Medical Mycology June 2010, **48**, 598–605

informa
healthcare

Proteomic analysis of proteins released from growth-arrested *Candida albicans* following exposure to caspofungin

JUDY KELLY & KEVIN KAVANAGH

Medical Mycology Unit, Department of Biology, National Institute for Cellular Biotechnology, National University of Ireland Maynooth, Co. Kildare, Ireland

The echinocandins (e.g., caspofungin) are a relatively new class of antifungal drugs that function by inhibiting the synthesis of β-1,3-glucan in the cell wall and thus lead to lysis of the cell. In this work the effect of caspofungin on the release of peptides from non-growing cells of the yeast *Candida albicans* that had been exposed to the drug was monitored. Exposure to 0.19 μg/ml caspofungin resulted in the release of amino acids from cells and of both small and large molecular weight proteins as demonstrated by 1- and 2-dimensional gel electrophoresis. Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-ToF) mass spectrometry was employed to identify a number of escaped peptides that were found to have increased in intensity upon exposure to the drug. A number of wall-associated proteins (e.g., phosphoglycerate kinase) and a number of glycolytic enzymes (phosphoglycerate mutase 1, fructose-bisphosphate aldolase) were identified. Importantly, several released proteins (e.g., pyruvate kinase, enolase 1, phosphoglycerate mutase, glyceraldehydes 3-phosphate dehydrogenase, fructose bisphosphate aldolase and alcohol dehydrogenase 1) are highly immunogenic in nature. The results presented here demonstrate that non-growing *C. albicans* cells are susceptible to the effect of caspofungin and that the caspofungin-mediated release of proteins from such cells could lead to a stronger immune response *in vivo*. This report illustrates that, in addition to hampering cell wall synthesis, caspofungin may also interfere with the permeability of the fungal cell wall.

Keywords antifungal, *Candida*, caspofungin, proteomics, proteins

Introduction

The incidence of nosocomial *Candida* infection continues to rise. Candidemia has become the fourth most common nosocomial bloodstream infection in the USA [1] and it has been suggested that as many as 10,000 deaths per year occur in the USA due to *Candida* infections [2]. *Candida albicans* is the principle opportunistic yeast pathogen of warm-blooded animals and humans [3] and is the most frequently isolated *Candida* species from both colonized and infected sites [4]. While the polyene and azole drugs have long been central to antifungal therapy, the echinocandins are a relatively new group of antifungal drug and caspofungin was the first member to be licensed for use [5]. Unlike polyenes or azoles that bind ergosterol or disrupt its biosynthesis, respectively, the echinocandins target the synthesis of β-1,3-glucan, the major polymer of the fungal cell wall [6]. Inhibition of β-1,3-glucan synthesis disrupts the structure of the growing cell wall, resulting in osmotic instability and ballooning out of the intracellular contents as a result of high osmotic pressure, which ultimately results in cell lysis [7,8]. It has been demonstrated previously that upon exposure to caspofungin, *C. albicans* cells increase their chitin content [9,10]. Chitin synthesis is controlled in part by the PKC pathway [11] and it has been found that caspofungin induces a set of genes under

Received 29 July 2009; Received in final revised form 2 October 2009; Accepted 12 October 2009

Correspondence: Kevin Kavanagh, Medical Mycology Unit, NICB, Department of Biology, NUI Maynooth, Co. Kildare, Ireland. Tel: +353 1708 3859; fax: +353 17083845; E-mail: kevin.kavanagh@nuim.ie

DOI: 10.3109/13693780903405782

control of the PKC pathway in *S. cerevisiae* [12] including the chitin synthase gene and genes involved in wall architecture. Therefore it is probable that in response to caspofungin exposure yeast cells undergo a wall re-modelling event where the synthesis of other wall polymers, notably chitin, is up-regulated. In addition it has been reported that sub-inhibitory concentrations of caspofungin unmask glucan in the cell wall [13,14].

The aim of this study was to analyse the effect of caspofungin on the release of peptides from *C. albicans* cells that were incubated in PBS, i.e., under conditions that did not support growth. One objective of this work was to establish whether caspofungin affects growth-arrested cells, since under these conditions the glucan synthase enzyme should not be as active as in growing cells. In addition it was the intention to establish the nature of peptides that were released from drug-treated cells. It was speculated that many of the released peptides could be highly immunogenic in nature and thus have the potential to lead to tissue inflammation *in vivo.*

Materials and methods

Organism and culture conditions

Candida albicans MEN (a gift from Dr D. Kerridge, Cambridge, UK) was used in this study. Cultures were grown in YEPD broth (2% (w/v) glucose (Sigma-Aldrich Chemical Co. Ltd, Dorset, UK), 2% (w/v) bacteriological peptone (Oxoid Ltd., Hampshire, England, UK) and 1% (w/v) yeast extract (Scharlau, Barcelona, Spain)) at 30°C and 200 rpm until the early stationary phase (approx. 1 x 10^8 cells/ml) was achieved. Stocks were maintained on YEPD containing 2% (w/v) agar (Scharlau), stored at 4°C and sub-cultured every 4-6 weeks.

Assessment of amino acid release

Stationary phase cultures of *C. albicans* were sub-cultured into YEPD broth and grown to the exponential phase (4 h). Cells (4×10^8) were harvested (2,056 *g* for 5 min on a Beckmann GS-6 bench centrifuge) and washed twice in sterile phosphate buffer (PBS; Sigma-Aldrich). Cell pellets were re-suspended in PBS (5 ml) with the addition of either 10% (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich (used as a positive control)) or caspofungin (0.19 µg/ml; Merck & Co. Inc., NJ, USA). A negative control consisted of PBS alone. The suspensions were incubated at 30°C and 200 rpm for 1 or 4 h. Cells were harvested as before and the supernatants collected. The release of amino acids was assessed using the ninhydrin colorimetric method as previously described [15]. A standard curve of amino acids (aspartic and glutamic acid (1:1) serially diluted in PBS) was prepared.

Trypan blue assay

Following incubation in the presence of caspofungin (0.19 µg/ml) cells (20 µl) were removed and added to 20 µl of 0.4% (w/v) trypan blue (Sigma-Aldrich) and 60 µl PBS. The mixture was allowed to rest for 5 min before examining under the light microscope. One hundred cells were counted and the number of stained and unstained cells was recorded. This assay was performed on three independent occasions.

Evaluation of protein release

Cultures of *C. albicans* were grown to the early stationary phase (1×10^8 cells/ml) in YEPD broth (100 ml), harvested and washed three times in sterile PBS. Cell pellets were weighed and all samples were adjusted to 1.5 g (wet weight). Cells were re-suspended in either PBS (5 ml), DMSO (10% (v/v); 5 ml) or caspofungin (0.19 µg/ml; 5 ml) and incubated at 30°C for 4 h. A caspofungin concentration of 0.19 µg/ml was chosen as this was previously established to be capable of inhibiting growth by 90% over 24 h (data not shown). PBS facilitated the extraction and identification by Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-ToF) of released peptides which could be masked by the presence of lipid, protein or other components in the growth medium (e.g., YEPD). Cells were harvested and the supernatants were placed on ice with protease inhibitors (100 µl pepstatin A (1 mg/ml; Sigma-Aldrich), 100 µl aprotinin (1 mg/ml; Sigma-Aldrich) and 100 µl phenylmethanesulfonyl fluoride (PMSF; 1 mM; Sigma-Aldrich)). Samples were diluted 1 in 10 in 100% (v/v) trichloroacetic acid (TCA; dissolved in sterile distilled water; Merck) and placed at 4°C overnight to precipitate protein. Samples were centrifuged at 17,950 *g* for 30 min at 4°C (Eppendorf centrifuge 5417R). Protein pellets were re-suspended in ice-cold acetone (300 µl; Sigma-Aldrich) and held at −20°C for at least 1 h to further precipitate protein. Protein was centrifuged at 17,950 *g* for 30 min at 4°C (Eppendorf centrifuge 5417R) and the acetone was discarded.

For one-dimensional SDS-PAGE gel analysis, released proteins were re-suspended in 5X sample buffer (10% (w/v) sodium dodecyl sulphate (SDS; Sigma-Aldrich); 5% (v/v) 2-mercaptoethanol (Sigma-Aldrich); 50% (v/v) glycerol (Sigma-Aldrich); 0.5M Tris-HCl, pH 6.8 (Sigma-Aldrich); 0.05% (w/v) bromophenol blue (Sigma-Aldrich)) and diluted 1 in 5 in this buffer before boiling. Samples were loaded into the wells of a 12.5% separating gel and run at 50 Volts for 10 min followed by 80 Volts for 120 min (Bio-Rad, CA, USA). Protein bands were revealed with Coomassie blue stain (0.2% (w/v) brilliant blue R (Sigma-Aldich), 10% (v/v) acetic acid (Sigma-Aldrich), 45% methanol (Sigma-Aldrich) in distilled water).

For two-dimensional SDS-PAGE gel analysis, released proteins were re-suspended in 250 µl IEF buffer (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 (DTT; added fresh; Sigma-Aldrich), 0.8% (v/v) IPG buffer pH 3-10 (G.E. Healthcare Bio-Sciences AB, Uppsala, Sweden)) with a few grains of molecular-grade bromophenol blue. The solution was applied to a 13 cm Immobiline™ DryStrip pH 3-10 (G.E. Healthcare) and iso-electric focusing was performed on an Ettan IPGphor II (Amersham Biosciences, NJ, USA). Following iso-electric focusing, strips were equilibrated in reducing buffer (50 mM Tris-HCl, 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 2% (w/v) DTT, pH 6.8) for 15 min 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 min. Strips were placed on top of homogeneous 12.5% SDS-PAGE gels (cast on the PROTEAN II casting unit (Bio-Rad); the dimensions of the gels were: 16 cm × 20 cm × 1 mm). 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 the PROTEAN PLUS Dodeca cell system (Bio-Rad) with temperature maintained at 6°C using a cooling system, for 20 h at 100 Volts and stained with Coomassie blue stain.

Protein was extracted from cultures on three separate occasions and gels were produced in triplicate per treatment on each occasion.

Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-ToF MS) Analysis

Mass spectrometric analysis was performed using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences). Protein samples for peptide mass determination were excised from gels, washed, digested with trypsin [16] and deposited (1 µl) with 1 µl alpha-cyano-4-hydroxy cinnamic acid (α-CHCA; LaserBio Labs, Sophia-Antipolis Cedex, France) onto mass spectrometry slides and allowed to dry prior to delayed extraction, reflection ToF analysis at 20kV. Internal calibrants used were Angiotensin III (Sigma-Aldrich) and ACTH fragment 18–39 (Sigma-Aldrich) at a final concentration of 20 pM which were used to calibrate all spectra. The mass lists generated were analyzed either by (i) NCBI nr database available as part of the mass spectrometer Evaluation Software Version 2.01, or (ii) *Candida albicans* custom database downloaded from The Broad Institute at: (http://www.broad.mit.edu/annotation/genome/candida_albicans/Multihome.html). The expectation value is a measure of the statistical significance of the identification being true and an expectation value cut-off of 0.05 was imposed. The lower the expectation value the greater the certainty of the identification. So an expectation value of zero would equate to a 100% certainty level. Mass lists were further blasted using the MASCOT protein search program available at http://www.matrixscience.com. The function and information about these proteins was determined using the UniProt data bank available at http://www.uniprot.org and/or the InterPro website available at http://www.ebi.ac.uk/interpro/ unless otherwise stated. Spots were located and analyzed with 'Progenesis SameSpots™' software by Nonlinear Dynamics in order to quantify the fold change between treatments.

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 1 and 2-Dimensional gels, representative examples are presented in Figs. 2 and 3 respectively.

Results and discussion

Effect of caspofungin on the leakage of amino acids of C. albicans

The ninhydrin colorimetric method was employed to quantify the release of amino acids from *C. albicans* cells incubated in PBS following caspofungin treatment. DMSO was employed as a positive control as it has been shown previously to alter the permeability of the cell membrane and lead to the release of amino acids [17,18]. The results of this experiment demonstrate that after 1 h, caspofungin leads to a small increase in amino acid released relative to that in the control, i.e., 20.0 µg/ml compared to 22.4 µg/ml in drug-treated cells (Fig. 1). Following 4 h exposure to caspofungin, amino acid release increased significantly from 22.2 µg/ml in the control to 33.5 µg/ml in the drug-treated cells ($P < 0.05$). DMSO also caused release of amino acids after 1 and 4 h exposure.

As a means of assessing cell viability following caspofungin exposure, a trypan blue exclusion assay was employed. After 1 h exposure to 0.19 µg/ml caspofungin, cellular viability was 95 ± 2%. This reduced to 91 ± 4% after 2 h and after 4 h exposure to the drug viability was 85 ± 9%. These results demonstrate that exposure to caspofungin increased the release of amino acids from the cell

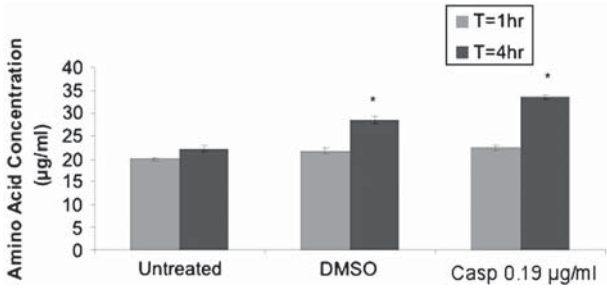

Fig. 1 Quantification of amino acid release from *Candida albicans* cells. Cells were exposed to caspofungin or DMSO for 1 or 4 h as described and the release of amino acids was quantified using the ninhydrin assay. DMSO was used as a positive control at a concentration of 10% (v/v). *Indicates statistically significant difference relative to control at $P < 0.05$.

possibly by interfering with the permeability of the cell wall and that the majority of cells remain viable after 4 h exposure to the drug as assessed by trypan blue exclusion. This result indicates that caspofungin is active against non-growing *C. albicans* cells.

Effect of caspofungin on the release of proteins from C. albicans

In order to ascertain whether caspofungin induced the release of proteins from *C. albicans*, cells were suspended in PBS, exposed to 0.19 µg/ml caspofungin or to 10% (v/v) DMSO for 4 h and the released constituents were precipitated as described. Proteins were resolved on a 1-D SDS PAGE gel and stained with Coomassie blue (Fig. 2). It was evident that several small molecular weight proteins (≤ 6.5 kDa) were released from the control cells during the 4-h incubation period. Cells treated with DMSO released more proteins, especially those of higher molecular weights (≥ 47.5 kDa). When cells were exposed to caspofungin, increased release of protein was evident especially in the range 25–50kDa, when compared to the control. It was noted that the pattern of proteins released from DMSO and caspofungin-treated cells differs in that there was a greater number of larger proteins released when cells were treated with the DMSO. This is possibly a result of the well established DMSO-permeabilising effect on membranes [17] leading to the release of large amounts of proteins from the cytoplasm.

Two–dimensional SDS-PAGE was employed in order to further separate the released proteins and thus facilitate their identification by mass spectrometry. Cells were exposed to caspofungin (0.19 µg/ml) for 4 h and the released proteins were collected, separated by 2-D electrophoresis and stained with Coomassie blue (Fig. 3). It is obvious that caspofungin induced the release of a large number of proteins as evident by the greater number of

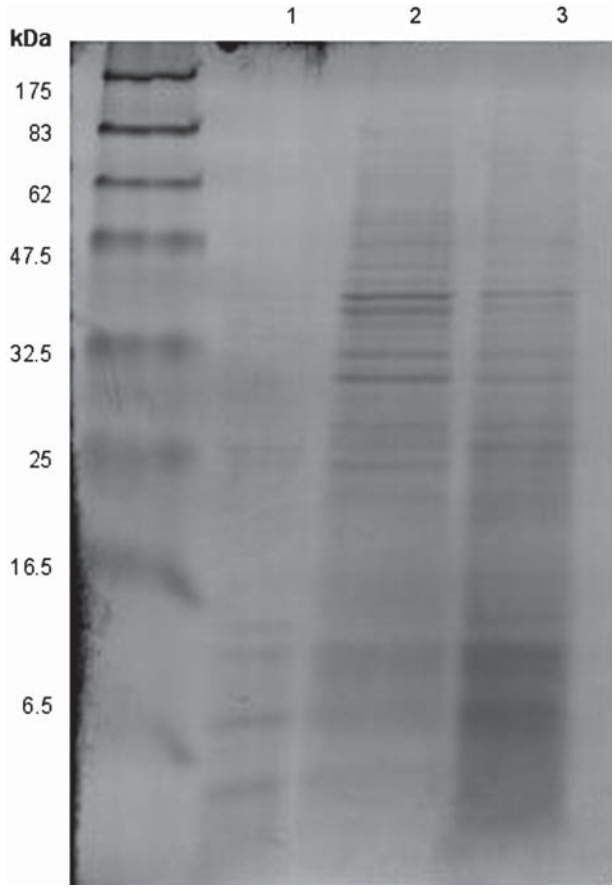

Fig. 2 Visualization of released proteins from *Candida albicans* following exposure to caspofungin. Cells were exposed to caspofungin or DMSO and released proteins were precipitated and separated by SDS-PAGE. Lane 1: Control, Lane 2: DMSO (10% (v/v)) treatment for 4 h, Lane 3: Caspofungin (0.19 µg/ml) treatment for 4 h.

protein spots (approximately 200) and an increase in spot intensity (Fig. 3). Selected spots were excised, washed, trypsin-digested and analyzed on a mass spectrometer as described (Table 1). The intensity of 13 peptide spots was increased following the treatment of cells with caspofungin and the intensity of these proteins was found to be increased by between 1.6 and 4.2 fold. Enolase 1, spot 10, is abundant in the cell and is predominantly found in the cytoplasm, however it may also be found in the cell wall as one of the glucan-associated cell wall proteins [19,20]. The release of enolase which has been described as an allergen [21,22] and as an immunodominant antigen [23,24] may be clinically relevant as it may induce tissue inflammation. Alcohol dehydrogenase, spot 12, has been found to play an immunogenic role during infections [25,26]. Although located in the cytoplasm, alcohol dehydrogenase has been found on the cell surface and has been implicated in adhesion to host extra-cellular matrix proteins [27,28].

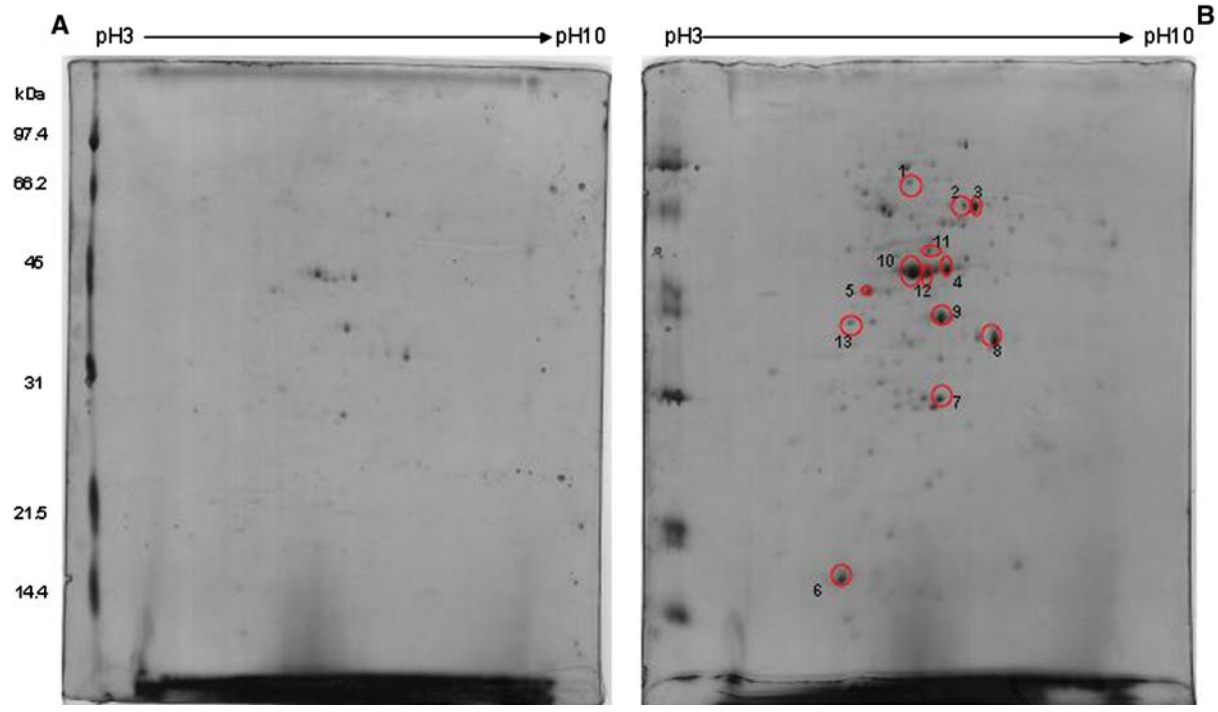

Fig. 3 Visualization by 2-D electrophoresis of proteins released from *Candida albicans* following exposure to caspofungin. Released proteins were collected and separated by 2-D SDS PAGE. (A) Proteins released from cells incubated in PBS for 4 h. (B) Protein released from cells exposed to 0.19 μg/ml caspofungin for 4 h. Numbered spots were subsequently removed and trypsin-digested prior to identification by MALDI-ToF mass spectrometry.

Phosphoglycerate kinase, spot 4, has been identified in the cell wall and at the surface [29] and is believed to be involved in cell wall biogenesis/degradation. The intensity of this protein increased 2.2 fold which may implicate this protein in the cell's response to caspofungin-induced cell wall damage. Pyruvate kinase, spot 1, has been identified as a wall protein [20] and was demonstrated to be highly immunogenic [30]. Here, caspofungin increased the release of this protein by 2.1 fold.

Glyceraldehyde-3-phosphate dehydrogenase, spot 8, is usually located in the cytoplasm but is also a surface antigen [31] and was demonstrated to be capable of binding to the extra-cellular matrix proteins fibronectin and laminin [32] thus implicating it in adherence. The intensity of this protein increased by 1.7 fold following caspofungin treatment. The glycolytic enzyme phosphoglycerate mutase 1, spot 7, binds to the extra-cellular matrix protein plasminogen [27] and has immunogenic properties [30,33].

Fructose-bisphosphate aldolase, spot 9, has also been implicated in plasminogen binding but with a low affinity [27] and has been isolated as a cell wall protein [20] although it is also known to reside in the cytoplasm. Previous studies have demonstrated fructose-bisphosphate aldolase to be immunogenic [30] and an IgE-binding antigen [21]. Importantly, when *C. albicans* cells are broken down in the human body, a large amount of glycolytic enzymes may be released and act as antigens [21]. Here it was found that caspofungin treatment resulted in a 1.8 fold increase in the intensity of fructose-bisphosphate aldolase from *C. albicans.*

The release of 6-phosphogluconate dehydrogenase, spot 11, was found to be increased by 4.2 fold in caspofungin-treated *C. albicans* cells. Six-phosphogluconate dehydrogenase, is an oxidative carboxylase that catalyzes the decarboxylating reduction of 6-phosphogluconate to ribulose 5-phosphate in the presence of NADP. The increased intensity of this protein may result from the cell wall remodelling known to be associated with the cell's response to caspofungin [9,10] and the associated requirement of the cell to degrade wall associated polymers in order to facilitate wall re-structuring.

Transaldolase, spot 13, is important for the balance of metabolites in the pentose-phosphate pathway and provides a link between the glycolytic and pentose-phosphate pathways and is believed to reside in the cytoplasm. Its intensity was increased by 2.4 fold by caspofungin. Citrate synthase, spot 2, which catalyzes the first reaction in the Krebs cycle, is located in the mitochondrial matrix and caspofungin increased the intensity of this protein by 1.9 fold in *C. albicans*. Glucan 1,3-β-glucosidase precursor, spot 5, catalyzes the hydrolytic removal of a glucose residue at the non-reducing end of β-1,3-glucan and to a lesser

Table 1 Identities of proteins observed to be increased in intensity following 2-D electrophoresis of samples from caspofungin-treated Candida albicans cells. Spots showing increased intensity in Fig. 3B were excised, digested and identified as described

Spot label	Identified protein	NCBI/Swiss Prot Accession Number	Function	Protein Size (kDa)	PI	Coverage (%)	Expectation (*p*) value	Fold Increase with Casp.
1	Pyruvate kinase (Pyk1)	XP_714997	Glycolysis, Magnesium/ potassium binding	55.48	6.4	39.2	0	+2.1
2	Citrate synthase, mitochondrial precursor (Cit1)	P79024	Carbohydrate metabolism, tricarboxylic acid cycle	51.98	7.8	18.6	0.003	+1.9
3	Hypothetical protein	XP_712412	Putative ATP binding	55.85	9.4	5	0.004	+2.5
4	Phosphoglycerate kinase (Pgk1)	P46273	Cell wall biogenesis/ degradation, Glycolysis	45.15	6.1	21.5	0.008	+2.2
5	Glucan, 1,3-*β*-glucosidase precursor (Xog1)	P29717	Metabolism of β-glucan, possible transglycosylase	49.9	5.4	13	0.028	+1.7
6	Hypothetical protein	XP_710838	Hypothetical LDG family protein 8	18.32	4.9	36.9	0.061	+2.8
7	Phosphoglycerate mutase 1 (Gpm1)	XP_721143	Glycolysis	27.44	5.8	41.4	0	+1.6
8	Glyceraldehyde-3-phosphate dehydrogenase (Gap1)	Q92211	Glycolytic enzyme, cell wall biogenesis, surface antigen	35.8	6.6	33	0	+1.7
9	Fructose-bisphosphate aldolase (Fba1)	XP_722836	Glycolysis, lyase	39.2	5.7	38.9	0	+1.8
10	Enolase 1 (Eno1)	P30575	Glycolysis	47.21	5.5	24.5	0	+1.6
11	6-Phosphogluconate dehydrogenase, decarboxylating (Gnd1)	O13287	Carbohydrate degradation, pentose phosphate pathway	38.85	5.5	22.4	0.005	+4.2
12	Alcohol dehydrogenase 1 (Adh1)	P43067	Oxidoreductase, allergen	46.14	8.8	43.8	0	+2.8
13	Transaldose (Tal1)	XP_715147	Balance of metabolites in tpentose-phosphate pathway	35.65	4.9	13.6	0.022	+2.4

extent, β-1,6-glucan [34] during wall metabolism. Importantly, caspofungin increased the intensity of this protein by 1.7 fold. As this enzyme is associated with the cell wall, perhaps its release is due to the alteration in wall organization in response to caspofungin.

A number of the above cell wall proteins (e.g., enolase1; alcohol dehydrogenase; phosphoglycerate kinase; glyceraldehyde-3-phosphate dehydrogenase; fructose-bisphosphate aldolase and 6-phosphogluconate dehydrogenase) were previously identified as 'atypical' in cell wall preparations [35]. The authors postulated that some proteins previously believed to be cytosolic in nature may become entrapped within the wall of the growing cell.

The results presented here suggest that caspofungin induces the release of a wide range of proteins from non-growing *C. albicans* cells and that these originate from different locations within the cell (e.g., cell wall, mitochondrion, cytoplasm). As caspofungin is known to interfere with β-1,3-glucan biosynthesis [7,8], it is not surprising to see the escape of many wall-associated proteins. A possible explanation for the release of intracellular proteins from caspofungin-treated *C. albicans* could be due to changes in membrane structure or composition as a result of the caspofungin-induced remodelling of the cell wall. It is interesting to note that caspofungin can induce these effects in non-growing cells. While viability was high after 4 h exposure to caspofungin (85 ± 9%), the possibility remains that the permeability of the cell may be altered sufficiently to allow the release of selected proteins. Interestingly, some of the proteins identified here are known to be highly immunogenic in nature (e.g., pyruvate kinase, phosphoglycerate mutase, enolase 1, glyceraldehydes 3 phosphate dehydrogenase, fructose bisphosphate aldolase and alcohol dehydrogenase 1). The increased release of these immunogenic proteins is noteworthy since from a clinical point of view they could lead to a stronger immune response and possibly inflammation.

The principal mode of action of caspofungin lies in its ability to inhibit β -1,3-glucan biosynthesis [7,8] which leads to osmotic instability and ultimately cell lysis. In addition to its direct effect on β -1,3-glucan biosynthesis, caspofungin also leads to the unmasking of β -1,3-glucan [13,14] which enhances the antifungal activity of human neutrophils [36] thus demonstrating that caspofungin makes the fungal cell more 'visible' to the host's immune system and thus aids in its destruction. The results presented here provide further insight into the effect of caspofungin on *C. albicans* and demonstrate that, as well as interfering with β -1,3-glucan synthesis, this agent induces the release of a range of proteins from a variety of cellular locations and that a number of these are known to be strongly immunogenic. This phenomenon may increase the visibility of *C. albicans* to the immune system and thus facilitate its destruction or alternatively challenge the host immune response and lead to increased inflammation.

Acknowledgements

This work was supported by funding from the Higher Education Authority of Ireland through the Programmes for Research in Third Level Institutes 3 and 4.

Declaration of interest: The authors have no conflicts of interest to disclose.

References

1 Pfaller MA, Jones RN, Messer SA, Edmond MB,Wenzel RP. National surveillance of nosocomial blood stream infection due to *Candida albicans*: frequency of occurrence and antifungal susceptibility in the SCOPE Program. *Diagn Microbiol Infect Dis* 1998; **31**: 327–332.
2 Sudbery P, Gow N, Berman J. The distinct morphogenic states of *Candida albicans*. *Trends Microbiol* 2004; **12**: 317–324.
3 Odds FC (ed.). Candida *and Candidosis A Review and Bibliography*, 2nd edn. London, UK: Bailliere Tindall, 1988.
4 Matthews R, Burnie J. The epidemiology and pathogenesis of candidiasis: applications in prevention and treatment. *Bull Inst Pasteur* 1998; **96**: 249–256.
5 Kartsonis NA, Nielsen J, Douglas CM. Caspofungin: the first in a new class of antifungal agents. *Drug Resist Update* 2003; **6**: 197–218.
6 Eggimann P, Garbino J, Pittet D. Management of *Candida* species infections in critically ill patients. *Lancet Infect Dis* 2003; **3**: 772–785.
7 Kurtz MB, Douglas CM. Lipopeptide inhibitors of fungal glucan synthase. *J Med Vet Mycol* 1997; **35**: 79–86.
8 Onishi J, Meinz M, Thompson J, *et al*. Discovery of novel antifungal (1,3)-β-D-glucan synthase inhibitors. *Antimicrob Agents Chemother* 2000; **44**: 368–377.
9 Walker LA, Munro CA, de Bruijn I, *et al*. Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. *PLoS Pathog* 2008; **4**: e1000040.
10 Stevens DA, Ichinomiya M, Koshi Y, Horiuchi H. Escape of *Candida* from caspofungin inhibition at concentrations above the MIC (Paradoxical Effect) accomplished by increased cell wall chitin: evidence for β-1,6-glucan synthesis inhibition by caspofungin. *Antimicrob Agents Chemother* 2006; **50**: 3160–3161.
11 Munro CA, Selvaggini S, deBruijn I, *et al*. The PKC, HOG and Ca2+ signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*. *Mol Microbiol* 2007; **63**: 1399–1413.
12 Reinoso-Martin C, Schuller C, Schuetzer-Muehlbauer M, Kuchler K. The yeast protein kinase C cell integrity pathway mediates tolerance to the antifungal drug caspofungin through activation of Slt2p mitogen-activated protein kinase signaling. *Eukaryot Cell* 2003; **2**: 1200–1210.
13 Wheeler RT, Fink GR. A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog* 2006; **2** (4, e35): 328–339.
14 Wheeler RT, Kombe D, Agarwala SD, Fink GR. Dynamic, morphotype-specific *Candida albicans* β-glucan exposure during infection and drug treatment. *PLoS Pathog* 2008; **4** (12): e1000227.
15 Ghosh A, Ghosh JJ. Release of intracellular constituents of *Candida albicans* in presence of polyene antibiotics. *Ann Biochem Exp Med* 1963; **23**: 611–626.
16 Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* 2006; **1**: 2856–2860.

17 Yu ZW, Quinn PJ. Solvation effects of dimethyl sulphoxide on the structure of phospholipid bilayers. *Biophys Chem* 1998; **70**: 35–39.
18 Reeves EP, Murphy T, Daly P, Kavanagh K. Amphotericin B enhances the synthesis and release of the immunosuppressive agent gliotoxin from the pulmonary pathogen *Aspergillus fumigatus*. *J Med Microbiol* 2004; **53**: 719–725.
19 Angiolella L, Facchin M, Stringaro A, *et al*. Identification of a glucan-associated enolase as a main cell wall protein of *Candida albicans* and an indirect target of lipopeptide antimycotics. *J Infect Dis* 1996; **173**: 684–690.
20 Pitarch A, Sanchez M, Nombela C, Gil C. Sequential fractionation and two-dimensional gel analysis unravels the complexity of the dimorphic fungus *Candida albicans* cell wall proteome. *Mol Cell Proteomics* 2002; **1**: 967–982.
21 Ishiguro A, Homma M, Torii S, Tanaka K. Identification of *Candida albicans* antigens reactive with immunoglobulin E antibody of human sera. *Infect Immun* 1992; **60**: 1550–1557.
22 Ito K, Ishiguro A, Kanbe T, Tanaka K, Torii S. Detection of IgE antibody against *Candida albicans* enolase and its cross-reactivity to *Saccharomyces cerevisiae* enolase. *Clin Exp Allergy* 1995; **25**: 522–528.
23 Strockbine NA, Largen MT, Zweibel SM, Buckley HR. Identification and molecular weight characterization of antigens from *Candida albicans* that are recognized by human sera. *Infect Immun* 1984; **43**: 715–721.
24 Pitarch A, Pardo M, Jimenez A, *et al*. Two-dimensional gel electrophoresis as analytical tool for identifying *Candida albicans* immunogenic proteins. *Electrophoresis* 1999; **20**: 1001–1010.
25 Shen HD, Choo KB, Lee HH, *et al*. The 40-kilodalton allergen of *Candida albicans* is an alcohol dehydrogenase: molecular cloning and immunological analysis using monoclonal antibodies. *Clin Exp Allergy* 1991; **21**: 675—681.
26 Swoboda RK, Bertram G, Hollander H, *et al*. Glycolytic enzymes of *Candida albicans* are non-ubiquitous immunogens during candidiasis. *Infect Immun* 1993; **61**: 4263–4271.

This paper was first published online on Early Online on 15 April 2010.

27 Crowe JD, Sievwright IK, Auld GC, *et al*. *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. *Mol Microbiol* 2003; **47**: 1637–1651.
28 Klotz SA, Pendrak ML, Hein RC. Antibodies to α5β1 and αvβ3 integrins react with *Candida albicans* alcohol dehydrogenase. *Microbiology* 2001; **147**: 3159–3164.
29 Alloush HM, Lopez-Ribot JL, Mastern BJ,Chaffin WL. 3-phosphoglycerate kinase: a glycolytic enzyme protein present in the cell wall of *Candida albicans*. *Microbiology* 1997; **143**: 321–330.
30 Pitarch A, Abian J, Carrascal M, *et al*. Proteomics-based identification of novel *Candida albicans* antigens for diagnosis of systemic candidiasis in patients with underlying hematological malignancies. *Proteomics* 2004; **4**: 3084–3106.
31 Gil-Navarro I, Gil ML, Casanova M, Martinez JP, Gozalbo D. The glycolytic enzyme glyceraldehyde-3-phosphate-dehydrogenase of *Candida albicans* is a surface antigen. *J Bacteriol* 1997; **179**: 4992–4999.
32 Gozalbo D, Gil-Navarro I, Azorin I, *et al*. The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is also a fibronectin and laminin binding protein. *Infect Immun* 1998; **66**: 2052–2059.
33 Pardo M, Ward M, Pitarch A, *et al*. Cross-species identification of novel *Candida albicans* immunogenic proteins by combination of two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. *Electrophoresis* 2000; **21**: 2651–2659.
34 Stubbs HJ, Brasch DJ, Emerson GW, Sullivan PA. Hydrolase and transferase activities of the β-1,3-exoglucanase of *Candida albicans*. *Eur J Biochem* 1999; **263**: 889–895.
35 Castillo L, Calvo E, Martinez AI, *et al*. A study of the *Candida albicans* cell wall proteome. *Proteomics* 2008; **8**: 3871–3881.
36 Lamaris GA, Lewis RE, Chamilos G, *et al*. Caspofungin-mediated beta-glucan unmasking and enhancement of human polymorphonuclear neutrophil activity against *Aspergillus* and non-*Aspergillus* hyphae. *J Infect Dis* 2008; **198**: 186–192.