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Exposure of *Aspergillus fumigatus* to caspofungin results in the release, and *de novo* biosynthesis, of gliotoxin

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Caspofungin is a member of the echinocandin class of antifungal agents that inhibit the synthesis of β 1,3 glucan thus disrupting fungal cell wall structure and function. Exposure of the *Aspergillus fumigatus* cultures to caspofungin (0.01, 0.1 or 1.0 µg/ml) resulted in a reduction in cell growth, but the production of the epipolythiodioxopiperazine toxin, gliotoxin, was comparable, or greater, in cultures exposed to caspofungin than untreated controls. Exposure of *A. fumigatus* hyphae to 1.0 µg/ml caspofungin for 4 h resulted in the release of amino acids ($P = 0.01$), protein ($P = 0.002$) and gliotoxin ($P = 0.02$). Cultures of *A. fumigatus* incubated in the presence of caspofungin for 4 or 24 h demonstrated enhanced gliotoxin release ($P = 0.04$ and 0.03, respectively) and biosynthesis ($P = 0.04$ and 0.03, respectively) compared to that by control cultures. The results presented here indicate that exposure of *A. fumigatus* to caspofungin results in increased cell permeability and an increase in the synthesis and release of gliotoxin. Since gliotoxin has well established immunosuppressive properties it is possible that exposure of *A. fumigatus* to caspofungin may potentiate the production of this toxin at the site of infection. Elevated gliotoxin biosynthesis may be an attempt by the fungus to restore the redox balance of the cell following exposure to the antifungal agent but the overall effect appears to be enhanced synthesis and release.

Keywords   *Aspergillus*, aspergillosis, caspofungin, gliotoxin, hyphae

Introduction

Caspofungin was the first member of the echinocandins to be licensed for use [1] and shows excellent *in vitro* and *in vivo* activity against *Candida* and *Aspergillus* species [2,3]. Caspofungin has an excellent safety profile and it is as effective as, and usually better tolerated than, liposomal amphotericin B [4]. Unlike polyenes and azoles (that target ergosterol or the ergosterol biosynthetic pathway, respectively) echinocandins function by inhibiting the synthesis of β-1,3-D-glucan, an essential component of the fungal cell wall. The caspofungin-mediated disruption of glucan synthesis leads to the formation of an osmotically fragile aberrant cell wall and osmotic lysis of the cell at high concentrations [1]. Caspofungin contains a long fatty acid side chain that may allow intercalation in the bi-layer of the fungal cell membrane [5] where it may interact with, and disrupt the function of, the β-1,3-D-glucan synthase.

Aspergillus fumigatus is an important fungal pathogen, particularly affecting those with pre-existing pulmonary malfunction (e.g., asthma, cystic fibrosis), disease (e.g., cancer, tuberculosis, chronic granulomatous disease), or undergoing immunosuppressive therapy prior to organ transplantation [6]. The fungus can induce a variety of diseases including allergic and invasive aspergillosis [7]. The latter is the most serious form of disease as it involves the invasion of viable tissue and may produce a mortality rate in excess of 80% [8,9]. Treatment

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can be difficult due to late diagnosis of disease and therapy with amphotericin B can induce a variety of negative side-effects [8].

A. fumigatus displays a number of virulence factors that may facilitate tissue colonization and persistence in the host [10–12]. One of these, gliotoxin ($C_{13}H_{14}N_2O_4S_2$, molecular weight 326.4), is an epipolythiodioxopiperazine toxin [13] which displays immunosuppressive properties *in vivo* [14]. Gliotoxin is capable of inhibiting macrophage function and may alter the immune response to *Aspergillus* as it can induce apoptotic cell death in macrophages [13,15], and disrupt the function of neutrophils [16]. Gliotoxin has been detected in humans where it may facilitate fungal persistence and colonization of tissue [17,18]. In addition, gliotoxin has been implicated in the destruction of lung parenchyma in invasive aspergillosis [15] and the penetration of blood vessels in angio-invasive aspergillosis [19].

We previously established that exposure of *A. fumigatus* to the polyene antifungal amphotericin B leads to membrane leakage, as measured by the release of amino acids and protein, and of the immunosuppressive toxin, gliotoxin [20]. It was established that amphotericin B induced the leakage and *de novo* biosynthesis of gliotoxin – a feature that could have significant implications for patients receiving amphotericin B therapy. Caspofungin has previously been shown to induce amino acid and protein leakage from growth-arrested stationary phase *Candida albicans* cells indicating that inhibition of glucan biosynthesis may not be the only mode of action exhibited by this drug [21]. It was postulated that caspofungin could have a secondary effect in altering membrane permeability. The aim of the work presented here was to establish if exposure of *A. fumigatus* to caspofungin induced leakage of gliotoxin and whether the fungus responded to this by synthesizing elevated levels of toxin intra-cellularly. The immunosuppressive effects of gliotoxin are well established [14–16] and any therapy that inadvertently induced the increased biosynthesis and release of this toxin could exacerbate inflammation and tissue damage at the site of infection.

Materials and methods

Aspergillus fumigatus *culture conditions*

Aspergillus fumigatus ATCC 26933 (obtained from the American Type Culture Collection, Maryland, USA) was used in this study. *Aspergillus* cultures were grown in RPMI1640 (GIBCO) medium supplemented with 5% (v/v) fetal calf serum (FCS, Sigma Aldrich) at 37°C and 200 rpm, for up to 4 days. *A. fumigatus* was maintained on malt extract agar (MEA) plates (Oxoid Ltd).

Preparation of caspofungin

Caspofungin (Cancidas, Merck & Co., Inc.) was dissolved in sterile water to give a stock concentration of 1,000 µg/ml prior to diluting in sterile water to working concentrations. Stock solutions were stored in 50 µl aliquots at − 80°C.

Effect of caspofungin on growth of A. fumigatus

MEA plates containing sporulating *Aspergillus* colonies were washed with 10 ml of 0.01% (v/v) Tween 80 (Merck) in Phosphate Buffered Saline (PBS, pH 7.2; Sigma Aldrich) to isolate conidia. The latter were washed twice in sterile PBS, harvested by centrifugation (1,500 *g*, 5 min in a Beckman GS-6 centrifuge) and enumerated using an haemocytometer. Sterile flasks containing RPMI (50 ml) were inoculated with *Aspergillus* conidia to give a concentration of 1×10^5 /ml and incubated at 37°C and 200 rpm. Cultures were supplemented with caspofungin (0.01, 0.1 or 1.0 µg/ml) at various times. It was previously determined that a concentration of 0.1 µg/ml caspofungin inhibited growth of *A. fumigatus* by approximately 50% (data not provided). Flasks were removed at each time point and the contents filtered through Whatman No. 1 filters in a Büchner funnel. A growth curve was constructed of fungal biomass versus incubation time.

Evaluation of amino acid and protein leakage from A. fumigatus *exposed to caspofungin*

Aspergillus fumigatus conidia were inoculated into 50 ml of RPMI to give a final concentration of 1×10^5 conidia/ml and incubated at 37°C for 96 h. Hyphae were collected by filtering through miracloth (Calbiochem) and washed twice with 10 ml sterile PBS. The hyphal mass (2 g) was re-suspended in 25 ml PBS containing 0.5% v/v Dimethyl sulfoxide (DMSO) (as positive control), or caspofungin (0.1 µg/ml or 1.0 µg/ml) and the cultures were incubated at 37°C and 200 rpm for another 0.5, 2 or 4 h. At each time point 750 µl of culture filtrate was removed and filtered through 0.45 µM syringe filters (Sarstedt). Free amino acids were measured using the ninhydrin colorimetric method [22] and expressed in terms of aspartic acid and glutamic acid, which were used as standards. To determine the quantity of protein released from the hyphal mass, samples were assayed using the Bradford assay (Bio-Rad), with BSA (Sigma Aldrich) as standard.

Extraction of gliotoxin from A. fumigatus *culture filtrate*

At each time point the culture contents were filtered through miracloth, weights were recorded and gliotoxin was

extracted from the culture filtrate by mixing with an equal amount of chloroform (Sigma-Aldrich) for 2 h. The extraction of gliotoxin from the chloroform layer was accomplished by rotary evaporation at 55°C. The dried extracts were dissolved in 250 µl of molecular-grade methanol (Sigma-Aldrich) and the level of gliotoxin determined by Reverse phase high performance liquid chromatography (RP-HPLC) [20].

Extraction of gliotoxin from hyphae of A. fumigatus

Intracellular gliotoxin was extracted from the hyphae of *A. fumigatus* by first recovering hyphae from RPMI culture media by filtration. The hyphal mass was then washed twice in PBS and ground to a fine powder using a pestle and mortar under liquid nitrogen. The crushed hyphal powders were re-suspended in 10 ml of 6 M HCl (Sigma-Aldrich). Chloroform (35 ml) was added and the mixture was stirred at room temperature for 2 h. The chloroform layer was separated and gliotoxin was extracted by evaporation of chloroform using a rotary evaporator at 55°C. The dried extracts were dissolved in 250 µl of molecular-grade methanol and the level of gliotoxin determined using RP-HPLC.

Quantification of gliotoxin by RP-HPLC

Gliotoxin was detected by Reversed Phase-HPLC (Spectra-Physics). The mobile phase was 34.9% (v/v)

acetonitrile (Hyper Solv, BDH), 0.1% (v/v) trifluoroacetic acid (Sigma Aldrich) and 65% (v/v) deionized-distilled water. Gliotoxin extract (10 ml) was injected onto a C18 Hewlett Packard column. A standard curve of peak area versus gliotoxin concentration was constructed using gliotoxin standards (0.1, 0.2, 0.5, 1.0 µg/10 µl) dissolved in methanol (Sigma Aldrich).

Statistical analysis

All assays were performed on three independent occasions. Results presented are the mean ± standard deviation. Statistical analysis were performed using Student's two-tailed *t*-test with values of $P \leq 0.05$ considered statistically significant and indicated with *.

Results

Effect of caspofungin on growth and gliotoxin production by A. fumigatus

Cultures of *A. fumigatus* were grown in RPMI 1640 medium supplemented with 0.01, 0.1 or 1.0 µg/ml caspofungin for 96 h. The biomass and gliotoxin concentration (µg/g hyphae) at different time points were determined. The results demonstrate that incubation of the fungus in the presence of caspofungin retarded hyphal growth (Fig. 1A). At 72 h, the control culture reached a hyphal
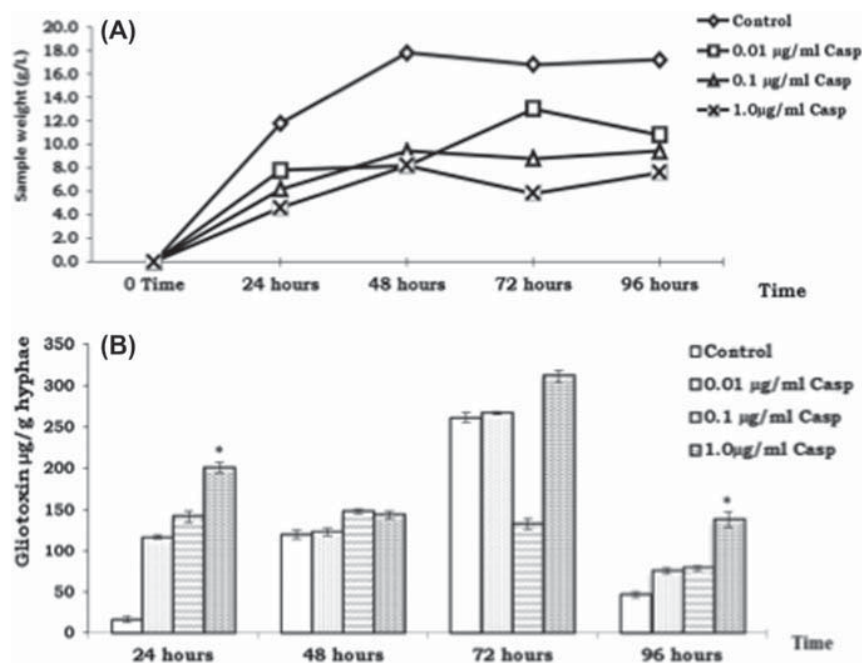

Fig. 1 (A) and (B) Effect of caspofungin on growth and gliotoxin production by *Aspergillus fumigatus*. Cultures of *A. fumigatus* were grown in the presence of caspofungin (0.01, 0.1 or 1.0 µg/ml) for 96 hours and fungal biomass (A) and gliotoxin production (µg/g hyphae) (B) was determined at 24-hour intervals. *$P = 0.01$.

mass of 17.8 g/l, whereas the culture supplemented with 0.01 µg/ml caspofungin had achieved a mass of 13 g/l. Cultures supplemented with 0.1 µg/ml caspofungin achieved a weight of 8.2 g/l at the same time point, while those cultures supplemented with caspofungin at a concentration of 1.0 µg/ml reached a hyphal mass of 5.8 g/l.

Analysis of the gliotoxin concentration in the culture medium revealed that peak levels were attained at 72 h and declined thereafter (Fig. 1B). The highest concentration of gliotoxin was found in the culture supplemented with 1.0 µg/ml caspofungin (311.71 µg/g of hyphae) at 72 h. The gliotoxin concentration of the control culture was 261.78 µg/g hyphae at this time (Fig. 1B). It is clear from these results that while exposure to caspofungin inhibited fungal growth, the level of gliotoxin production, as a function of hyphal weight, was similar to, or greater than, that produced by the control.

Effect of caspofungin on release of amino acids and protein from A. fumigatus

Cultures of *A. fumigatus* were grown at 37°C for 96 h, harvested, washed and resuspended in PBS supplemented with caspofungin (0.1 or 1.0 µg/ml) or 0.5% DMSO, as the positive control due to its well known ability to solubilize membranes. Cultures were incubated at 37°C for 4 h and the release of amino acids and protein was

quantified as described above. Cultures exposed to 1.0 µg/ml caspofungin were found to have an elevated release of amino acids compared to negative and positive controls at 2 and 4 h ($P = 0.01$; Fig. 2A). A similar trend was noted in protein release from the hyphae in caspofungin supplemented especially after 4 h exposure of hyphae to the drug where the greatest level of protein release was from the hyphae exposed to 1.0 µg/ml caspofungin ($P = 0.002$; Fig. 2B). These results demonstrate the increased leakage of amino acids and protein from *A. fumigatus* hyphae when exposed to caspofungin.

Effect of caspofungin on release of gliotoxin from A. fumigatus

Cultures of *A. fumigatus* were grown at 37°C for 96 h, harvested, washed and resuspended in PBS supplemented with caspofungin (0.1 or 1.0 µg/ml) or 0.5% DMSO. The release of gliotoxin was quantified by RP-HPLC after 4h exposure to the drug or DMSO. The results indicate that the culture supplemented with 1.0 µg/ml caspofungin released a significantly greater amount of gliotoxin (15.56 ± 0.52 µg/ml) than the untreated control (5.86 ± 0.3 µg/ml) ($P = 0.02$; Fig. 3). Gliotoxin leakage from DMSO treated cells was also evident (11.05 ± 1.45 µg/ml). Exposure of stationary phase *A. fumigatus* hyphae leads to increased leakage of gliotoxin.
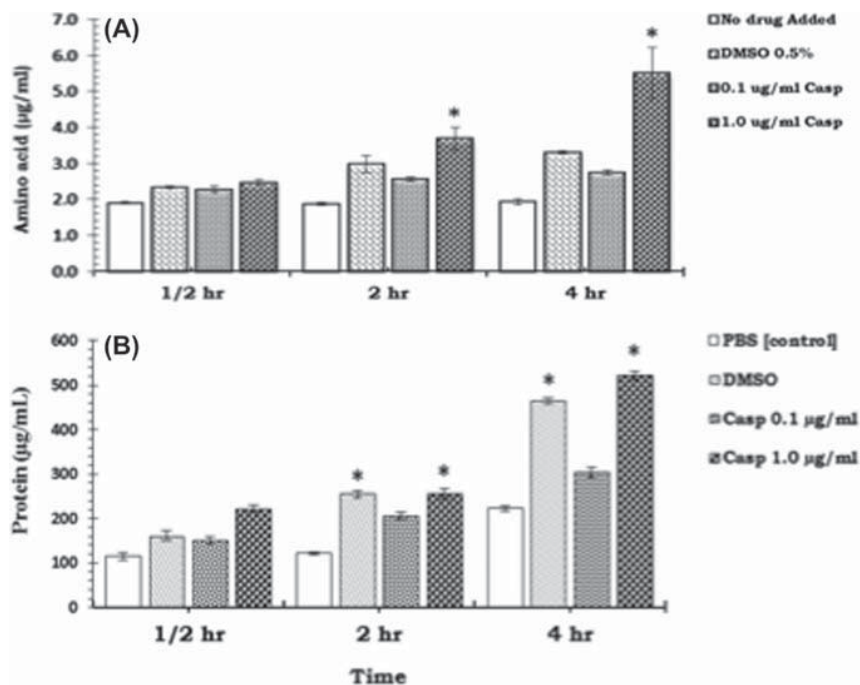

Fig. 2 (A) and (B) Caspofungin exposure induces leakage of amino acids and protein from *Aspergillus fumigatus*. The release of amino acid (A) and protein (B) from 96 h old cultures of *A. fumigatus* following exposure of hyphae to two different concentrations of caspofungin for 4 hour in PBS was measured. Statistically significant difference compared to control indicated by $*P < 0.05$.
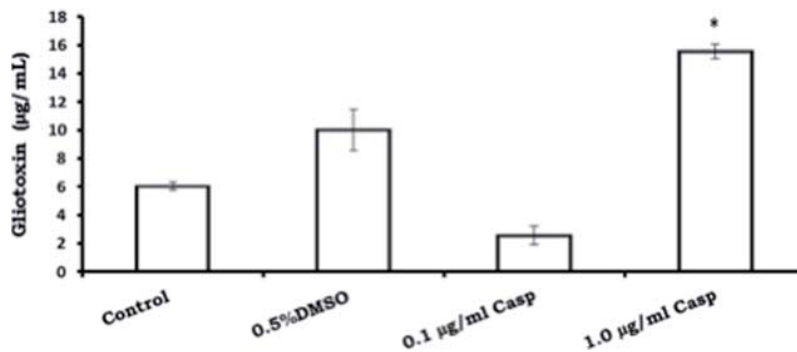
Fig. 3 Effect of caspofungin on release of gliotoxin from *Aspergillus fumigatus* following 4 hour exposure to the drug. The level of gliotoxin released by *A. fumigatus* after 4 h exposure to 0.1 or 1.0 µg/ml caspofungin was measured. $*P = 0.02$.

Short-term (4 h) exposure to caspofungin potentiates gliotoxin production and release by A. fumigatus

Cultures of *A. fumigatus* were grown for 96 h in RPMI medium at 37°C. Cultures were supplemented with caspofungin (0.01, 0.1 or 1.0 µg/ml) for 4 h. After this time hyphae were harvested, washed, and resuspended in the same volume of fresh RPMI medium as previously. The concentration of gliotoxin in the culture medium and within the hyphae (intracellular) was assessed for up to 48 h after the short-term (4 h) exposure to caspofungin. Cultures exposed to 1.0 µg/ml caspofungin had significantly higher gliotoxin concentrations in the medium at 24 h (7.94 ± 1.88 µg/ml) and 48 h (11.19 ± 0.57 µg/ml) post-exposure to caspofungin compared to the relevant control ($P = 0.04$; Fig. 4A).

Internal concentrations of gliotoxin were also measured, as described, 48 h post-exposure to caspofungin (Fig. 4B). Internal gliotoxin levels were significantly higher (224.8 µg/g of hyphae) in the cultures that had been exposed to 1 µg/ml caspofungin for 4 h compared to the control ($P = 0.04$). These results reveal that short-term

(4 h) exposure to caspofungin has the ability to potentiate the production of gliotoxin when cells are placed in fresh medium.

Exposure of cultures of A. fumigatus to caspofungin for 24 hours leads to increased internal and external concentrations of gliotoxin

Cultures of *A. fumigatus* were grown in RPMI medium for 96 h at 37°C at which time caspofungin was added to cultures to give final concentrations of 0.1 or 1.0 µg/ml. The cultures were then incubated for a further 24 h in RPMI medium at which time the internal and external gliotoxin concentrations were ascertained as described. The results reveal an increase in the external gliotoxin concentration in cultures that were supplemented with caspofungin. The culture supplemented with 1.0 µg/ml caspofungin had a significantly higher level of gliotoxin in culture medium at 8 h (8.29 ± 0.08 µg/ml), 16 h (6.09 ± 0.5 µg/ml) and 24 h (6.78 ± 0.29 µg/ml) (Fig. 5A) compared to the relevant controls ($P = 0.03$). In addition, the intracellular concentration of gliotoxin at 24 h also increased in the cultures
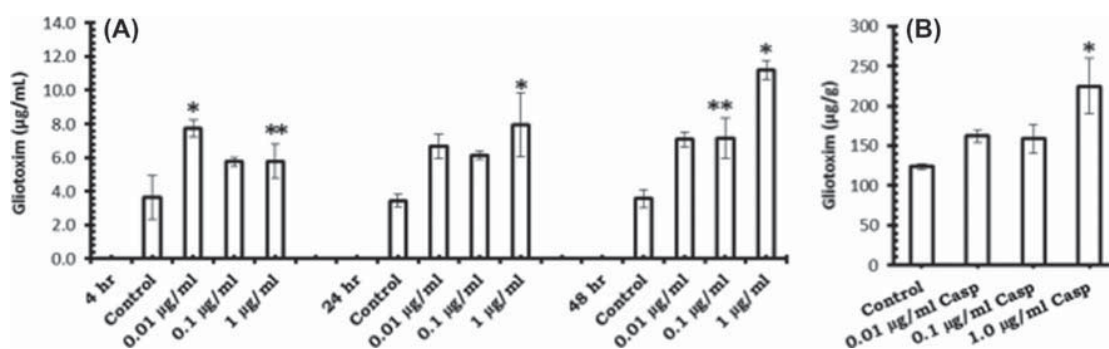

Fig. 4 Effect of short-term caspofungin exposure (4 hour) on gliotoxin release and biosynthesis in *Aspergillus fumigatus*. Extracellular gliotoxin concentration (A) and internal concentration of gliotoxin (B) in *A. fumigatus* 4, 24 and 48 hours after exposure of hyphae to caspofungin for 4 hours was measured. The intracellular concentration of gliotoxin was measured at 48 hours. Statistically significant ($P < 0.05$) difference compared to control indicated by $*P = 0.04$. ** indicates $P = 0.023$).
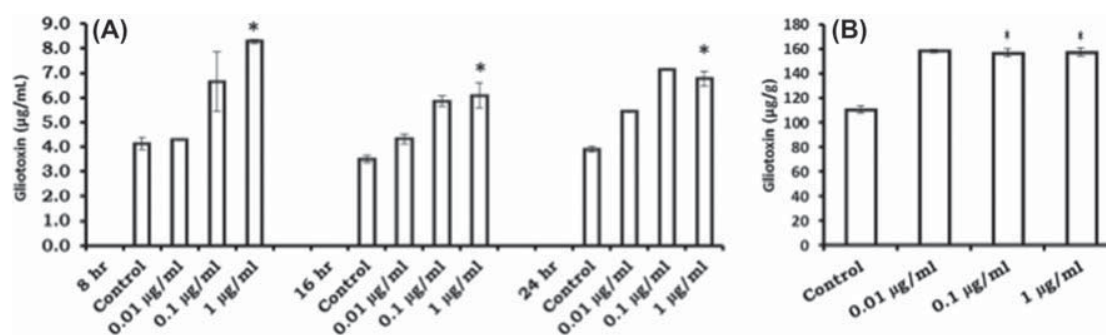
Fig. 5   Effect of long-term caspofungin exposure (24 hour) on gliotoxin release and biosynthesis in *Aspergillus fumigatus*. Extracellular and intracellular gliotoxin levels in 96-hour-old cultures after exposure to caspofungin for 24 hours. Gliotoxin release was measured at 8, 16 and 24 hours after addition of caspofungin to cultures (A). The intracellular concentration of gliotoxin was measured at 24 hours (B). Statistically significant difference compared to control indicated by $*P = 0.03$.

supplemented with 0.1 or 1.0 µg/ml caspofungin relative to the control ($P = 0.03$; Fig. 5B). These results indicate that incubation of stationary phase cultures of *A. fumigatus* with caspofungin for 24 h leads to increased extracellular and intracellular levels of gliotoxin.

Discussion

Caspofungin is a highly effective antifungal agent with proven efficacy against a wide range of fungal pathogens [2]. It is well tolerated *in vivo* and no significant instances of clinical resistance have been documented [2,23]. Its low toxicity makes it a more attractive agent for antifungal therapy than amphotericin B which has well characterized toxic side-effects. The results presented here demonstrate that exposure of *A. fumigatus* to caspofungin results in increased membrane permeability as measured by the elevated release of amino acids, protein and gliotoxin. In addition, there are greater levels of gliotoxin within cells that are exposed to caspofungin for a short (4 h) or long (24 h) period. Exposure of *A. fumigatus* to caspofungin leads to enhanced internal and external gliotoxin concentrations up to 48 h after the beginning of the exposure period (Fig. 4A and 4B). Interestingly exposure of growing cultures of *A. fumigatus* to caspofungin results in reduced biomass but the production of gliotoxin is comparable to that in control cultures (Fig. 1A and 1B).

While gliotoxin has well established cytotoxic properties [24], its primary function may be to act as an antioxidant within the fungal cell and so protect it from the effects of exogenous and endogenous oxidants [25]. We have previously demonstrated the activation of the Cap and Hog pathways in *C. albicans* when exposed to caspofungin indicating the drug induces oxidative and osmotic stress within the cells [26]. The increased level of gliotoxin within *A. fumigatus* following exposure to caspofungin as evident by this study may be an attempt by the cell to restore the redox balance disrupted by exposure to caspofungin, or by the loss of toxin from within the cell. While gliotoxin may represent a means by which *A. fumigatus* regulates its environment, the net effect of increased biosynthesis and release following exposure to caspofungin is the possibility of enhanced tissue damage and inflammation at the site of infection.

We previously established that exposure of growth arrested stationary phase *C. albicans* cell to caspofungin induced the release of amino acids and a variety of intracellular peptides [21]. Caspofungin inhibits glucan biosynthesis which results in disruption of fungal cell wall synthesis and ultimately cell lysis when high concentrations are employed [1]. In the results presented here, and in those of the earlier work [21], stationary phase cultures of *A. fumigatus* or *C. albicans* were exposed to caspofungin so *de novo* wall formation would not have been a significant. Consequently, it is possible that in addition to inhibiting glucan biosynthesis, caspofungin may have a secondary effect on membrane permeability and thus facilitate the leakage of amino acids, protein and toxins from fungal cells. Depletion of the intracellular gliotoxin concentration may stimulate increased biosynthesis of the toxin to replenish the lost gliotoxin in order to restore the redox balance within the cell [25]. This phenomenon of caspofungin-induced gliotoxin leakage and *de novo* biosynthesis has the potential to contribute to elevated levels of pulmonary damage and immunosuppression [16,24]. It is possible that administration of caspofungin to patients may lead to eventual fungal cell death but that before this is achieved internal gliotoxin would be released from cells and the cells could have the opportunity to commence synthesizing more toxin to replenish that which is lost into surrounding tissue. Previous work that examined the response of *C. albicans* to amphotericin B illustrated the ability of cells to recover from antifungal therapy even though they appeared incapable of replicating in culture

thus highlighting the possibility of continued cell survival in the presence of 'fungicidal' concentrations of drug [27]. This finding illustrates the possibility of continued gliotoxin biosynthesis in the presence of caspofungin concentrations that would inhibit fungal cell growth.

Acknowledgements

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