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Exposure of Aspergillus fumigatus to caspofungin induces an oxidative stress response.

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Abbreviations
DMSO: Dimethyl sulfoxide. GLR: glutathione reductase. IA: Invasive aspergillosis. PBS: Phosphate buffered saline. SOD: superoxide dismutase

Declaration: This work was conducted independently of any pharmaceutical company with interests in anti-fungal drug development or marketing.

ABSTRACT

Caspofungin is widely used for the treatment of systemic fungal infections and exerts its effect by disrupting β-glucan biosynthesis. The aim of the work presented here was to establish how Aspergillus fumigatus reacted following exposure to capsofungin. Exposure of A. fumigatus to 0.1 or 1.0 µg/ml caspofungin for 4 hours resulted in protein leakage from hyphae and the increased expression of a variety of proteins involved in the oxidative stress response (e.g. catalase, antibiotic response protein), virulence (e.g. Asp F3, 18kDa antigen) and homeostasis (e.g. glyceraldehyde 3-phosphate, translation elongation factor, ATP synthase F1). In addition there was an increase in the activity of catalase, glutathione reductase and superoxide dismutase in hyphae exposed to 0.1 µg/ml caspofungin for 1 hour. The results presented here indicate that when A. fumigatus is exposed to caspofungin there is increased expression of a number of proteins associated with virulence and the oxidative stress response, and the activity of detoxifying enzymes possibly in an attempt to circumvent the intracellular effects of the antifungal agent.

1. INTRODUCTION:

Aspergillus fumigatus is a significant cause of morbidity and mortality in those with pre-existing disease (e.g. leukaemia, chromic granulomatous disease) or pulmonary malfunction (e.g. asthma, cystic fibrosis) (Vonberg & Gastmeier, 2006). The fungus can induce a variety of diseases and invasive aspergillosis can produce a mortality rate in excess of 85% (Brakage & Langfelder, 2002; Rementeria *et al.,* 2005; Singh *et al*., 2009). Conventional treatment for aspergillosis has relied upon the use of polyene (e.g. amphotericin B, liposomal amp B) and azole (e.g. itraconazole, voriconazole) anti-fungal agents but the cost and toxicity of some of these drugs together with the emergence of resistance to some azoles (Verweij *et al*., 2009), prompted the search for an effective but non-toxic antifungal for the treatment of systemic fungal infections such as aspergillosis.

Caspofungin is a member of the echinocandin group of antifungal agents and was the first of this group to be licenced for clinical use (Deresinski & Stevens, 2003). Caspofungin is widely used and is highly effective against a range of fungal pathogens including *A. fumigatus* (Maertens *et al*., 2004; Sarria *et al*., 2004; Betts *et al.*, 2006; Walsh *et al*., 2004; Abruzzo *et al*., 2000; Petraitiene *et al.*, 2000). The echinocandins function by inhibiting the activity of β-1,3-D glucan synthase and, at high concentrations, induce the formation of an osmotically sensitive cell wall and cell lysis (Denning, 2003). Caspofungin contains a long fatty acid side chain that may enable intercalation in the lipid bilayer of the fungal cell membrane where it may disrupt the activity of the β-1,3 D-glucan synthase (Denning, 2003). In response to the caspofungin-induced inhibition of glucan synthesis, *C. albicans* has been shown to increase the chitin content of cell walls (Walker *et al.,* 2008; Stevens *et al.,* 2006). The PKC pathway, which controls the expression of chitin synthase genes, was activated when *Saccharomyces cerevisiae* was exposed to caspofungin thus suggesting that cell wall remodeling occurs in response to this agent (Reinoso-Martin *et al.,* 2003). Exposure to sub-inhibitory concentration of caspofungin also leads to the unmasking of β-glucan in the cell wall of *C. albicans* (Wheeler *et al*., 2006, 2008) which may provoke an enhanced immune response and possibly lead to increased cell-mediated killing of the fungus

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in the body. Analysis of the proteomic changes that occur in *A. fumigatus* following exposure to caspofungin revealed decreased expression of proteins associated with the mitochondrial hypoxia response (Cagas et al., 2011). In addition decreased expression of Asp F1 was also observed. While echinocandins are effective antifungal agents (Walsh *et al.,* 2004), they also demonstrate negligible toxicity to humans (Denning, 2003) and no cases of clinical resistance have been detected.

The aim of the work presented here was to analyse the response of *A. fumigatus* hyphae to caspofungin in order to determine whether the fungus could mount a protective response against the effects of this antifungal agent.

MATERIALS AND METHODS

Aspergillus fumigatus culture conditions.

Stocks of *A. fumigatus* ATCC 26933 were grown on malt extract agar (Oxoid Ltd) plates at 37 °C and maintained on the same medium at 4°C for short term storage. *A. fumigatus* liquid cultures were grown in RPMI 1640 (GIBCO) medium supplemented with 5% (v/v) foetal bovine serum (FBS, Sigma Aldrich) at 37°C and 200 rpm, for up to 4 days.

CASPOFUNGIN

Caspofungin (Cancidas™, Merck & Co., Inc.) was dissolved in sterile water to give a stock concentration of 1000 µg/ml prior to diluting in sterile water to working concentrations. Stock solutions were stored in 50 µl aliquots at -80C. Drug concentrations of 0.1 or 1.0 µg/ml caspofungin were chosen as the test concentrations (Eshwika et al., 2012).

EVALUATION OF PROTEIN RELEASE.

MEA plates containing sporulating *A. fumigatus* 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. Isolated conidia were resuspended in 10 ml PBS and inoculated into 50 ml of RPMI plus 5% (v/v) FBS medium to give a final concentration of 1 $x10^5$ conidia/ml and incubated at 37°C for 96 hours. Following incubation hyphae were harvested by partial filtration using Mira-cloth and were washed three times with sterile PBS. Hyphae (1.5g) were re-suspended in either PBS (5 ml), DMSO (10% (v/v); 5 ml) or caspofungin (0.1 or 1.0 µg/ml; 5 ml) and incubated at 37 °C for 240 minutes. To determine the quantity of protein released from the hyphal mass supernatants at 0, 30, 60, 120, 180 and 240 minutes were assayed for their protein content using a Bradford assay (Bio-Rad), with BSA (Sigma Aldrich) as standard.

EXTRACTION OF PROTEINS FROM HYPHAE

Cultures were grown for 96 hours at 37°C, hyphae were harvested as described and washed with PBS. Hyphae (1.5g) were exposed to caspofungin (0.1 or 1.0 µg/ml) for 4 hours. Hyphae were harvested, washed with PBS and ground to a fine powder using a pestle and mortar by temporary freezing in liquid nitrogen. Protein extraction buffer (4 mls, 0.4 M NaCl, 10mM Tris HCl, 2 mM EDTA) was added. All samples were centrifuged at 1500 g, 4°C for 5 minutes. The supernatant was retained and the pellets were discarded. Protein was precipitated from the supernatant and retained.

PROTEIN ELECTROPHORESIS

For two-dimensional SDS-PAGE analysis, precipitated proteins were re-suspended in IEF buffer (250µl) (8 M urea (Sigma-Aldrich), 1% (v/v) triton X-100 (Sigma-Aldrich), 4% (w/v) CHAPS (Sigma-Aldrich), 10 mM Tris-HCl, 2 M thiourea (Sigma-Aldrich), 65 mM dithiothreitol (Sigma-Aldrich), 0.8% (v/v) IPG buffer along with a few grains of molecular-grade bromophenol blue. The solution was applied to a 13 cm (pH 3 – 10) Immobiline™ DryStrip (G.E. Healthcare) and iso-electric focusing was performed using an Ettan IPGphor II (Amersham Biosciences, NJ, USA). Following iso-electric focusing, strips were equilibrated in reducing buffer (10 mls) (50 mM Tris-HCl, 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 2% (w/v) DTT, pH 6.8) for 15 minutes at room temperature, followed by equilibration in alkylation buffer (50 mM Tris-HCl, 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 2.5% (w/v) iodoacetamide (IAA; Sigma-Aldrich) and trace bromophenol blue, pH 6.8) for 15 minutes. Strips were placed on top of homogeneous 12.5% SDS-PAGE gels. Strips were overlaid with hand-warm sealing solution (1% (w/v) agarose (Sigma-Aldrich); 0.5% (w/v) bromophenol blue; 2.5 mM Tris-HCl, 25 mM glycine (Sigma-Aldrich), 0.01% (w/v) SDS). Gels were electrophoresed on a PROTEAN PLUS Dodeca cell system (Bio-Rad) with temperature maintained at 6°C using a cooling system, for 20 hours at 100 Volts and stained with Coomassie blue stain. Protein spots were located and analysed with 'Progenesis SameSpots™' software by Nonlinear Dynamics

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in order to quantify the fold change between treatments. All gels were performed in triplicate on independent occasions and the average fold change in protein intensity was calculated relative to the expression in the control (i.e. PBS treated sample).

LC-MS ANALYSIS

In gel digestion of peptides was performed according to the method of Schevchenko *et al.,* (2006). The protein spot of interest was excised and washed for approximately 60 minutes in 150 µl 100 mM ammonium bicarbonate/acetonitrile (1:1 v:v). Following removal of the wash buffer gel pieces were incubated in 500 µl acetonitrile until the gel pieces became white and reduced in size. Following removal of acetonitrile the excised spots were digested overnight in trypsin (13 ng/µl). Peptides were extracted and vacuum dried prior to LC/MS analysis on an Agilent 6340 Ion Trap. Resulting data were analysed using the mascot search engine, (www.matrixscience.com). The mass error tolerance was 1 Da allowing for a maximum of no more than 2 missed cleavages. Verification of protein sequences was confirmed by blasting the protein sequence on the Uniprot, (www.uniprot.org), and NCBI, (www.ncbi.nlm.nih.gov), websites. Mascot score values greater than 68 were considered significant at $p < 0.05$.

ASSESSMENT OF ENZYMATIC ACTIVITY

A. fumigatus hyphae (96 hr culture, 1 g) were exposed to caspofungin (0.1 µg/ml) for 15, 30 or 60 minutes or to hydrogen peroxide (0.5 mM) for 15 minutes. At each time point hyphae were harvested, washed in PBS and resuspended in 8 ml of lysing buffer (4 ml Tris-HCl (100 mM, pH 7.5); 4 ml EDTA (1mM, Sigma-Aldrich); 100 µl pepstatin A (1 mg/ml), 100 µl aprotinin (1 mg/ml), 100 µl PMSF (1 mM) and 5 mM DTT). Cells were disrupted by vigorous vortexing on ice with glass beads (4g, size: 425-600 µm, Sigma-Aldrich). Cellular debris and glass beads were removed by centrifugation (250 x g for 5 minutes at 4°C, Eppendorf centrifuge 5417R) and the supernatant was used for evaluation of enzymatic activity.

Catalase activity was measured as described (Larsen & White, 1995) with slight modifications as detailed previously (Kelly *et al.,* 2009). The absorbance at 240 nm was obtained on a Beckman DU640 spectrophotometer. A blank consisted of 17 mM H_2O_2.

For analysis of the glutathione reductase (GLR) activity of cells the method described previously (Foster & Hess, 1980) was used. The absorbance was read at 340 nm for 2 minutes at 20 second intervals (Cary varian UV-Visible Spectrophotometer). The GLR activity was calculated using the following equation:

$$\text{GLR (units/µl)} = \frac{\text{(Rate of change of sample – Rate of change of blank)}}{\text{6.22 mM/cm X Concentration of protein (µg/µl)}}$$

Superoxide dismutase (SOD) activity was measured using a SOD Assay Kit (Fluka Biochemika) in accordance with the manufacturer's instructions, using a concentration of 1 µg/µl of protein extract as described (Foster & Hess, 1980). The absorbance at 450 nm was read using a microplate reader (Synergy HT, Bio-Tek).

STATISTICAL ANALYSIS

All assays were preformed on three independent occasions. Results are expressed as the mean ± SE and were compared by *t* test using Sigma Stat Statistical analysis Package Version 1.00 (SPSS Inc, Chicago, IL, USA). Differences were considered significant at $p \leq 0.05$. In the case of the 2-Dimensional gels, representative examples are presented in Figure 2.

RESULTS.

EXPOSURE TO CASPOFUNGIN INDUCES PROTEIN RELEASE FROM A. FUMIGATUS.

A. fumigatus hyphae were exposed to caspofungin (0.1 or 1.0 ɥ g/ml) for 240 minutes and the quantity of protein released was evaluated as described. Protein was released from hyphae throughout the incubation period but the greatest release occurred from hyphae exposed to 1.0 ɥ g/ml caspofungin for 240 mins (593±14.2 µg/ml) ($p < 0.05$) (Figure 1). Protein was also released from hyphae exposed to 0.1ɥ g/ml caspofungin (473±16 ɥ g/ml at 240 minutes) and the level of release was comparable to that induced by exposure to DMSO (423±17 ɥ g/ml, 240 minutes) which was employed as a positive control (Reeves *et al.,* 2004).

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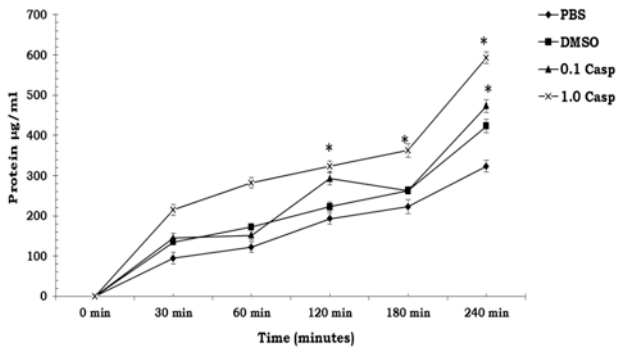

Figure 1: Exposure to caspofungin induces the release of protein from *A. fumigatus* hyphae.
A. fumigatus hyphae were exposed to 0.1 or 1.0 µg/ml caspofungin for 4 hours and the amount of protein released at 30 minute intervals was measured by Biorad assay as described. * indicates p <0.05 relative to the control (PBS).

EFFECT OF CASPOFUNGIN ON INTRACELLULAR PROTEIN EXPRESSION IN A. FUMIGATUS

A. fumigatus hyphae were harvested, washed, exposed to 0.1 or 1.0 µg/ml caspofungin (for 4 hours), and the internal proteome was extracted and resolved by 2D SDS-PAGE as described (Figure2). Proteins showing alteration in intensity were excised and identified by LC/MS. A wide range of proteins demonstrated altered expression following exposure of *A. fumigatus* to caspofungin (Table 1). Strong identities were recorded for nineteen proteins. A range of proteins showed homology to proteins involved in stress responses (e.g. spot B18 (catalase), spot B16 (Vacuolar protease A), spot B19 (Hsp70), spot B2 (Cip-like antibiotic response protein), and spot B12 (thioredoxin reductase)). A number of spots showed homology to proteins associated with the virulence of *A. fumigatus* (e.g. spot B1 (18kDa antigen), spot B3 (allergen Asp F3)), and with cell homeostasis (e.g. spot B7 (glyceraldehyde 3-phosphate), spot B9 (translation elongation factor), and spot B17 (ATP synthase F1)).

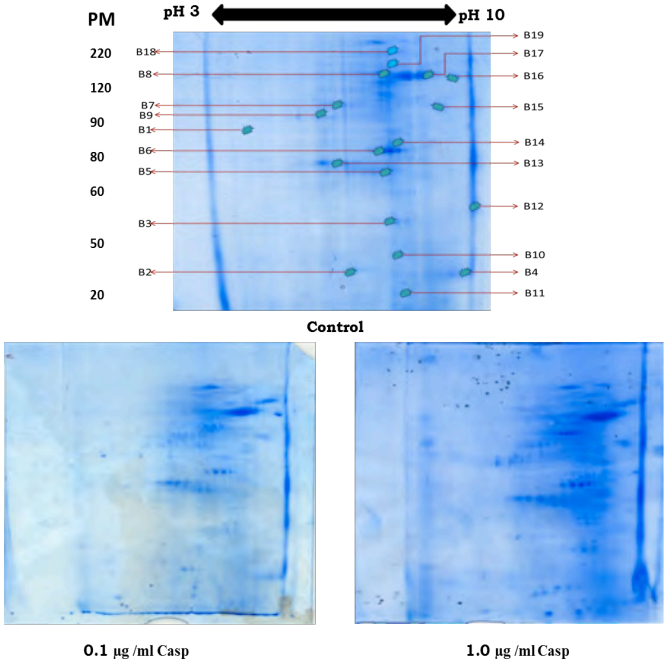

Figure 2: Representative two-dimensional SDS-PAGE of protein extracted from *A. fumigatus* hyphae exposed to caspofungin for 4 hours.
Visualisation by 2-D SDS-PAGE of proteins extracted from *A. fumigatus* hyphae following exposure 0.1 or 1.0 µg/ml caspofungin for 4 hours.

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Table 1.

Spot no.	Protein name	Score	Seq. cover	Accession No.	Source	Function	Fold Change		
							Control	0.1 Casp	1.0 Casp
B1	18kDa antigen	84	11%	CAA41217	*A. fumigatus*	Ribonuclease	1.0	1.8	1.5
B2	CipC-like antibiotic response protein	270	40%	XP_753706	*A. fumigatus*	Antibiotic response protein	1.0	4.7	0.8
B3	Asp F3	278	48%	XP_747849	*A. fumigatus*	Oxidoreductase activity	1.0	1.4	1.8
B4	Chitonase	175	26%	AAD26111	*A. fumigatus*	Chitosanase activity	1.0	1.9	0.9
B5	Dienelactone hydrolase	148	10%	XP_751152	*A. fumigatus*	Hydrolase activity	1.0	1.5	1.5
B6	FG-GAP repeat protein	276	30%	XP_750162	*A. fumigatus*	Calcium ion binding	1.0	0.7	0.8
B7	Glyceraldehyde 3-phosphate dehydrogenase	250	27%	XP_748238	*A. fumigatus*	Oxidation-reduction	1.0	1.2	1.4
B9	Translation elongation factor1-alpha	219	19%	ABF50913	*Zygozyma suomiensis*	GTP dependent binding	1.0	1.2	0.8
B10	Actin-depolymerising factor	84	21%	XP_002420345	*C. dubliniensis*	Polypeptide binding	1.0	1.1	1.3
B12	Thioredoxin reductase GliT	88	10%	XP_750863	*A. fumigatus*	Posttranslational , protein turnover	1.0	2.0	2.1
B13	Short chain dehydrogenase	73	6%	XP_748339	*A. fumigatus*	NADP binding site	1.0	1.8	1.2
B14	GliN	130	11%	AAW03301	*A. fumigatus*	Methyltransferase domain	1.0	1.2	1.2
B15	Aspartic endopeptidase Pep2	148	11%	XP_754479	*A. fumigatus*	Aspartyl protease	1.0	1.1	1.3
B16	Vacuolar protease A	90	11%	XP_001399855	*A. niger*	Proteinase A	1.0	1.8	1.3
B17	ATP synthase F1	291	17%	XP_753589	*A. fumigatus*	F1 ATP synthase	1.0	0.8	0.8
B18	Catalase	724	24%	AAB71223	*A. fumigatus*	Inorganic ion transport and metabolism	1.0	0.8	0.9
B19	Hsp70	322	13%	XP_750490	*A. fumigatus*	Nucleotide binding site	1.0	1.2	1.4

EXPOSURE TO CASPOFUNGIN INDUCES AN OXIDATIVE STRESS RESPONSE IN A. FUMIGATUS.

Analysis of the activity of various enzymes associated with the oxidative stress response in *A. fumigatus* was performed as described. Exposure of *A. fumigatus* to 0.1 ɥ g/ml caspofungin for 30 minutes induced a six-fold increase in catalase activity while exposure to the drug for 60 minutes induced an eight-fold increase in activity ($p < 0.05$) (Figure 3.A). Exposure of hyphae to hydrogen peroxide for 15 minutes resulted in a four fold increase in catalase activity. Catalase demonstrates anti-oxidant properties and is essential in maintaining the redox balance of the cell. Exposure of *A. fumigatus* to 0.1 ɥ g/ml caspofungin for 15 – 60 minutes resulted in a two fold increase in GLR activity ($p < 0.02$) (Figure3.B). GLR is essential for the recycling of oxidized glutathione to its reduced form and plays a key role in detoxifying the cell. SOD is an anti-oxidant enzyme that catalyses the dismutation of superoxide radicals into dioxygen and hydrogen peroxide. SOD activity was increased significantly ($p < 0.05$) when *A. fumigatus* was exposed to hydrogen peroxide. SOD activity was increased by approximately two fold following exposure of *A. fumigatus* to caspofungin for 15 – 60 minutes (Figure3.C).

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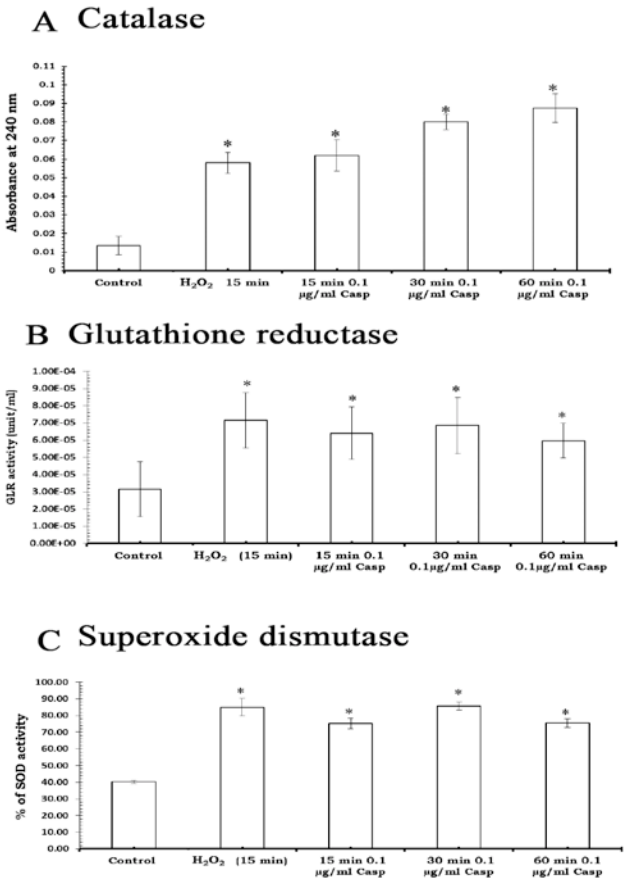

Figure 3: Activity of catalase, glutathione reductase and superoxide dismutase in *A. fumigatus* hyphae following exposure to caspofungin.
A.fumigatus hyphae were exposed to 0.1 µg/ml caspofungin for 60 minutes. Cells were harvested, disrupted and the activity of A. catalase, B. glutathione reductase and C. superoxide dismutase were measured as described. Exposure to hydrogen peroxide was the positive control in all cases. * indicates $p<0.05$

DISCUSSION.

Caspofungin is widely used in the treatment of systemic fungal infections and has the advantage of being highly effective but of negligible toxicity to humans (Denning, 2003). The aim of the work presented here was to establish how *A. fumigatus* responded to caspofungin and to determine whether the fungus mounted a response aimed at minimizing the effect(s) of the drug. Previous work established that exposure of *C. albicans* to caspofungin lead to the induction of oxidative and osmotic stress responses as evidenced by the activation of the Cap and Hog pathways (Kelly *et al.,* 2009).
The release of protein from *A. fumigatus* following exposure to caspofungin could indicate a general increase in cell permeability thus suggesting the need for the cell to counteract the elevated permeability of the cell. Analysis of the changes in the expression of proteins following exposure of *A. fumigatus* to 0.1 µg/ml caspofungin indicated the increased expression of a number of proteins associated with the oxidative stress response including spot B18 (catalase), spot B19 (Hsp70), and spot 12 (thioredoxin reductase). Protein spot B1 (18 kDa antigen) and spot B3 (allergen Asp F3) which are associated with virulence were also increased in expression. Cagas el al. noted an increase in the secretion of Asp F3 (3.5 fold) from susceptible *A. fumigatus* strains exposed to caspofungin. This allergen is a thioredoxin peroxidase and is increased in expression in cells experiencing oxidative stress as a result of exposure to hydrogen peroxide (Lessing e t al., 2007 – ref 25 cagas). Exposure of *A. fumigatus* to caspofungin resulted in the elevated activity of catalase, glutathione reductase and superoxide dismutase which are associated with the oxidative stress response. Increased activity of these enzymes has also been observed in *C. albicans* exposed to caspofungin or hydrogen peroxide (Kelly et al., 2009).
While caspofungin is a highly effective antifungal agent and has proven clinical efficacy, the response of fungal cells to it may not be fully elucidated. The primary action of caspofungin lies in the inhibition of glucan synthase and the consequent disruption of cell wall formation in fungi. However it has been established that exposure of *C. albicans* to this agent leads to increased chitin content (Walker *et al.,* 2008) and the induction of a number of genes associated with the PKC pathway in *C. albicans* leading to alterations in cell wall architecture (Munro *et al.,* 2008). In addition, caspofungin has been shown to unmask glucan in the cell wall and thus may lead to a potentially elevated immune response (Wheeler & Fink, 2006; Wheeler *et al.,* 2008).
The results presented here indicate that exposure of *A. fumigatus* to caspofungin leads to the release of protein and the induction of an oxidative stress response as indicated by the increased expression and activity of a number of stress–response proteins and enzymes. Previous work demonstrated the enhanced leakage and *de novo* biosynthesis of gliotoxin from caspofungin-treated *A. fumigatus* (Eshwika, 2013). Elevated production of this toxin by *A. fumigatus* following its amphotericin B- or caspofungin-mediated release (Reeves et al., 2004; Eshwika et al. 2012) may be associated with restoring the redox balance within the fungus (Schrettl et al., 2010). Cagas et al. have demonstrated altered expression of a range of proteins in *A. fumigatus* following exposure to caspofungin. The work presented here demonstrates

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the increased expression of a number of protein associated with the oxidative response and the elevated activity of enzymes associated with this response.

While the primary mode of action of caspofungin is the inhibition of glucan biosynthesis (Denning, 2003) this work and that of others (Walker *et al.,* 2008; Wheeler *et al.,* 2008; Stevens *et al.,* 2006; Kelly *et al.,* 2009 Cagas2011) demonstrate other effects including the stimulation of chitin synthesis and the induction of an oxidative stress response as the fungal cell attempts to counteract the direct and indirect effects of the antifungal.

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