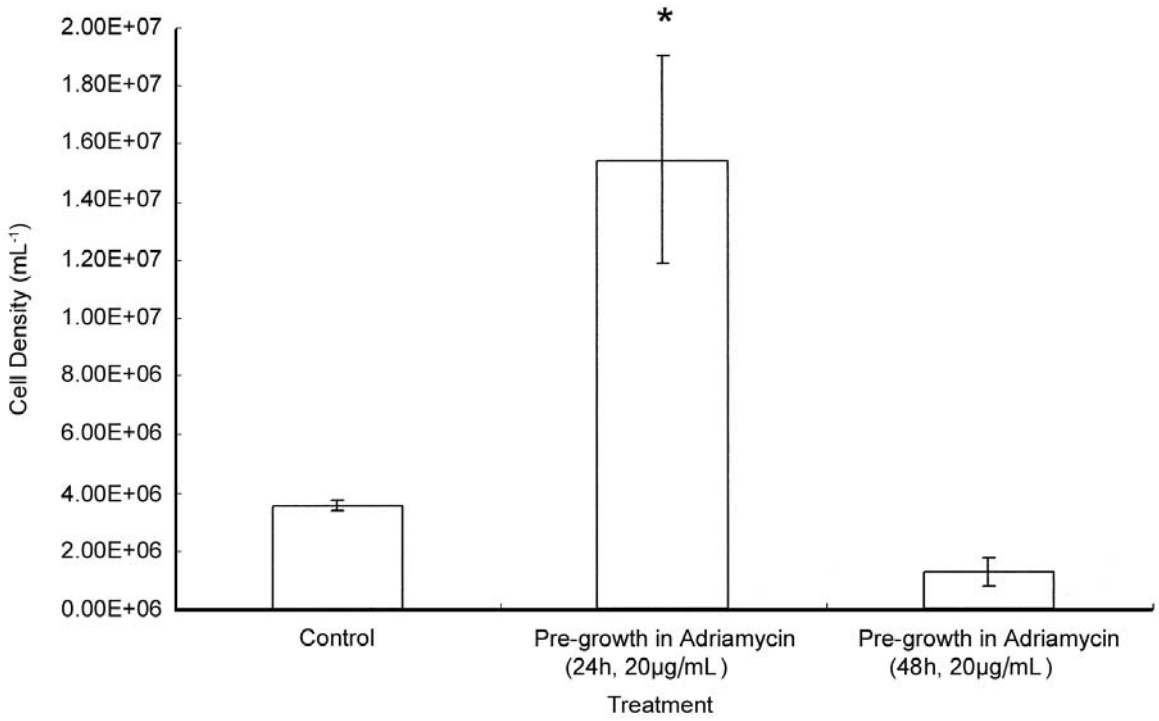

Figure 1. *Growth of C. albicans in AB3 medium supplemented with amphotericin B (0.625 µg/mL) following pre-growth of cells in adriamycin (20 µg/mL) for 24 or 48 hours. Experiments were performed on three independent occasions and the means are expressed±SE.*
** indicates a statistically significant difference relative to the control at p=0.05.*

medium (supplemented with 2% (w/v) glucose and adriamycin (20 µg/mL) was inoculated with *C. albicans* at a density of 5 x 10^5 mL and grown at 37˚C for 24 h or 48 h. Cells were harvested by centrifugation and re - suspended in AB3 containing amphotericin B (0.625 µg/mL) at a density of 5 x 10^5 cells/mL. 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).

Protein extraction. AB3 medium containing either 20 µg/mL adriamycin, or a drug-free control was inoculated with a late exponential phase culture of *C. albicans* ATCC 10231 and incubated in a static 30˚C incubator for 24h. Samples were pelleted at 2056 x g and washed in PBS. To each sample 3g of glass beads (0.45µm) were added along with 4.85ml extraction buffer (100mM Tris, pH 7.5, 1mM EDTA), 25 µl (1M DDT), 12.5 µl (2 mg/mL pepstatin A), 12.5 µl (2 mg/mL Aprotinin), 100µl (100mM PMSF (in methanol)-Sigma Aldrich). Samples were vortexed on ice at full speed for 20 minutes. Protein concentration was determined using the Bradford Protein Assay (10) and samples were concentrated by adding 7 volumes of cold absolute acetone, to a density of 1.25 µg/µL and left at -70˚C for 3h. The acetone was decanted and the samples were re-suspended in 2% SDS (sodium do-deceyl sulphate). Samples were aliquoted and stored at -70˚C.

Western blot analysis. Samples were loaded on a 10% SDS polyacrylamide gel and electrophoresed in a Mini-PROTEAN III electrophoresis cell (Bio-Rad). Proteins were transferred to nitrocellulose membranes by Western blotting using a Trans-Blot Semi-Dry transfer cell (Bio-Rad). Immunodetection on CDR1p was performed as outlined (11). Analysis of the role of CDR was also performed on similar treatments using the human anti *mdr*1 MAb Clone 6/1C. Immunodetection with anti *mdr*1 MAb Clone 6/1C was performed as described in (12). Both signals were developed using the Supersignal chemiluminescent substrate (Pierce). Membranes were exposed to X-ray film (Kodak) overnight for analysis.

Statistical analysis. Confirmation of statistical significance was performed using the SigmaStat Statistical Analysis Package Version 1.00. As a non-parametric alternative, the Kruskal-Wallis test was performed on all data. Experiments were performed on three independent occasions and the means are expressed ±SE.

Results

Amphotericin B is reserved for the treatment of systemic fungal infections in immuno-compromised patients such as cancer patients and those receiving anti-neoplastic therapy (7,8). Consequently, it is not uncommon for patients receiving

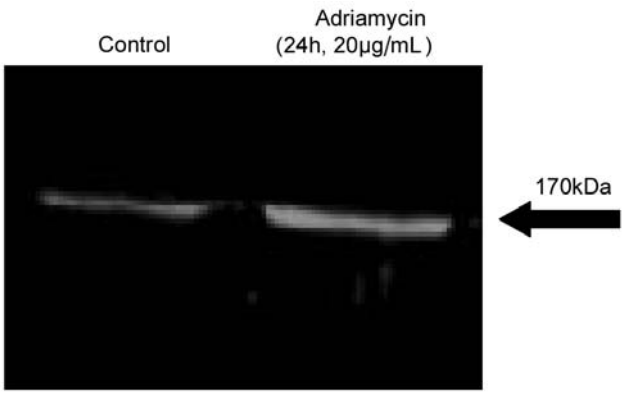

Figure 2. *Western blot using anti-CDR1p showing increased CDR1 expression in C. albicans cells that had been pre-exposed to adriamycin (24h, 20 µg/mL).*

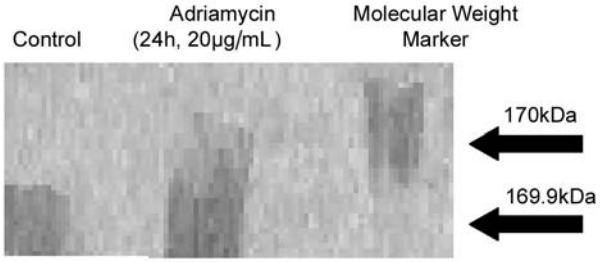

Figure 3. *Western blot using anti-MDR1 MAb Clone 6/1C showing increased CDR1 detection in C. albicans cells that had been pre-exposed to adriamycin (24h, 20 µg/mL).*

adriamycin as part of an anti-neoplastic regime to develop fungal infections that are treated with amphotericin B.

Experiments were performed to determine whether a correlation existed between the enhanced tolerance of *C. albicans* for amphotericin B and cell growth. *C. albicans* was cultivated in AB3 medium supplemented with adriamycin (20 µg/mL) for 24 or 48 hours. Cells were harvested, washed and inoculated into AB3 medium containing amphotericin B (0.625 µg/mL) at an initial cell density of 5 x 10^5 mL. Cultures were grown for 24 hours and the final cell density determined. The cell density attained in the culture that had been pre-grown in adriamycin was 1.55 x 10^7 mL whereas the cell number attained in the control was 3.57 x 10^6 mL (Figure 1). Cultures that had been exposed to adriamycin for 48 hours showed a lower final cell density (1.28 x 10^6 mL) than the control.

Protein extraction was performed on cells exposed to 20 µg/mL adriamycin, or a drug-free control and SDS gel electrophoresis was performed on protein samples that had been concentrated to 15 µg/mL.

Gels were blotted and probed with anti-CDR1p. Following detection with anti-CDR1p, the Western blot showed significantly increased CDR1 detection in the adriamycin pre-treated sample compared to control *C. albicans* cells (Figure 2). The band occurred in the region of 170 kDa, which is the expected size for the CDR1 protein.

Secondary analysis using an alternative antibody (anti-MDR1 MAb Clone 6/1C) was also performed. This antibody is directed against human MDR1, which is 41% homologus with fungal CDR1 (http://www.ncbi.nlm.nih.gov/blast). Adriamycin pre-treated *C. albicans* yielded a band in the region of 169.9 kDa, which is slightly more intense than control cells (Figure 3).

Discussion

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 (8). Amphotericin B is reserved for the treatment of systemic fungal infections frequently seen in leukaemic and solid tumour patients (13). The work presented here demonstrates that exposure of *C. albicans* to the anti-neoplastic agent adriamycin leads to an increase in tolerance to amphotericin B (Figure 1).

There could be several reasons for adriamycin - induced tolerance to amphotericin B in *C. albicans*. The work presented here examined the role of the membrane bound pump in conferring increased tolerance to amphotericin B when *C. albicans* is pre-exposed to the anti-neoplastic agent adriamycin. A comparison of both CDR1 and MDR1 was performed at the protein level using a pairwise BLAST (http://www.ncbi.nlm.nih.gov/blast) program with both sequences sharing an identity of 41%. CDR1 expression appears to be increased in both Western blots (Figures 2 and 3); although more pronounced when the specifically designed anti - CDR1 probe as opposed to the human anti MDR1 MAb Clone 6/1C is used. Alteration in CDR1 expression in *C. albicans* may mediate this short term tolerance to amphotericin B since the effect appears to be lost at 48 hours (Figure 1). Western blot analysis of cultures grown in adriamycin for 48 hours showed CDR1 levels similar to the controls.

The work presented here demonstrates that adriamycin anti-neoplastic therapy has the potential to increase the tolerance of *C. albicans* for amphotericin B by increasing CDR1 expression. 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. This work highlights the need to monitor the occurrence and treatment of fungal infections in cancer patients receiving

adriamycin chemotherapy to ensure that anti-fungal tolerance does not emerge and compromise recovery.

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