JOURNAL OF MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Ajdidi *et al.*, *Journal of Medical Microbiology* 2019;68:1497–1506

DOI 10.1099/jmm.0.001065

MICROBIOLOGY SOCIETY

Assessment of the *in vitro* and *in vivo* activity of atorvastatin against *Candida albicans*

Ahmad Ajdidi[1], Gerard Sheehan[1], Khaled Abu Elteen[2] and Kevin Kavanagh[1,*]

Abstract

Aim. The aim of this work was to characterize the response of *Candida albicans* to atorvastatin, and to assess its *in vivo* antifungal capability.

Methodology. The effect of atorvastatin on the growth and viability of *C. albicans* was assessed. The ability of the statin to alter cell permeability was quantified by measuring amino acid and protein leakage. The response of *C. albicans* to atorvastatin was assessed using label-free quantitative proteomics. The *in vivo* antifungal activity of atorvastatin was assessed using *Galleria mellonella* larvae infected with *C. albicans*.

Results. Atorvastatin inhibited the growth of *C. albicans*. The atorvastatin-treated cells showed lower ergosterol levels than the controls, demonstrated increased calcofluor staining and released elevated quantities of amino acids and protein. Larvae infected with *C. albicans* showed a survival rate of 18.1±4.2% at 144 h. In contrast, larvae administered atorvastatin (9.09 mg kg^{-1}) displayed a survival rate of 60.2±6.4% (P<0.05). Label-free quantitative proteomics identified 1575 proteins with 2 or more peptides and 465 proteins were differentially abundant (P<0.05). There was an increase in the abundance of enzymes with oxidoreductase and hydrolase activity in atorvastatin-treated cells, and squalene monooxygenase (4.52-fold increase) and lanosterol synthase (2.84-fold increase) were increased in abundance. Proteins such as small heat shock protein 21 (−6.33-fold) and glutathione peroxidase (−2.05-fold) were reduced in abundance.

Conclusion. The results presented here indicate that atorvastatin inhibits the growth of *C. albicans* and is capable of increasing the survival of *G. mellonella* larvae infected with *C. albicans*.

INTRODUCTION

The yeast *Candida albicans* induces a wide range of superficial and life-threatening systemic infections in susceptible patients [1]. *C. albicans* possesses an extensive repertoire of virulence factors that allow successful colonization and dissemination in the host [1] and these include the ability to adhere to host tissue, produce a range of enzymes (e.g. secreted aspartyl proteinase, phospholipase), form biofilms and alter phenotype to overcome the host's immune response [2, 3]. Conventional antifungal therapy relies upon the use of polyene, azole or echinocandin drugs, but the delivery of appropriate therapy is often problematic due to the appearance of resistance and the inherent toxicity of some antifungal therapies [4].

Statins (e.g. atorvastatin, lovastatin, pravastatin and simvastatin) are one of the most widely prescribed medications and lower cholesterol levels in blood by inhibiting the action of 3-hydroxy-3-methylglutaryl-CoA (HMG-Co A) reductase [5, 6]. Fungal HMG-CoA reductases are also inhibited by statins, which results in a reduction in ergosterol content and the inhibition of fungal growth [5]. Statins inhibit the growth of a wide range of medically important fungi (e.g. *Aspergillus fumigatus* and *C. albicans*), but not *Candida krusei* [6], and supplementation of statin-containing growth medium with ergosterol leads to the recovery of growth, indicating that statins specifically inhibit the mevalonate pathway [7]. Prolonged exposure of *Candida glabrata* cells to statins leads to an increase in the percentage of petite (respiratory-deficient)

Received 10 April 2019; Accepted 07 August 2019; Published 28 August 2019

Author affiliations: [1]SSPC Research Centre, Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland; [2]Department of Biology and Biotechnology, Faculty of Science, The Hashemite University, Zarqa, Jordan.

***Correspondence:** Kevin Kavanagh, kevin.kavanagh@nuim.ie

Keywords: atorvastatin; *Candida*; ergosterol; *Galleria mellonella*; infection; statin.

Abbreviations: AMP, antimicrobial peptide; BP, biological process; DEP, differentially expressed proteins; FDR, false discovery rate; GO, gene ontology; MP, molecular function; SSDA, statistically significant differentially abundant.

Three supplementary figures are available with the online version of this article.

mutations in the population, suggesting that statins may also affect the integrity of mitochondrial DNA [8].

Patients on statin therapy experience fewer fungal infections [9], suggesting that statins may be capable of inhibiting the growth of fungi *in vivo* and so may represent a novel, non-toxic antifungal treatment option [10, 11]. In addition, the administration of statins to patients with severe sepsis [12] or bacteraemia [13] results in increased survival, possibly due to a reduction in inflammatory responses [14]. The aim of the work presented here was to characterize the effect of atorvastatin on *C. albicans* and establish its *in vivo* antifungal activity using *Galleria mellonella* larvae.

METHODS

Fungal cultures

C. albicans MEN was maintained on yeast extract–peptone–dextrose (YEPD) agar and grown for approximately 24 h in YEPD broth [2% (w/v) glucose, 2% (w/v) bacteriological peptone (Difco Laboratories), 1% (w/v) yeast extract (Oxoid Ltd, Basingstoke, UK] to the stationary phase (approximately 2×10^8 ml^{-1}). *C. albicans* cells were washed twice in sterile phosphate-buffered saline (PBS) before use and enumerated by haemocytometry.

Atorvastatin

Atorvastatin (20 mg film-coated; Rowex Ltd) tablets were ground to a fine powder and dissolved in sterile PBS. The solution was filter-sterilized using 0.45 and 0.02 µm pore filters (Sarstedt). The tablets contained the following excipients – sodium lauryl sulfate, microcrystalline cellulose, silica, colloidal anhydrous, pregelatinized maize starch, trometamol, yellow iron oxide (E 172), magnesium stearate and talc sodium starch glycolate (coating: carmellose sodium, glycerol, trometamol, sodium lauryl sulfate and hydroxyethyl cellulose) – and *in vitro* testing revealed no antifungal activity of these agents at the concentrations used in the tablets.

Assessment of effect of atorvastatin on growth of *C. albicans*

The effect of atorvastatin on the growth of the *C. albicans* was assessed using the broth microdilution technique (Document M27, Clinical and Laboratory Standards Institute) [15]. Stationary-phase *C. albicans* cells were harvested, washed in PBS (5 ml) and resuspended in medium at a concentration of 5×10^6 cells ml^{-1}. The cell suspension (100 µl) was added to each well of a 96-well plate containing various concentrations of statin in 100 µl of YEPD. The plate was incubated at 30 °C for 24 h. The optical density at 540 nm was determined using a microplate reader (Beckman DU 640 spectrophotometer) and growth was quantified as a percentage of the control [15].

Effect of atorvastatin on the viability of *C. albicans*

C. albicans cells were incubated in 10 ml PBS supplemented with atorvastatin (6, 12 and 24 µg ml^{-1}) for 2, 4 and 24 h at 30 °C. Aliquots (20 µl) were removed and serially diluted, and 100 µl of diluted sample was spread onto YEPD agar plates. The resulting colonies were enumerated and expressed as % survival relative to the control (n=3).

Ergosterol extraction and quantification

Ergosterol was extracted from *C. albicans* cells as described [16]. Ergosterol quantification was performed using a gas chromatograph (Hewlett Packard 5890 series II) with a flame ionization detector and a chrompack capillary column (Chrompack International BV, Middleburg, The Netherlands). The carrier gas was N_2, and the injector and detector temperatures were set at 320 °C. Ergosterol standards were used to calibrate the instrument.

Determination of amino acid leakage

Amino acid leakage from *C. albicans* cells exposed to atorvastatin (12 µg ml^{-1}) for 0 to 4 h at 30 °C was determined using the ninhydrin colorimetric method and expressed in terms of aspartic acid and glutamic acid [17]. Ninhydrin (Sigma-Aldrich) was dissolved in ethanol to give a final concentration of 0.35% (w/v) and 250 µl was added to each sample (1 ml) and heated to 95 °C for 4 min followed by cooling on ice. The absorbance at 570 nm was recorded on a spectrophotometer (Beckman DU 640).

Assessment of protein release

Stationary-phase *C. albicans* cells were exposed to atorvastatin (12 µg ml^{-1}) for 0 to 4 h at 30 °C. Cells were harvested by centrifugation at 3000 ***g*** and the supernatants were collected. The quantity of protein released from the cells in the supernatant was assayed using the Bradford assay (Bio-Rad), with bovine serum albumin (BSA) (Sigma-Aldrich) as standard.

Fluorescence microscopy

C. albicans cells were grown in the presence of atorvastatin (6, 12 and 24 µg ml^{-1}) for 24 h, harvested by centrifugation, washed in PBS and stained with calcofluor white (Sigma) for 15 min at 16 °C on a glass slide. Cells were washed twice (PBS) and a cover slide was placed on top. Cells were viewed with an Olympus BX51 fluorescence microscope.

Adherence assay

The adherence of *C. albicans* to buccal epithelial cells (BECs) was analysed as described [18]. Cells pre-grown overnight in atorvastatin-supplemented medium (12 µg ml^{-1}) were harvested, washed in PBS and resuspended at a density of 5×10^5 ml^{-1}. *C. albicans* cells were mixed with BECs at a ratio of 50:1 in a final volume of 2 ml and incubated at 30 °C and 200 r.p.m. for 90 min. The BEC–yeast cell mixture was harvested by passage through a polycarbonate membrane and placed on glass slides to air-dry overnight. Cells were heat-fixed and stained using 0.5% (w/v) crystal violet. The number of *C. albicans* cells adhering to each of 200 BECs per treatment was assessed microscopically.

Assessment of *in vivo* toxicity of atrovastatin in *G. mellonella* larvae

G. mellonella larvae were obtained from the Meal Worm Company (Sheffield, UK) and stored in wood shavings in the dark at 15 °C prior to use as described [19, 20]. Larvae weighing 0.22±0.03 g were used within 2 weeks of receipt and 10 healthy larvae per treatment and controls (n=3) were placed in sterile 9 cm Petri dishes containing some wood shavings. Larvae of *G. mellonella* were inoculated with atrovastatin solution (20 µl of 50 or 100 µg ml^{-1}) through the last left proleg and survival was monitored over 144 h at 30 °C.

Assessment of *in vivo* anti-fungal activity of atorvastatin using *G. mellonella* larvae

Larvae were administered 20 µl solution containing 1×10^6*C. albicans* cells ml^{-1} through the last left proleg as described [19]. Treated larvae were administered 20 µl of atorvastatin (4.55 or 9.09 mg kg^{-1}) 30 min post-infection with *C. albicans* and the survival of larvae was monitored over 144 h at 30 °C. The aim of this work was to replicate the situation in practice where the administration of agent(s) occurs after infection.

Label-free quantitative proteomics analysis of *C. albicans*

Label-free shotgun semi-quantitative proteomics was conducted on *C. albicans* cells pre-grown in the presence of atrovastatin (12 µg ml^{-1}) for 24 h (n=4). Protein was extracted as described [19, 20] and protein (75 µg) was reduced with dithiothreitol (DTT; 200 mM) (Sigma-Aldrich), alkylated with iodoacetamide (IAA; 1 M) (Sigma-Aldrich) and digested with sequence grade trypsin (Promega, Ireland) at a trypsin:protein ratio of 1:40, overnight at 37 °C. Tryptic peptides were purified for mass spectrometry using C18 spin filters (Medical Supply Company, Ireland) and 1 µg of peptide mix was eluted onto a Q-Exactive (Thermo Fisher Scientific, USA) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID) using a 65 min reverse-phase gradient at a flow rate of 250 nl min^{-1}. All data were acquired with the mass spectrometer operating in automatic data-dependent switching mode. A high-resolution mass spectrometer scan (300–2000 Dalton) was performed using the Orbitrap to select the 15 most intense ions prior to tandem mass spectrometry.

Protein identification from the tandem mass spectrometry data was performed using the Andromeda search engine in MaxQuant (version 1.2.2.5; http://maxquant.org/) to correlate the data against the proteome of *C. albicans* obtained from Uniport. The following search parameters were used: first search peptide tolerance of 20 p.p.m., second search peptide tolerance of 4.5 p.p.m., carbamidomethylation of cysteines set as a fixed modification, with oxidation of methionines and acetylation of N-terminals set as variable modifications, and a maximum of two missed cleavage sites allowed. False discovery rates (FDRs) were set to 1% for both peptides and proteins and the FDR was estimated following searches against a target–decoy database. Peptides with a minimum length of seven amino acids were considered for identification and proteins were only considered to have been identified when more than one unique peptide for each protein was observed.

Results processing, statistical analyses and graphics generation were conducted using Perseus v. 1.5.5.3. Label-free quantification (LFQ) intensities were $\log_2$-transformed and analysis of variance (ANOVA) of significance and t-tests between the proteome of the 24 h control and the atrovastatin-treated (12 µg ml^{-1}) *C. albicans* were performed using a P-value of 0.05 and significance was determined using FDR correction (Benjamini–Hochberg). Proteins that had non-existent values (indicative of absence or very low abundance in a sample) were also used in statistical analysis of the total differentially expressed group following imputation of the zero values using a number close to the lowest value of the range of proteins plus or minus the standard deviation. After data imputation these proteins were included in subsequent statistical analysis. Blast2GO software was applied to determine gene ontology (GO) terms relating to biological processes (BPs) and molecular function (MF).

Statistical analysis

All experiments were performed on three independent occasions and the results are expressed as the mean±SD. Statistical significance in the growth assay, viability assay, ergosterol quantification and leakage experiments was assessed by t-test. Larval survival was assessed by Kaplan–Meier log rank. All statistical analysis was performed using GraphPad Prism. Differences were considered significant at P≤0.05.

Data availability

The MS proteomics data and MaxQuant search output files have been deposited at the ProteomeXchange Consortium [21] via the PRIDE partner repository with the dataset identifier PXD013365.

RESULTS

Analysis of the effect of atorvastatin on the growth and viability of *C. albicans*

Cells of *C. albicans* were exposed to atorvastatin and growth was assessed after 24 h. The results revealed that a dose of 96 µg ml^{-1} reduced growth by 74.6±0.3% at 24 h (P<0.0001) (Fig. 1). Concentrations of 12 and 24 µg ml^{-1} reduced growth by 11.9±1.1 % and 21.8±0.5% (P<0.05), respectively, at 24 h. Exposure of *Escherichia coli* or *Staphylococcus aureus* to atorvastatin resulted in no inhibition of growth (Fig S1, available in the online version of this article). The effect of atorvastatin on the viability of *C. albicans* was also assessed and the results revealed a small fungicidal effect (Fig S2). Exposure of cells to 24 µg ml^{-1} atorvastatin reduced viability by 19±1.6% at 4 h and by 22.5±0.9% at 24 h.

Exposure of *C. albicans* to atorvastatin (12 µg ml^{-1}) for 24 h led to a reduction in the ergosterol content of cells [control=0.063±0.008 µg ml^{-1} and treatment=0.027±0.003

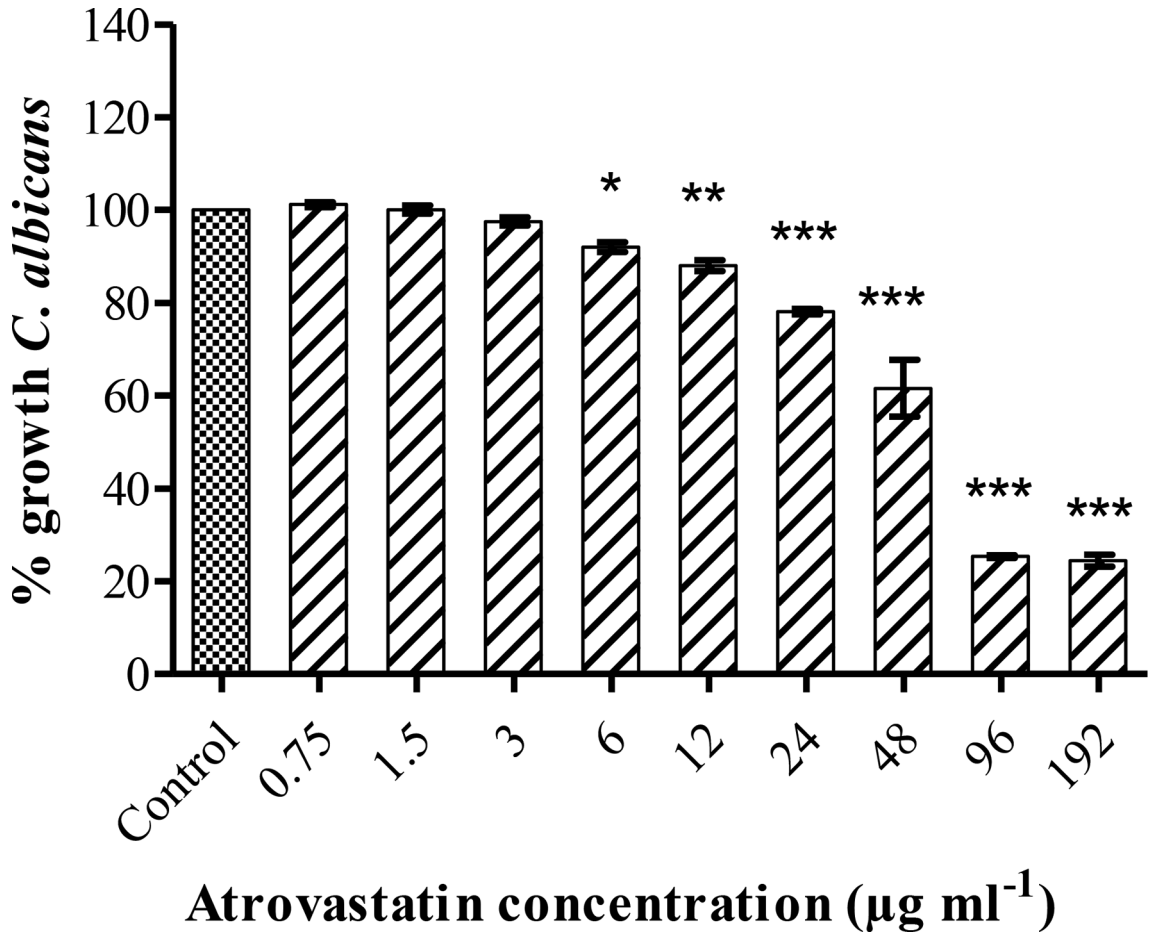

Fig. 1. Effect of atorvastatin on growth of *C. albicans*. *C. albicans* (initial concentration 10^4 per well) was exposed to atorvastatin (0.75 – 195 µg ml^{-1}) in YEPD. Growth (%) was calculated by comparing atorvastatin-treated *C. albicans* to control cells after 24 h of growth (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

µg ml^{-1} ($P=0.04$) (Fig. 2]. Experiments to assess the extent of leakage from atorvastatin-treated cells were performed. Exposure of cells to atorvastatin (12 µg ml^{-1}) for up to 4 h led to an increase in amino acid (Fig. 3a) and protein (Fig. 3b) leakage from cells. Exposure to atorvastatin for 2 and 4 h led to an amino acid concentration of 1.97±0.09 µg ml^{-1} and 1.78±0.1 µg ml^{-1}, respectively. Protein release at 2 and 4 h was 147.7±10.8 and 132.7±18.1 µg ml^{-1}, respectively, while for the control it was 57.3±6.2 and 35.67±4.6 µg ml^{-1}, respectively ($P<0.05$).

Altered membrane permeability could lead to distortions in the fungal cell wall. Staining cells with calcofluor following exposure to atorvastatin revealed increased fluorescence, indicating an increase in the chitin content of cells. Atorvastatin-treated cells also appeared to be larger and demonstrated incomplete cell separation (Fig. 4). Atorvastatin-treated cells demonstrated a reduced ability to adhere to exfoliated buccal epithelial cells, indicating a possible disruption to the surface of the yeast cell (Fig S3). Control cells showed 5.55±0.6 yeast cells adhering per BEC, while cells exposed to atorvastatin showed 3.02±0.5 adhering per BEC ($P=0.009$)

Assessment of toxicity and *in vivo* antifungal efficacy of atrovastatin

The administration of doses of atorvastatin (4.55 or 9.09 mg kg^{-1}) to *G. mellonella* larvae by intrahaemocoel injection resulted in no adverse effect over 144 h (Fig. 5). Larvae infected with *C. albicans* were subsequently administered a dose of atorvastatin (4.55 or 9.09 mg kg^{-1}) 30 min post-infection (Fig. 5). The results indicate increased survival in those larvae given a dose of atorvastatin (4.55 or 9.09 mg kg^{-1}). Larvae infected with *C. albicans* showed a survival rate of 76.8±3.5% at 72 h and one of 18.1±4.2% at 144 h. In contrast, those larvae administered a 9.09 mg kg^{-1} dose of atorvastatin displayed a survival rate of 94.3±1.96% at 72 h and one of 60±6.4% at 144 h ($P<0.05$).

Proteomic analysis of the effect of atorvastatin on *C. albicans*

Comparative analysis of differential protein abundance in atorvastatin-treated *C albicans* was performed using label-free proteomics. A principal component analysis (PCA) was

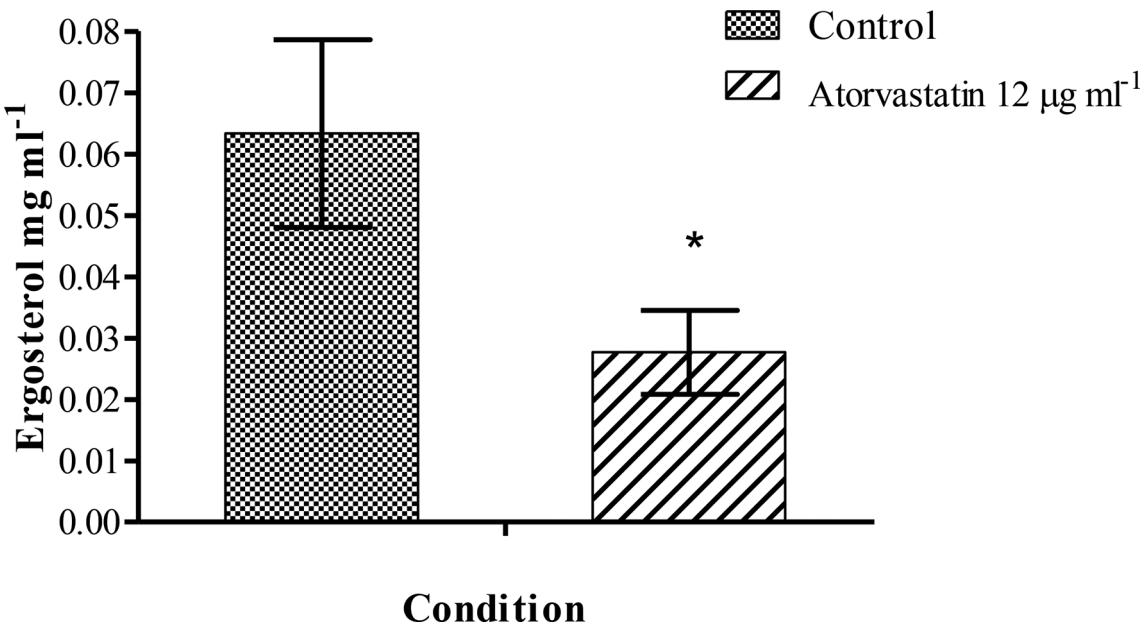

Fig. 2. Atorvastatin exposure reduced the ergosterol content of *C. albicans*. Ergosterol quantification was performed using a gas chromatogram with a flame ionization detector and a chrompack capillary column. Ergosterol standards were used to calibrate the instrument. Ergosterol content was expressed in terms of μg ml^{-1} (*, P<0.05).

performed on all filtered proteins and identified distinct proteomic differences between the control and statin-treated samples (Fig. 6a). The heat maps also show major differences in the relative abundance of proteins in the control and atorvastatin-treated cells (Fig. 6b). In total, 1575 proteins with 2 or more peptides were identified and 465 proteins were determined to be differentially abundant (ANOVA, P<0.05) with a fold change of >1.5. Of these, 231 were found in higher abundance in the atorvastatin-treated cells and 234 were present in lower abundance in the treated cells. A total of 74 proteins were present in all 3 control samples and absent in all 3 treated samples, and a further 57 were present in each replicate of the atorvastatin treatment and absent in all 3 control samples.

The volcano plot (Fig. 6c) shows the relative increase or decrease in the abundance of proteins in the atorvastatin-treated cells compared to the control cells. A variety of proteins were increased in abundance in atorvastatin-treated cells and these included squalene monooxygenase ERG1 (4.52-fold), lanosterol synthase (3.30-fold), 3-keto-steroid reductase (2.78-fold), lanosterol 14-alpha demethylase (2.57-fold) and acetyl-CoA C-acetyltransferase (2.23-fold). A number of proteins were decreased in abundance in the

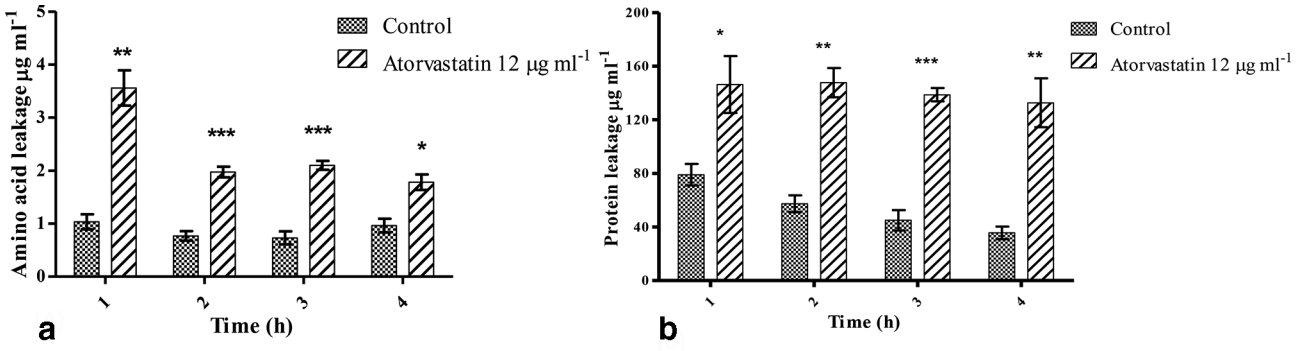

Fig. 3. Effect of atorvastatin on the release of amino acids (a) and protein (b) from *C. albicans*. The effect of atorvastatin (12 μg ml^{-1}) on *C. albicans* amino acid and protein release was determined by ninhydrin colorimetric assay and Bradford protein assay. Atorvastatin treatment resulted in increased amino acid and protein release relative to control cells (*, P<0.05; **, P<0.01; ***, P<0.001).

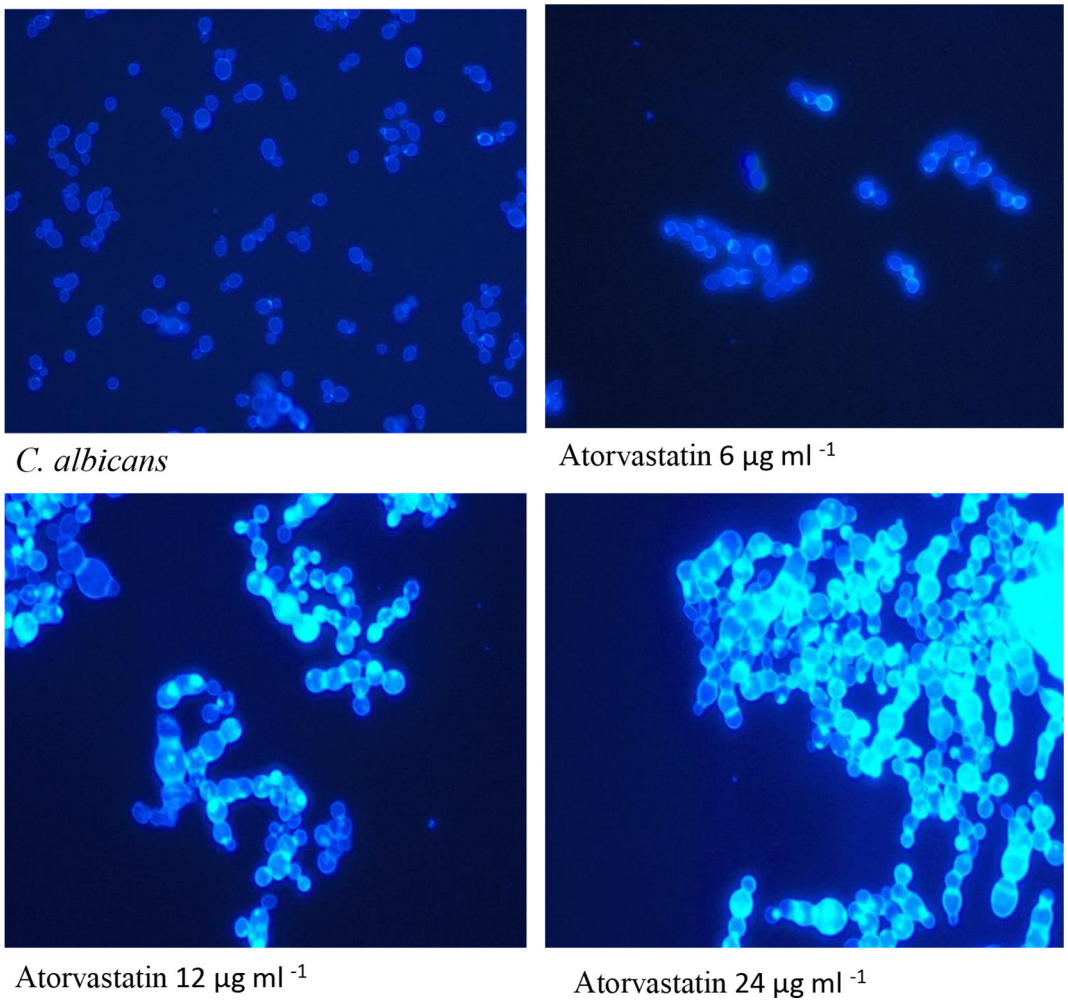

Fig. 4. Calcofluor staining of *C. albicans* following exposure to atorvastatin. (Magnification x 400). *C. albicans* cells were exposed to atorvastatin (6, 12 and 24 µg ml^{-1}) for 24 h, stained with calcofluor white and visualized with an Olympus BX51 fluorescence microscope. Increased fluorescence indicated an increase in the chitin content of cells.

treated cells, including Hsp12p (−6.33-fold), small heat shock protein 21 (-6.26-fold), yeast-form wall protein 1 (−5.68-fold), Wh11p (−4.93-fold), Acyl-CoA desaturase (−4.88-fold) and heat shock protein SSA1 (−4.40-fold). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of proteins that showed altered abundance in atorvastatin-treated cells revealed seven proteins that showed increased abundance in the fungal steroid pathway (Fig. 7). This suggests that the cell may be attempting to increase ergosterol biosynthesis or redirect biosynthesis following the inhibition of the action of the fungal 3-hydroxy-3-methylglutaryl-CoA reductase.

DISCUSSION

Statins are widely used for the control of cholesterol and function by inhibiting the action of HMG-Co A (3-hydroxy-3-methylglutaryl-CoA) reductase [22]. The long term use of statins is generally regarded as safe [23], but there is evidence to suggest immune modulatory effects in certain patients [24, 25] and, in particular, the induction of alterations in the activity of regulatory T-cells [26].

Cholesterol in mammals and ergosterol in fungi share a similar structure and biosynthetic pathways and consequently both pathways are sensitive to the same inhibitory effects of statins [27, 28]. Patients on statin therapy display fewer fungal infections, thus prompting the suggestion that statins may have potential applications as anti-fungal agents *in vivo* [6, 9]. This raises the possibility that statins, in addition to being used to control cholesterol levels, may also be used in the control of fungal infections either as sole therapies or in combination with existing antifungal therapies [6]. For example, statins could be useful in the treatment of fungal pathogens showing resistance to conventional antifungal agents (e.g. azoles). The work presented here evaluated the interaction of atorvastatin

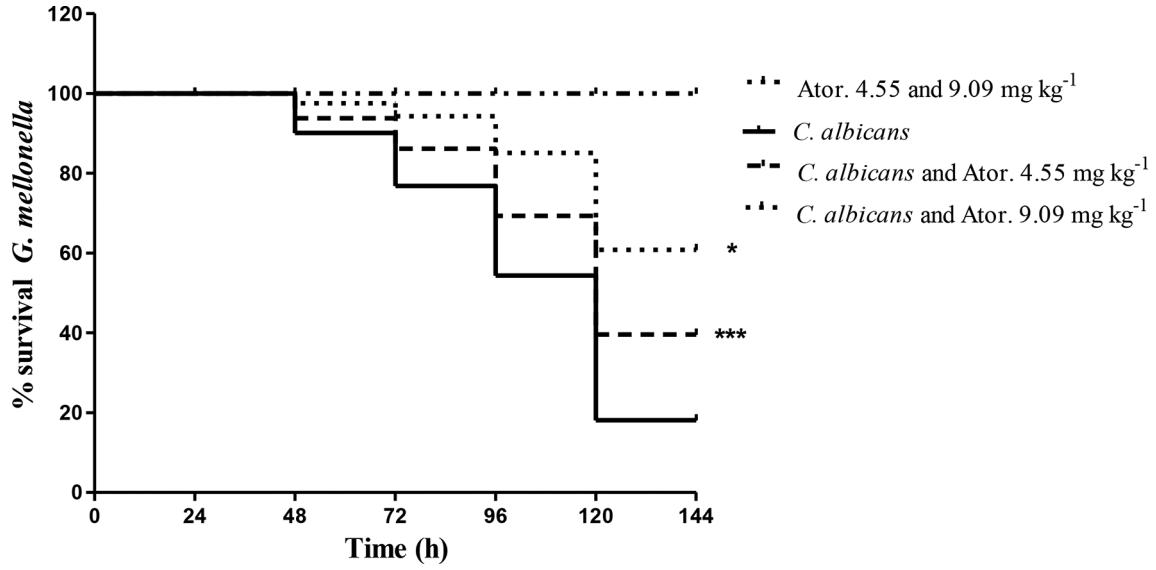

Fig. 5. Effect of atorvastatin on viability of *G. mellonella* larvae infected with *C. albicans*. Larvae injected with atorvastatin (4.55 or 9.09 mg kg^{-1}) displayed no decrease in survival. Larvae were inoculated with *C. albicans* (1×10^6 $larva^{-1}$) and then treated with atorvastatin (4.55 or 9.09 mg kg^{-1}) 30 min later, and survival was monitored over 144 h. Atorvastatin (4.55 or 9.09 mg kg^{-1}) treatment enhanced larval survival relative to non-treated larvae.

with *C. albicans* and demonstrated the *in vivo* activity of the statin against *C. albicans* infection in *G. mellonella* larvae.

In this work atorvastatin tablets were ground to a fine powder, added to a volume of water and filter-sterilized prior to use. Some loss of product could have occurred during the filtration process, but this was not considered to be significant. The exposure of *C. albicans* to atorvastatin reduced growth and also caused a small reduction in viability. Statins reduce cholesterol biosynthesis *in vivo* [22] and also reduced the ergosterol content in *C. albicans*. Ergosterol plays a key role in maintaining the integrity and permeability of the fungal cell membrane and reduced levels of ergosterol could indicate a more porous membrane [9, 23]. Statin exposure did lead to an increase in the release of amino acid and proteins from cells, indicating increased permeability.

Atorvastatin-treated cells displayed elevated levels of chitin, were larger than untreated cells and demonstrated incomplete cell division. Interestingly, proteomic analysis revealed that glutamine-fructose-6-phosphate aminotransferase, an enzyme that plays a role in chitin synthesis and hyphal growth, was increased in abundance (+2.29-fold) in atorvastatin-treated cells. Adherence to host cells is an important virulence factor of *C. albicans* [29] and is mediated by a variety of specific and non-specific mechanisms. The dominant adherence mechanism is mediated by cell wall-bound adhesins that attach to host cell receptors and anchor the cell in hostile environments to form a focal point for tissue invasion. The exposure of *C. albicans* to atorvastatin led to a reduction in the adherence ability of cells, possibly as a consequence of the altered cell wall composition, as indicated by the increased calcofluor staining. Proteomic analysis revealed decreased abundance of yeast-form wall protein (−6.26-fold), which plays a role in adherence and the promotion of dispersal and in Wh11p (−5.68-fold), a protein involved in biofilm formation and pathogenesis.

A number of proteins involved in the ability of the cell to respond to stress were reduced in abundance. Heat shock proteins such as Hsp12p (−6.33-fold), small heat shock protein 21 (-6.33-fold) and heat shock protein SSA1 (−4.40-fold) were reduced in abundance and these play roles in the pathogenicity of the yeast and in its ability to respond to oxidative and osmotic stress. Reduced abundance of these proteins may have an impact on the cell's ability to grow and proliferate *in vivo*. Proteins such as Cip1p (−3.73-fold), Pst2p (−3.01-fold), pleiotropic ABC efflux transporter (−2.85-fold) were also reduced in abundance and all play a role in the cell's response to oxidative stress [30].

Proteomic analysis revealed an increase in the abundance of proteins involved in ergosterol biosynthesis in atorvastatin-treated cells. In particular, squalene monooxygenase (+4.52-fold), lanosterol synthase (+3.30-fold), keto-steroid reductase (+2.78-fold) and lanosterol 14-alpha demethylase (+2.57-fold) were all increased in abundance. Atorvastatin-treated cells displayed reduced ergosterol content and perhaps the cells responded to statin exposure by attempting to increase its biosynthesis. Statins inhibit the synthesis of important isoprenoids, e.g. farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which are lipid attachments for the γ subunit of heterotrimeric G-proteins [31], guanosine triphosphate-binding protein Ras and Ras-like proteins (Rho, Rab, Rac, Ral, or Rap) [31–33]. Moreover, statins act as inhibitors of some G-protein actions and Ras or Ras-like signalling, which affect several important bioprocesses [34].

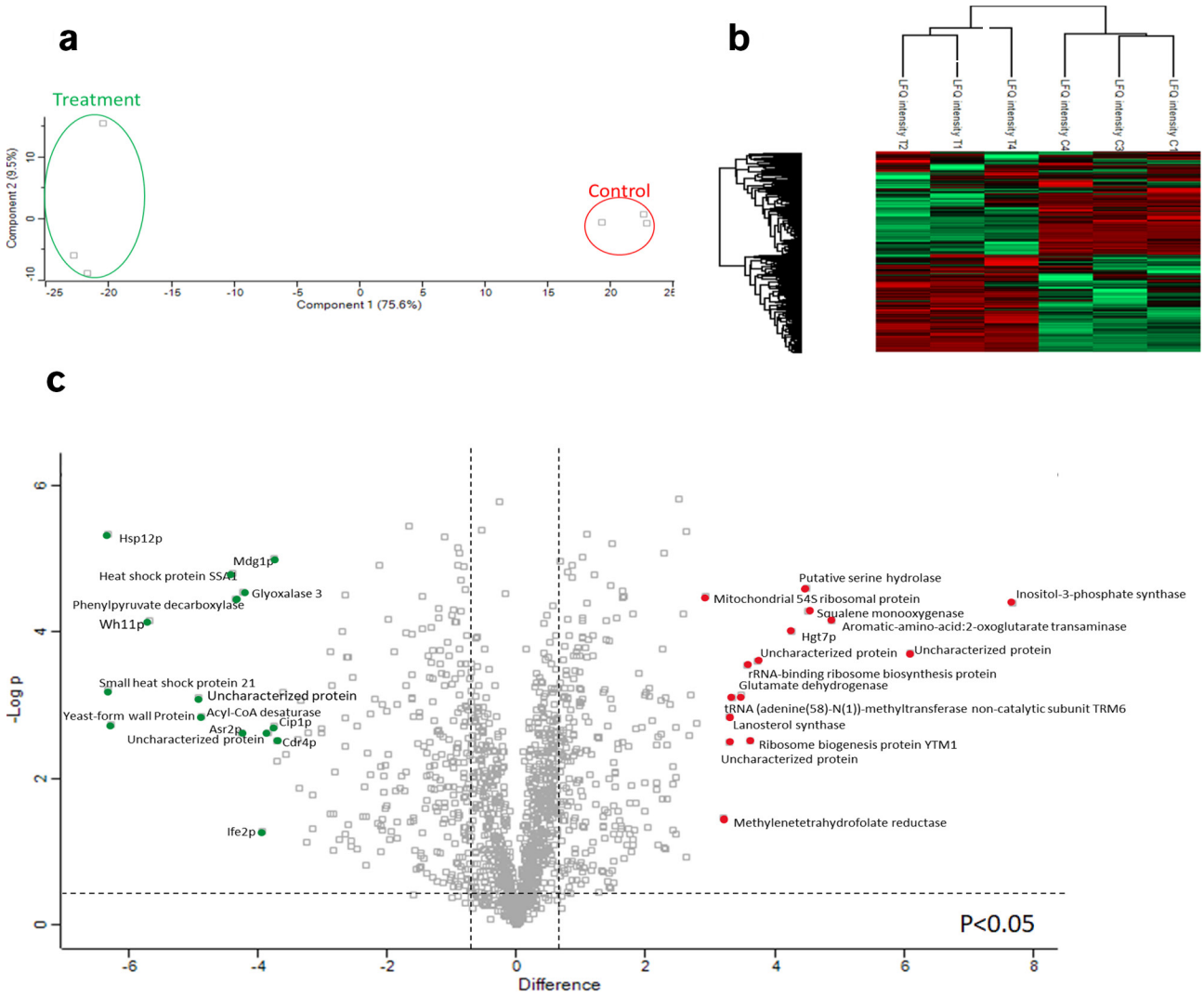

Fig. 6. Shotgun proteomics of responses of *C. albicans* to atorvastatin (12 µg ml^{-1}) after 24 h at 30 °C. (a) Principal component analysis of the control and atorvastatin-treated *C. albicans* over 24 h showed a clear distinction between the control and treated *C. albicans*. (b) Two-way unsupervised hierarchical clustering of the median protein expression values of all statistically significant differentially abundant proteins from the *C. albicans* control (C1, C3 and C4) and the atorvastatin-treated (T1, T2 and T4) *C. albicans*. (c) Volcano plot showing the distribution of quantified proteins according to *P*-value (–log$_{10}$ *P*-value) and fold change (log$_2$ mean LFQ intensity difference). Proteins above the horizontal line are considered statistically significant (*P*-value <0.05) and those to the right and left of the vertical lines indicate relative fold changes ±1.5.

G. mellonella larvae can be employed to assess the virulence of fungal pathogens [35, 36] and determine the *in vivo* activity of antifungal agents [37, 38]. Their use offers the possibility of quickly establishing the *in vivo* toxicity and efficacy of antimicrobial agents without the need to resort to the use of mammals in initial screening studies. In the work presented here atorvastatin was shown to be non-toxic to larvae and to be able to protect larvae following infection with *C. albicans*. The doses of 50 and 100 µg ml^{-1} used in the larvae are equivalent to 4.55 and 9.09 mg kg^{-1} in humans, respectively. Interestingly, atorvastatin had a negative effect on the survival of mice infected with *C. albicans* and followed up with atorvastatin (40 mg kg^{-1}), and this was due to decreased INF-gamma and IL-4 levels in response to *C. albicans*, while the LD_{50} for atorvastatin is 5000 mg kg^{-1} in mice [39]. However, other work has demonstrated that pravastatin enhanced survival and led to a decreased fungal burden in C3H/HeN mice infected with *C. albicans* and the potential benefits of statins in the clinic against *Candida* have been described [10, 40–42]. The proteomic results indicated reduced abundance of proteins associated with virulence and stress response (e.g. Hsp12p and ABC efflux transporter) in *C. albicans* and this may have made the cells more susceptible to killing by the larval immune response.

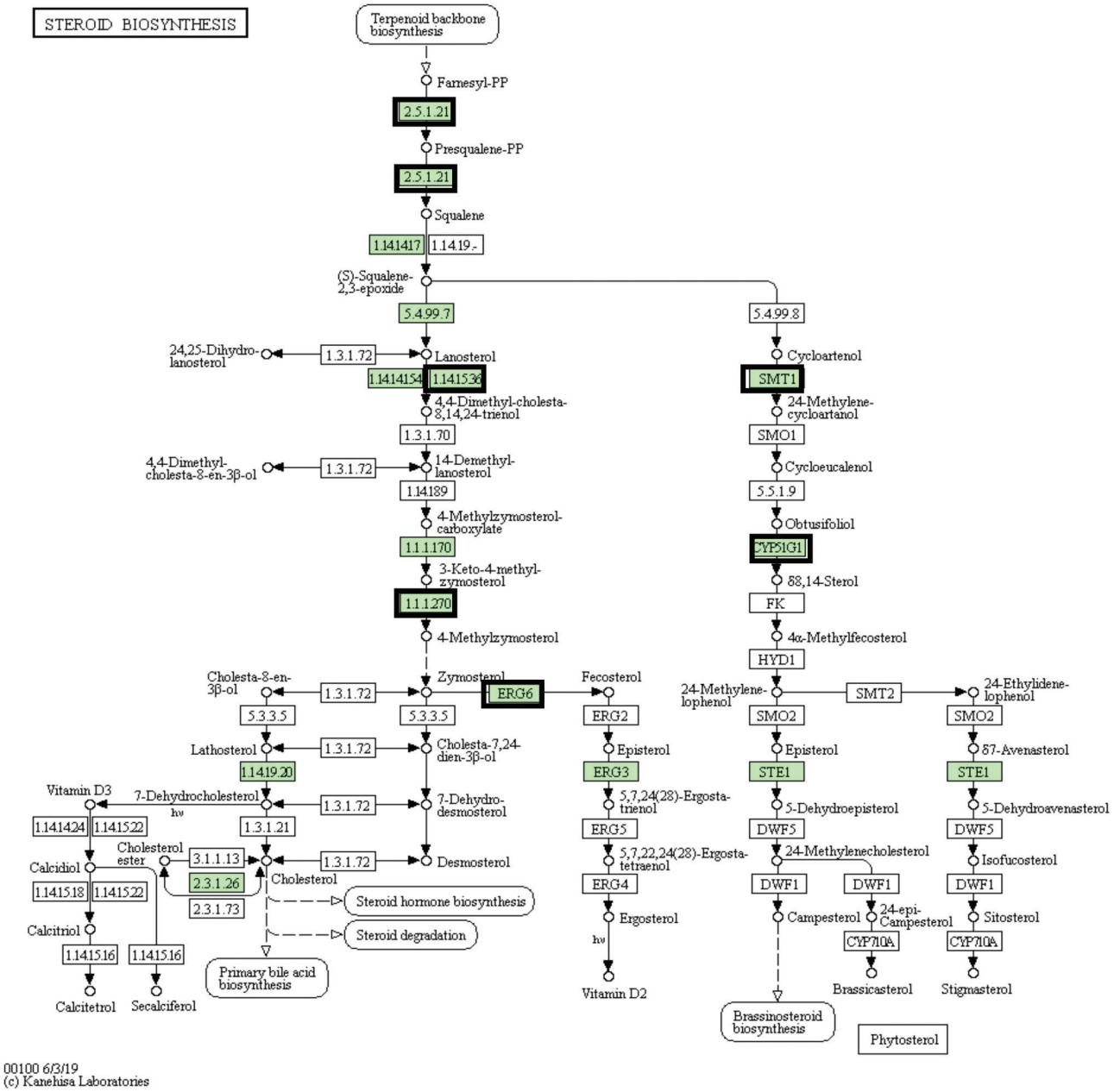

Fig. 7. KEGG analysis of proteins changed in abundance in the steroid biosynthesis pathway in *C. albicans*. Proteins that are highlighted in black are elevated in abundance in *C. albicans* treated with atorvastatin.

The results presented here indicate that atrovastatin has a profound effect on *C. albicans*, reducing its ability to grow and increasing its susceptibility to killing in the *G. mellonella* larval model system. While the anti-microbial activity of statins are well established, this work demonstrates how they interact with the fungal proteome to induce their effects and highlights their ability to retard proliferation *in vivo*. The continuing problems associated with existing antifungal therapies (e.g. toxicity and drug resistance) make the possibility of employing an active, non-toxic therapy such as atorvastatin to supplement existing therapies particularly attractive [5, 6].

Funding information

A. A. is the recipient of a PhD Scholarship from the Libyan Ministry of Education and G. S. is the recipient of a Maynooth University Doctoral studentship. The Q-exactive mass spectrometer was funded under the SFI Research Infrastructure Call 2012; Grant Number: 12/RI/2346 (3).

Acknowledgements
A. A. acknowledges the support of the Libyan Ministry of Education and G. S. is the recipient of a Maynooth University Doctoral studentship. This publication has emanated from research supported in part by a research grant from Science Foundation Ireland (SFI) and is co-funded under the European Regional Development Fund under grant number 12/RC/2275_P2.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Mayer FL, Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. *Virulence* 2013;4:119–128.
2. Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev* 2003;67:400–428.
3. Calderone RA, Fonzi WA. Virulence factors of *Candida albicans*. *Trends Microbiol* 2001;9:327–335.
4. Scorzoni L, de Paula e Silva ACA, Marcos CM, Assato PA, de Melo WCMA *et al*. Antifungal therapy: new advances in the understanding and treatment of mycosis. *Front Microbiol* 2017;08.
5. Nyilasi I, Kocsubé S, Krizsán K, Galgóczy L, Pesti M *et al*. *In vitro* synergistic interactions of the effects of various statins and azoles against some clinically important fungi. *FEMS Microbiol Lett* 2010;307:175–184.
6. Galgóczy L. Statins as antifungal agents. *World J Clin Infect Dis* 2011;1:4.
7. Macreadie IG, Johnson G, Schlosser T, Macreadie PI. Growth inhibition of *Candida* species and *Aspergillus fumigatus* by statins. *FEMS Microbiol Lett* 2006;262:9–13.
8. Westermeyer C, Macreadie IG. Simvastatin reduces ergosterol levels, inhibits growth and causes loss of mtDNA in *Candida glabrata*. *FEMS Yeast Res* 2007;7:436–441.
9. Sun H-Y, Singh N. Antimicrobial and immunomodulatory attributes of statins: relevance in solid-organ transplant recipients. *Clin Infect Dis* 2009;48:745–755.
10. Spanakis EK, Kourkoumpetis TK, Livanis G, Peleg AY, Mylonakis E. Statin therapy and decreased incidence of positive Candida cultures among patients with type 2 diabetes mellitus undergoing gastrointestinal surgery. *Mayo Clin Proc* 2010;85:1073–1079.
11. Donnino MW, Cocchi MN, Howell M, Clardy P, Talmor D *et al*. Statin therapy is associated with decreased mortality in patients with infection. *Acad Emerg Med* 2009;16:230–234.
12. Dobesh PP, Klepser DG, McGuire TR, Morgan CW, Olsen KM. Reduction in mortality associated with statin therapy in patients with severe sepsis. *Pharmacotherapy* 2009;29:621–630.
13. Kruger PS. Statins: the next anti-endotoxin. *Crit Care Resusc* 2006;8:223–226.
14. Weitz-Schmidt G. Statins as anti-inflammatory agents. *Trends Pharmacol Sci* 2002;23:482–487.
15. Sheehan G, Nagl M, Kavanagh K. Exposure to N-chlorotaurine induces oxidative stress responses in *Aspergillus fumigatus*. *J Med Microbiol* 2019;68:279–.
16. Arthington-Skaggs BA, Jradi H, Desai T, Morrison CJ. Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*. *J Clin Microbiol* 1999;37:3332–3337.
17. Sheehan G, Bergsson G, McElvaney NG, Reeves EP, Kavanagh K. The human cathelicidin antimicrobial peptide LL-37 promotes the growth of the pulmonary pathogen *Aspergillus fumigatus*. *Infect Immun* 2018;IAI:00097–18.
18. Murphy AR, Kavanagh KA. Adherence of clinical isolates of Saccharomyces cerevisiae to buccal epithelial cells. *Med Mycol* 2001;39:123–127.
19. Sheehan G, Clarke G, Kavanagh K. Characterisation of the cellular and proteomic response of Galleria mellonella larvae to the development of invasive aspergillosis. *BMC Microbiol* 2018;18:1–11.
20. Sheehan G, Kavanagh K. Analysis of the early cellular and humoral responses of *Galleria mellonella* larvae to infection by *Candida albicans*. *Virulence* 2018;9:163–172.
21. Côté RG, Griss J, Dianes JA, Wang R, Wright JC *et al*. The proteomics identification (pride) converter 2 framework: an improved suite of tools to facilitate data submission to the pride database and the ProteomeXchange Consortium. *Mol Cell Proteomics* 2012;11:1682–1689.
22. Manzoni M, Rollini M. Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs. *Applied Microbiology and Biotechnology* 2002;58:555–564.
23. Ramkumar S, Raghunath A, Raghunath S. Statin therapy: review of safety and potential side effects. *Acta Cardiologica Sinica* 2016;32:631–639.
24. Chow SC. Immunomodulation by statins: mechanisms and potential impact on autoimmune diseases. *Arch Immunol Ther Exp* 2009;57:243–251.
25. Zeiser R. Immune modulatory effects of statins. *Immunology* 2018;154:69–75.
26. Forero-Peña DA, Gutierrez FRS. Statins as modulators of regulatory T-cell biology. *Mediators Inflamm* 2013;2013:167086–.
27. Stancu C, Sima A. Statins: mechanism of action and effects. *J Cell Mol Med* 2001;5:378–387.
28. Song J, Zhai P, Zhang Y, Zhang C, Sang H *et al*. The *Aspergillus fumigatus* damage resistance protein family coordinately regulates ergosterol biosynthesis and azole susceptibility. *MBio* 2016;7.
29. Pendrak ML, Klotz SA. Adherence of *Candida albicans* to host cells. *FEMS Microbiology Letters* 1995;129:103–113.
30. Brown AJP, Leach MD, Nicholls S. The relevance of heat shock regulation in fungal pathogens of humans. *Virulence* 2010;1:330–332.
31. Pella D, Rybar R, Mechirova V. Pleiotropic effects of statins. *Acta Cardiologica Sinica* 2005;21:190–198.
32. Liao JK. Isoprenoids as mediators of the biological effects of statins. *J Clin Invest* 2002;110:285–288.
33. Ghittoni R, Patrussi L, Pirozzi K, Pellegrini M, Lazzerini PE *et al*. Simvastatin inhibits T-cell activation by selectively impairing the function of Ras superfamily GTPases. *Faseb J* 2005;19:605–607.
34. Cordle A, Koenigsknecht-Talboo J, Wilkinson B, Limpert A, Landreth G. Mechanisms of statin-mediated inhibition of small G-protein function. *J Biol Chem* 2005;280:34202–34209.
35. Brennan M, Thomas DY, Whiteway M, Kavanagh K. Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae. *FEMS Immunol Med Microbiol* 2002;34:153–157.
36. Fuchs BB, Mylonakis E. Using non-mammalian hosts to study fungal virulence and host defense. *Curr Opin Microbiol* 2006;9:346–351.
37. Maurer E, Browne N, Surlis C, Jukic E, Moser P *et al*. *Galleria mellonella* as a host model to study *Aspergillus terreus* virulence and amphotericin B resistance. *Virulence* 2015;6:591–598.
38. Kavanagh K, Sheehan G. The use of *Galleria mellonella* larvae to identify novel antimicrobial agents against fungal species of medical interest. *J Fungi* 2018;4:113.
39. Rahal EA, Constantin WN, Zeidan N, Abdelnoor AM. Atorvastatin reduces the survival of *Candida albicans*-Infected BALB/c mice. *Front Microbiol* 2015;6:1474.
40. Tashiro M, Kimura S, Tateda K, Saga T, Ohno A *et al*. Pravastatin inhibits farnesol production in *Candida albicans* and improves survival in a mouse model of systemic candidiasis. *Med Mycol* 2012.
41. Cuervo G, Garcia-Vidal C, Nucci M, Puchades F, Fernández-Ruiz M *et al*. Effect of statin use on outcomes of adults with candidemia. *PLoS One* 2013;8:e77317.
42. Forrest GN, Kopack AM, Perencevich EN. Statins in candidemia: clinical outcomes from a matched cohort study. *BMC Infect Dis* 2010;10:152.