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Exposure to N-chlorotaurine induces oxidative stress responses in *Aspergillus fumigatus*

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Abstract

Purpose. The neutrophil-derived oxidant N-chlorotaurine (NCT) displays remarkable *in vivo* tolerability and efficacy against a range of pathogens. The aim of this study was to characterize the response of the pulmonary pathogen *Aspergillus fumigatus* to NCT.

Methodology. The effect of NCT on the growth and viability of *A. fumigatus* was characterized. NCT-induced alteration of amino acids and gliotoxin from *A. fumigatus* mycelium was assessed. Label-free shotgun quantitative proteomic analysis was performed on *A. fumigatus* exposed to NCT for 24 h.

Results. Incubation of *A. fumigatus* with NCT at concentrations ranging from 6.8 to 55 mM decreased conidial growth and viability, and mycelium biomass relative to the controls. Exposure to NCT (13.77 mM) resulted in increased amino acids and gliotoxin levels from *A. fumigatus* mycelium. Exposure of *A. fumigatus* mycelium to NCT (6.8 mM) revealed an enrichment in proteins associated with the ribosome, transcription and translation and non-ribosomal peptide biosynthesis (e.g. Pes1, Pes3), which play an essential role in oxidative stress resistance in *A. fumigatus*. A decrease in the abundance of proteins associated with fumagillin and pseurotin biosynthesis highlighted the anti-virulence activity of NCT.

Conclusion. These results indicate that NCT induces an oxidative stress response in *A. fumigatus* as evidenced by alterations in the proteome and inhibits conidial and mycelial growth. Clinical investigations of topical application of NCT to treat *Aspergillus* infections are encouraged.

INTRODUCTION

Aspergillus fumigatus is a ubiquitous filamentous fungus that produces small (3–5 µm) airborne conidia, which are readily dispersed into the surrounding environment and inhaled [1]. *A. fumigatus* is capable of inducing a wide range of diseases, which are dependent upon the host immune status. Colonization, saprophytic and allergic disease are generally observed in the immune-defective host [e.g. asthmatic, cystic fibrosis (CF)], while invasive aspergillosis is observed in neutropenic and immune-suppressed patients, and carries a high mortality rate (30–95 %) if quick and effective anti-fungal therapy is not commenced [2, 3].

In the immunocompetent host, non-specific mucociliary clearance and a robust and efficient humoral (Pentraxin 3, complement proteins, collectins, ficolins, antimicrobial peptides) and cellular (neutrophils, alveolar macrophages, type II pneumocytes) response eliminate the fungal threat [2]. However, when the immune system becomes compromised *A. fumigatus* conidia germinate, produce hyphae and grow between and through alveolar epithelial cells by the production of a range of tissue-degrading enzymes and immunosuppressive toxins. *A. fumigatus* virulence is multifaceted and includes the production of low molecular weight toxins, proteolytic enzymes, thermotolerance, immune detoxification mechanisms and metabolic adaptations [1, 4]. The hyphae of *A. fumigatus* produce gliotoxin and fumagillin and these have been shown to inhibit fungicidal activity of neutrophils [5, 6]. Gliotoxin has pleotropic immune-suppressive activities and can induce macrophage apoptosis, inactivation of neutrophil NADPH-oxidase and has been implicated in destruction of lung tissue during invasive aspergillosis [7, 8]. *A. fumigatus* also produces several non-ribosomal peptides (NRP) such as pes1 and pes3 [9, 10].

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Keywords: N-chlorotaurine; antiseptic; Antimicrobial; anti-infective; *Aspergillus fumigatus*.

Abbreviations: CF, cystic fibrosis; CGD, chronic granulomatous disease; FCS, fetal calf serum; FDR, false discovery rate; HOCl, hypochlorous acid; LFQ, label-free quantification; MEM, minimal essential media; NCT, N-chlorotaurine; NRP, non-ribosomal peptide; PCA, principle component analysis; ROS, reactive oxygen species.

Five supplementary figures and two supplementary tables are available with the online version of this article.

The former (pes1) plays a role in fungal tolerance to oxidative stress, mediated by the conidial phenotype [9].

The importance of an effective neutrophil response to *A. fumigatus* was characterized in several studies that demonstrated that neutrophil depletion in mice resulted in higher mortality rates [11, 12]. Neutrophils employ both oxidative and non-oxidative mechanisms to eradicate conidia, germlings and hyphae of *A. fumigatus* by phagocytosis, degranulation of proteolytic enzymes and antimicrobial peptides and the production of NADPH-oxidase-mediated reactive oxygen species (ROS) [13]. The importance of a functional NADPH oxidase is highlighted in chronic granulomatous disease, characterized by mutations in $p47^{phox}$ and thus defective cellular oxidative killing mechanisms. NADPH-oxidase defective ($gp91^{phox-/-}$) mice are unable to inhibit conidial germination and their lung tissue displays expansive hyphal proliferation and tissue invasion as well as neutrophil aggregates, not observed in wild-type mice [14, 15].

Activated neutrophils through a series of enzymatic reactions produce superoxide and hydrogen peroxide with NADPH oxidase and superoxide dismutase, respectively, and hypochlorous acid (HOCl) is formed from hydrogen peroxide (H_2O_2) and chloride by myeloperoxidase [16]. HOCl in the presence of taurine results in the formation of N-chlorotaurine (NCT; Cl-HN-CH_2-CH_2-SO_3H) [17, 18]. NCT is the most abundant representation of this class of compounds and is a long-lived oxidant [19]. Chloramine has been detected in supernatants from stimulated granulocytes at concentrations ranging from 30 to 100 µM [17]. NCT is the main constituent of these compounds, reaching concentrations of between 10 to 50 µM [19, 20]. NCT is bactericidal, fungicidal, virucidal and vermicidical at concentrations ranging from 100 µM to 55 mM and non-cytotoxic up to 0.5 mM [21, 22]. Bronchial exudates from CF patients display chloramine concentrations of 118±25 µM [23], indicating a high level of NCT present extracellularly *in vivo*, which is continually replenished by activated neutrophilic and eosinophilic granulocytes, monocytes and, in small amounts, probably macrophages [24, 25]. The microbicidal activity of NCT is enhanced in CF sputum medium against a range of common CF pathogens [26]. NCT is non-toxic and well tolerated. For example, inhalation and topical application to the skin, eye, middle ear, oral cavity and bladder with 1 % (10 mg ml^{-1}; 55 mM) appears to be safe [21]. NCT eradicated bacterial conjunctivitis within 5 days in a Phase IIa open pilot study [27]. Furthermore, NCT was superior to a combination of neomycin, polymyxin B and hydrocortisone at decreasing inflammation and infection of external otitis [28]. NCT (1 %; 55 mM) results in a>5 log_{10}-fold reduction in the c.f.u. of MRSA, *Streptococcus pyogenes, Escherichia coli and Pseudomonas aeruginosa* after 1 h incubation [21]. NCT significantly deceased the viability of medically important fungi including *Candida albicans* (up to 4.7 log_{10}-fold reduction in c.f.u.) and *A. fumigatus* (up to 2.6 log_{10}-fold reduction in c.f.u.) after 4 h exposure [21]. NCT decreased virulence factor production and activity from *C. albicans* (secreted aspartyl proteinases) and *A. fumigatus* (gliotoxin), respectively, before viability of these fungi was impaired probably as a result of oxidative stress interfering with protein synthesis [29, 30]. Moreover, *Scedosporium spp*. lost virulence in the *Galleria mellonella* model after sublethal incubation time in NCT [31]. A similar effect has been observed with *Staphylococci* and *Streptococci* [26, 32]. The activity of NCT is augmented in the presence of hydrogen peroxide [33].

Neutrophils are the first line of defense against *A. fumigatus* and this is demonstrated by the high incidence of invasive aspergillosis in chronic granulomatous disease (CGD) patients who lack functional neutrophils [34]. *A. fumigatus* is engulfed into the phagosome and encounters NCT during the oxidative killing process. *A. fumigatus* comes into contact with NCT in CF bronchoalveolar lavage fluid. *A. fumigatus* has extensive mechanisms to circumvent and detoxify PMNs killing including oxidative mechanisms [35]. In order to elucidate the role of NCT in the human defense system and its potential for topical application as an anti-infective, it was the aim of this study to determine its mode of action on *A. fumigatus* and the response of this pulmonary pathogen.

METHODS

Chemicals and reagents

N-chlorotaurine (NCT) (molecular mass 181.57 g mol^{-1}, lot 2015-02-05) was prepared as a crystalline sodium salt at pharmaceutical grade in our laboratory, as reported previously [36], stored at −20 °C, and freshly dissolved in sterile distilled water to a concentration of 0.68, 6.8, 13.77 mM etc. for each experiment.

A. fumigatus culture conditions

A. fumigatus ATCC 26933, ATCC 46645, Af293 were obtained from the American Type Culture Collection and grown in sabouraud dextrose broth (SAB) or minimal essential media supplemented with 5 % v/v fetal calf serum at 37 °C and 200 r.p.m. Stocks were grown (for 3 days) and maintained on malt extract agar (MEA) (Oxoid) and used within 5 days.

Impact of N-chlorotaurine on the viability of *A. fumigatus* conidia (quantitative killing assay)

A. fumigatus (ATCC 26933) conidia (5×10^7) were incubated in 1 ml PBS supplemented with NCT (final concentrations; 0.68–68 mM) for 0, 1, 4, 24 h at 37 °C. Aliquots (20 µl) were taken and serially diluted at each time point, and 100 µl of diluted sample was spread onto MEA agar plates. Resulting colonies were counted and expressed in terms of % growth relative to the control, (n=3). The detection limit was 10^2 c.f.u. ml^{-1} after 1 h incubation. Because of rapid inactivation of NCT after the dilution on the plates within

3 min [31], no inactivation substance was necessary after these long incubation times.

Assessment of effect of N-chlorotaurine on *A. fumigatus* conidia

MEA plates containing sporulating *A. fumigatus* (ATCC 26933, ATCC 46645, Af293) colonies were washed with 0.1 % (v/v) Tween 80 (Merck) in PBS (pH 7.2) (Sigma Aldrich) to isolate conidia. Conidia were washed twice in sterile PBS, centrifuged (1500 ***g***, 5 min in a Beckman GS-6 centrifuge) and enumerated using a haemocytometer. NCT was serially diluted in growth medium (SAB or MEM +5 % FCS; 0.21 mM to 55 mM) on a 96-well plate (Corning Costar) and *Aspergillus* conidia (1×10^4 well^{-1}) were added to each well. Plates were incubated at 37 °C and growth was quantified by measuring readings at OD 570 (Bio-Tek Synergy HT) at 48 h, (n=3).

Inhibition of growth of *A. fumigatus* hyphae by N-chlorotaurine (biomass evaluation)

Flasks containing SAB (25 ml) were inoculated with 2.5×10^6 *A. fumigatus* (ATCC 26933, ATCC46645, Af293) conidia to give a density of 1×10^5 ml^{-1} and incubated at 37 °C and 200 r.p.m. for 24 h. Cultures were supplemented with 1 ml of NCT PBS solution or 1 ml PBS (control) for a further 24 h when hyphal wet weights were determined, (n=4).

Effect of N-chlorotaurine on *A. fumigatus* mycelium membrane permeability

Flasks containing SAB (25 ml) were inoculated with 2.5×10^6 *A. fumigatus* (ATCC 26933, ATCC46645, Af293) conidia to give a density of 1×10^5 ml^{-1} and incubated at 37 °C and 200 r.p.m. for 24 h. Cultures were supplemented with NCT (0.68–13.77 mM) or PBS (control) and incubated for a further 24 h, (n=4). *A. fumigatus* cultures were filtered using mira-cloth and 0.22 µm cellulose filters (Sarstedt). Amino acid leakage by *A. fumigatus* was determined using the ninhydrin colorimetric method and expressed in terms of aspartic acid and glutamic acid [37]. Ninhydrin (Sigma-Aldrich) was dissolved in ethanol to give a final concentration of 0.35 % (w/v) and 250 µl was added to each culture filtrate 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). Gliotoxin extraction from culture medium was performed as described [37]. Filtered culture supernatants were mixed with an equal volume (20 ml) of chloroform (Hyper Solv; BDH) for 2 h. For extraction of intracellular gliotoxin, mycelium was washed in PBS, ground to a fine powder with liquid N_2 in a pre-chilled pestle and mortar, resuspended in 10 ml 6 M HCl for 10 mins followed by the addition of chloroform (20 ml) to the mixture for 2 h, stirring at room temperature. The chloroform fraction was collected and evaporated to dryness in a Büchi (Brinkmann Instruments; Westbury, NY) rotor evaporator. Dried extracts were dissolved in 500 µl methanol (Hyper Solv, BDH) and stored at −70 °C.

Quantification of gliotoxin by RP-HPLC

Gliotoxin was detected by reversed phase-HPLC (Spectra-Physics). The mobile phase was 34.9 % (v/v) acetonitrile (Hyper Solv, BDH), 0.1 % (v/v) trifluoroacetic acid (Sigma Aldrich) and 65 % (v/v) ddH_2O. Gliotoxin extract (20 µl) was injected onto a C18 Hewlett Packard column. A standard curve of peak area versus gliotoxin concentration was constructed using gliotoxin standards (0.1, 0.2, 0.5, 1.0 µg 10 µl^{-1}) dissolved in methanol (Sigma Aldrich).

Whole cell protein extraction from *A. fumigatus*

Mycelium (1 g) was ground to a fine powder in a pestle and mortar with liquid nitrogen followed by the addition of 6 ml of protein extraction buffer [0.4 M NaCl, 10 mM Tris HCl, 2 mM EDTA, 10 % (v/v) glycerol] supplemented with aprotinin (10 µg ml^{-1}), peptstatin A (10 µg ml^{-1}), TLCK (10 µg ml^{-1}) leupeptin (10 µg ml^{-1}). The suspension was disrupted using a sonication probe (Bandelin Sonopuls, Bandelin electronic, Berlin) at 20 % power, cycle 6 for 10 s. This was repeated twice with the sample being cooled on ice between each sonication. Protein supernatants were obtained by centrifugation (10 000 ***g***, 4 °C for 20 min).

Label-free quantitative proteomics of *A. fumigatus* protein lysate

Label-free shotgun quantitative proteomic analysis was performed on protein extracted from control and NCT (6.8 mM) treated *A. fumigatus* (ATCC 26933) hyphae for 24 h at 37 °C in SAB medium, (n=3). As described elsewhere [37], protein (75 µg) was reduced with dithiotreitol (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 (ThermoFisher Scientific, USA) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system.

Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.6.0.16; http://maxquant.org/) to correlate the data against an annotated database derived from the *A. fumigatus* Af293 Reference proteome downloaded from Uniprot [38, 39].

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. Statistical differences between the NCT treated and control *A. fumigatus* hyphal proteomes were tested using ANOVA and t-tests with false discovery rate (FDR) correction (Benjamini-Hochberg). P-values of <0.05 were considered significant. Proteins that had non-existent values (indicative of absence or very low abundance in a sample) were included in the study only when they were completely absent from one group and present in at least two of the three replicates in the second group. Identified proteins were grouped into

functional categories based on the FunCat (Functional Catalogue) and GO (Gene Ontology) annotations, using the FungiFun application [40].

Statistical analysis

All experiments were performed on at least three independent occasions and results are expressed as the mean±standard error. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test for susceptibility and flask assays (wet weight and gliotoxin) with values of $P<0.05$ considered statistically significant as compared to controls. All statistical analysis listed was performed using GraphPad Prism [v 6.0].

Data availability

The MS proteomics data and MaxQuant search output files have been deposited to the ProteomeXchange Consortium [41] via the PRIDE partner repository with the dataset identifier PXD009853.

RESULTS

Effects of N-chlorotaurine on the growth and viability of *A. fumigatus*

The growth response of *A. fumigatus (*ATCC 26933) to NCT was assessed *in vitro* using a susceptibility assay. Exposure of *A. fumigatus* to 6.8 and 55 mM NCT reduced growth to 6.42±0.43% and 5.22±0.43 %, respectively, relative to the control in SAB after 48 h ($P<0.001$). A concentration of 3.44 mM reduced growth by 9.83±3.64 %, ($P<0.05$) (Fig. 1). NCT at 55 mM reduced growth of *A. fumigatus* in minimal essential media (MEM) +5 % fetal calf serum (FCS) to 70.67 ±1.29 %, and 3.4 mM NCT reduced growth to 78.17 ±11.01 %, but this was less in MEM +5 % FCS as compared to SAB, (Fig. S1a, available in the online version of this article). NCT also reduced the growth of other *A. fumigatus* strains (ATCC46645 and Af293) at concentrations ranging from 3.44 to 55 mM (Fig. S1b and c). The effect of NCT on the growth *A. flavus* was also determined and 55 mM NCT reduced growth to 6.84±0.04 % ($P<0.0001$) and 3.44 mM NCT reduced growth to 28.37±1.81 % ($P<0.0001$) compared to the control (98.74±4.49 %), (Fig. S1d).

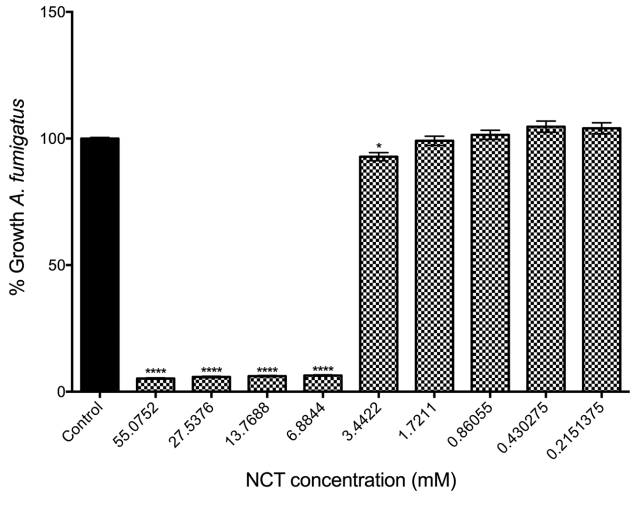

Fig. 1. Susceptibility of *A. fumigatus* ATCC 26933 conidia (initial concentration 10^4 well^{-1}) to N-chlorotaurine at concentrations ranging from 0.21 to 55 mM after 48 h growth in Sabouraud medium as determined by photometric analysis. N-chlorotaurine significantly reduced the growth of *A. fumigatus* at concentrations between 55 and 6.8 mM as compared to the control, (*, $P<0.05$; ****, $P<0.0001$).

To assess the effect of NCT on mycelium biomass accumulation, 24 h preformed mycelium was supplemented with NCT (0.68 to 13.77 mM) for a further 24 h. A NCT concentration of 0.68 mM reduced biomass formation (0.75 ±0.06 g/ (25 ml culture)$^{-1}$, $P<0.01$). A concentration of 6.8 (0.64±0.04 g/ (25 ml culture)$^{-1}$, $P<0.001$) and 13.77 mM (0.18±0.009 g, $P<0.0001$) resulted in a statistically significant decrease in mycelium wet weight compared to the control (1.03±0.055 g), (Fig. 2). This decrease in mycelium biomass was also noted with *A. fumigatus* strains (ATCC 46645 and Af293) and *A. flavus*. A concentration of 13.77 and 0.68 mM reduced mycelium biomass of *A. fumigatus* ATCC 46645 to 0.138±0.004, ($P<0.05$) and 0.637±0.03 g/ (25 ml culture)$^{-1}$, ($P<0.0001$), respectively, as compared to the control (0.741±0.008), (Fig. 2a). NCT (13.77 mM) significantly reduced the biomass accumulation of *A. fumigatus* Af293 to 0.319±0.05 g/ (25 ml culture)$^{-1}$, ($P<0.01$) as compared to the relative control (0.682±0.03 g), (Fig. S2b). Furthermore, NCT also reduced the mycelium wet weight of *A. flavus* at 13 mM (0.531±0.03, $P<0.01$) and 6.8 mM (0.656±0.01, $P<0.01$) as compared to control mycelium ([0.857±0.003 g/ (25 ml culture)$^{-1}$]), (Fig. S2c).

Incubation of *A. fumigatus* (ATCC 26933) conidia in NCT for 1, 4 and 24 h at concentrations ranging from 6.8 to 68 mM decreased the viability of conidia in a dose- and time-dependent manner. Incubation of conidia in PBS supplemented with 68 mM NCT reduced viability at 1 (28.73 ±7.78 %, $P<0.05$), 4 (0±0.0007 %, $P<0.05$) and 24 (0 % ±0.0034 %, $P<0.05$) h and incubation in 6.8 mM NCT reduced viability at 1 (78.4±2.89 %), 4 (54.67±3.46 %, $P<0.05$) and 24 (15.78±2.32 %, $P<0.05$) h (Fig. 3).

N-chlorotaurine induces alterations in amino acids and gliotoxin from *A. fumigatus*

To assess the effect of NCT on membrane permeability, the amount of extracellular amino acids and gliotoxin levels following treatment with varying concentrations of NCT (0.68–13.77 mM) were determined via the ninhydrin colorimetric assay and RP-HPLC, respectively. NCT (13.7 mM) significantly increased amino acid leakage (8.82±1.72 µg mg^{-1} mycelium, $P<0.0001$) as compared to the control (0.24±0.01 µg mg^{-1} mycelium), (Fig. 4).

A. fumigatus (ATCC 26933) mycelium treated with 6.8 mM NCT [25.67±0.43 ng (mg mycelium)$^{-1}$, $P<0.0001$] and 0.68 mM [38.38±1.55 ng (mg mycelium)$^{-1}$ $P<0.001$] NCT for 24 h displayed significantly less extracellular gliotoxin as compared to control cells [64.15±3.34 ng (mg mycelium)$^{-1}$]

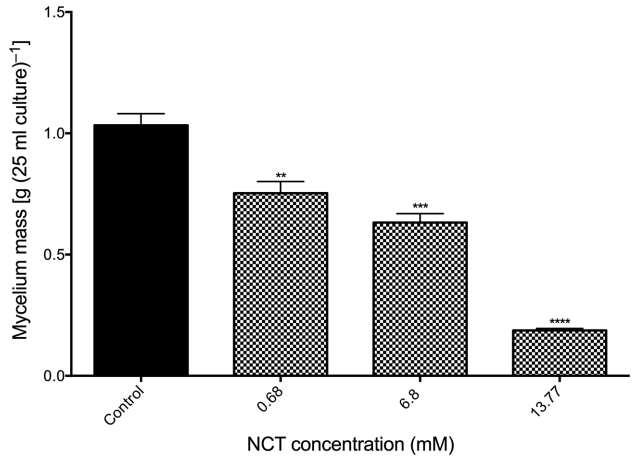

Fig. 2. The effect of N-chlorotaurine on growth of *A. fumigatus* ATCC 26933. A 24 h culture of *A. fumigatus* (10^5 ml^{-1}) in Sabouraud dextrose broth was supplemented with N-chlorotaurine (0.68, 6.8 and 13.77 mM) or PBS (control). After 24 h, mycelia were weighed. N-chlorotaurine dose dependently decreased hyphal mass significantly after 24 h exposure, (**; P<0.01, ***; P<0.001, ****; P<0.0001).

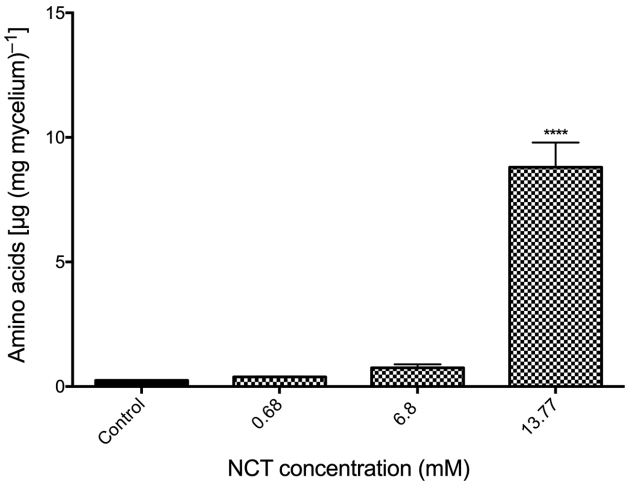

Fig. 4. Amino acid quantification in *A. fumigatus* ATCC 26933 supernatants exposed to N-chlorotaurine (0.68, 6.8 and 13.77 mM) or PBS (control) for 24 h was determined by ninhydrin colorimetric assay. N-chlorotaurine (13.77 mM) significantly increased extracellular amino acids as compared to the control, (****; P<0.0001).

. However, exposure to 13.77 mM NCT resulted in a statistically significant increase in extracellular gliotoxin [88.19 ±1.98 ng (mg mycelium)$^{-1}$, P<0.001] (Fig. 5). Furthermore,

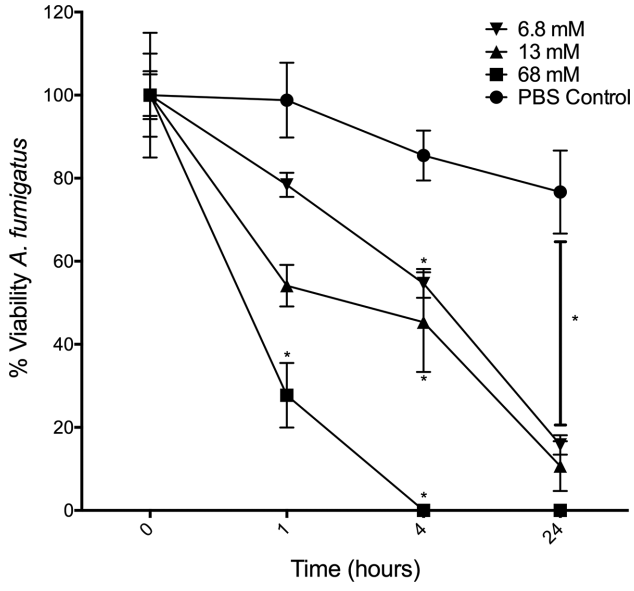

Fig. 3. The viability of *A. fumigatus* ATCC 26933 conidia (initial concentration; 5×10^7 ml^{-1}) after incubation in varying concentrations of N-chlorotaurine [6.8, 13, 68 mM (PBS)]) and PBS (control) for 0, 1, 4, 24 h. Cells were removed, diluted, plated at each time point and % viability calculated relative to the PBS 0 h control. N-chlorotaurine statically significantly and dose dependently reduced the viability of *A. fumigatus* conidia at a variety of concentrations over time as compared to the control, (*; P<0.05).

NCT (0.68 and 6.8 mM) also reduced extracellular gliotoxin levels in *A. fumigatus* (ATCC 46645) supernatants to 9.70 ±8.19 ng (mg mycelium)$^{-1}$, (P<0.01) and 18.56±11.78 ng (mg mycelium)$^{-1}$, (P<0.05) respectively, as compared to the control mycelium [77.78±12.35 ng (mg mycelium)$^{-1}$], (Fig. S3a). This effect was observed with *A. fumigatus* (Af293) with the same concentrations of NCT reducing extracellular gliotoxin levels to 2.64±1.49 ng (mg mycelium)$^{-1}$ and 13.81 ±4.13 ng (mg mycelium)$^{-1}$, respectively, as compared to the control [65.87±13.79 ng (mg mycelium)$^{-1}$, P<0.05], (Fig. S3b). For intracellular gliotoxin quantification a similar effect was observed. Treatment of *A. fumigatus* mycelium with 13.77 mM NCT resulted in a significant increase in intracellular gliotoxin [9.42±1.50 ng (mg mycelium)$^{-1}$, P<0.01] relative to control mycelium [2.39±1.18 ng (mg mycelium)$^{-1}$]. Interesting, no gliotoxin was detected within mycelium treated with 6.8 mM, (Fig. 5).

The whole cell proteomic response of *A. fumigatus* to N-chlorotaurine

Shotgun quantitative proteomic analysis was performed on *A. fumigatus* (ATCC 26933) treated with 6.8 mM NCT for 24 h as described. In total, 30 538 peptides were identified representing 1932 proteins with two or more peptides and 210 (*A. fumigatus* NCT-treated versus control *A. fumigatus*) proteins were determined to be differentially abundant (ANOVA, P<0.05) with a fold change of >1.5. A total of 44 proteins were deemed exclusive (i.e. with LFQ intensities present in all three replicates of one treatment and absent in all threereplicates of the other treatment). These proteins were also used in statistical analysis of the total differentially expressed group following imputation of the zero values as described. After data imputation, these proteins were

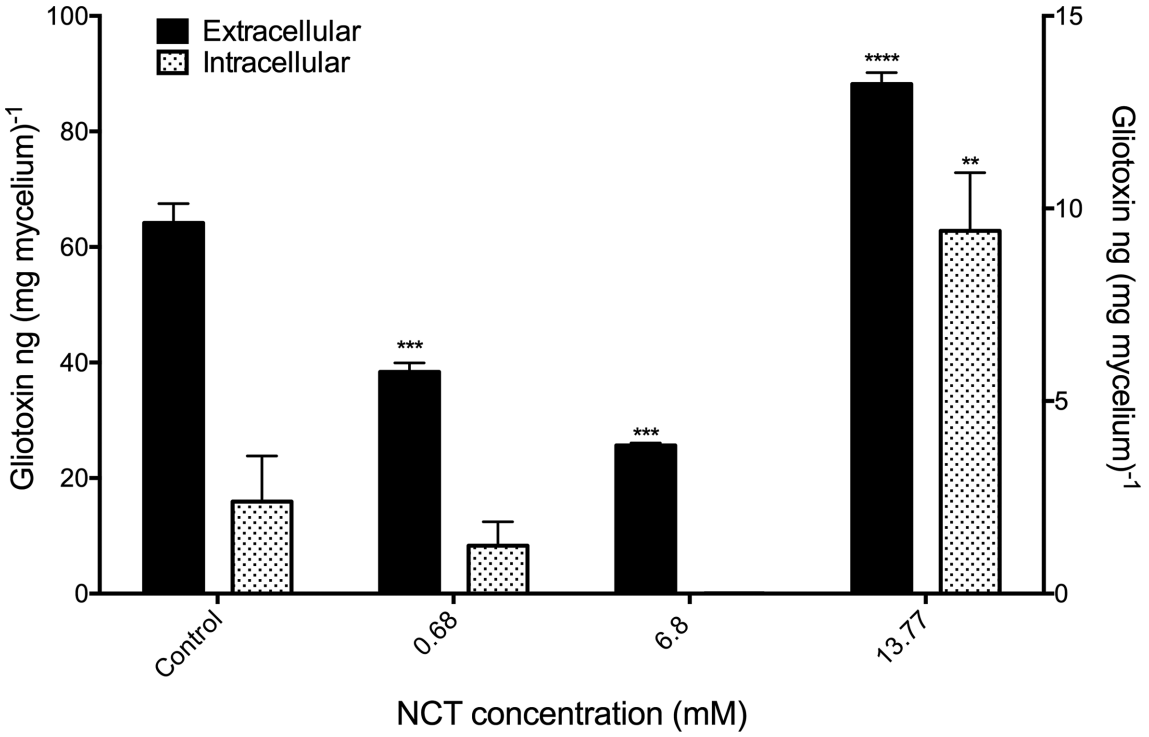

Fig. 5. Gliotoxin quantification (extracellular; closed bars and left *Y*-axis, intracellular; open bars and right *Y*-axis) in *A. fumigatus* ATCC 26933 exposed to N-chlorotaurine (0.68, 6.8 and 13.77 mM) or PBS (control) for 24 h was determined by RP-HPLC. N-chlorotaurine decreased gliotoxin levels at a concentration of 0.68 and 6.8 mM. However, N-chlorotaurine (13.77 mM) significantly increased both extracellular and intracellular gliotoxin levels as compared to the respective controls, (**; $P<0.01$, ***; $P<0.001$, ****; $P<0.0001$).

included in subsequent statistical analysis, (Fig. 6). A principal component analysis (PCA) performed on all filtered proteins distinguished the NCT-treated *A. fumigatus* and control *A. fumigatus* samples indicating a clear difference between each proteome (Fig. S4).

Functional (GO term and FunCat) analysis revealed enrichment for biological processes such as oxidation-reduction process, proteolysis and translation, while molecular functions such as nucleotide binding and oxidoreductase activity were significantly enriched within the SSDA dataset (Fig. S5a). FunCat categories associated with electron transport, nitrogen, sulfur and selenium metabolism, catabolism of nitrogenous compounds and sulfate assimilation confirm *A. fumigatus* mycelium responds to NCT by activation of a variety of processes (Fig. S5b).

Proteins that were increased in abundance in NCT-treated mycelium were enriched for ribosomal proteins [Ribosomal protein S28e (23.1-fold), ribosomal biogenesis protein gar2 (5.5-fold), ribosome assembly factor mrt4 (5.1-fold), 40S ribosomal protein S26 (2.2-fold), 60S ribosomal protein L35Ae (1.7-fold), 60S ribosomal protein L12 (1.6-fold), ribosomal protein L16a (1.6-fold), 60S ribosomal protein L5 (1.5-fold), 60S ribosomal protein L27 (1.5-fold)] and transcription and translation associated proteins [small nucleolar ribonucleoprotein complex subunit (4.6-fold), rRNA processing protein pwp1 (threefold), mRNA splicing factor (prp17) (2.9-fold), RNA cytidine acetyltransferase (2.3-fold), ATP-dependent RNA helicase has1 (2.2-fold), eEF-3 (1.9-fold), eEF-1B (1.8-fold), pre-mRNA-splicing factor (1.7-fold), rRNA biogenesis protein RRP5 (1.6-fold)]. There was also increased abundance of proteins associated with cell response to stress/drug [oxidoreductase, zinc-binding dehydrogenase family protein (11-fold), superoxide dismutase (Cu-Zn); (7.7-fold), hsp30-like (5.6-fold), hsp30/hsp42 (2.4-fold), nitroreductase family protein (2.2-fold), glutathione S-transferase GstA (1.7-fold), ecm33 protein (1.6-fold), MFS multidrug transporter (1.5-fold)], mitochondrion-associated proteins [cytochrome c oxidase subunit VIa (12.4-fold), cytochrome c oxidase subunit 2 (1.7-fold), cytochrome P450 phenylacetate 2-hydroxylase, putative (1.5-fold)] and secondary metabolite biosynthesis proteins (nonribosomal peptide synthetase 8, nonribosomal peptide synthetase 1, NRPS-like enzyme, fumipyrrole biosynthesis protein C), Table S1. Proteins that were decreased in abundance in response to NCT were associated with sterol biosynthesis [14-alpha sterol demethylase Cyp51A (1.5-fold)] and secondary metabolism [NRPS-like enzyme, putative (7.3-fold), fumagillin dodecapentaenoate synthase (6.5-fold), fumagillin biosynthesis methyltransferase (5.2-fold), fumagillin biosynthesis acyltransferase (fivefold), pseurotin

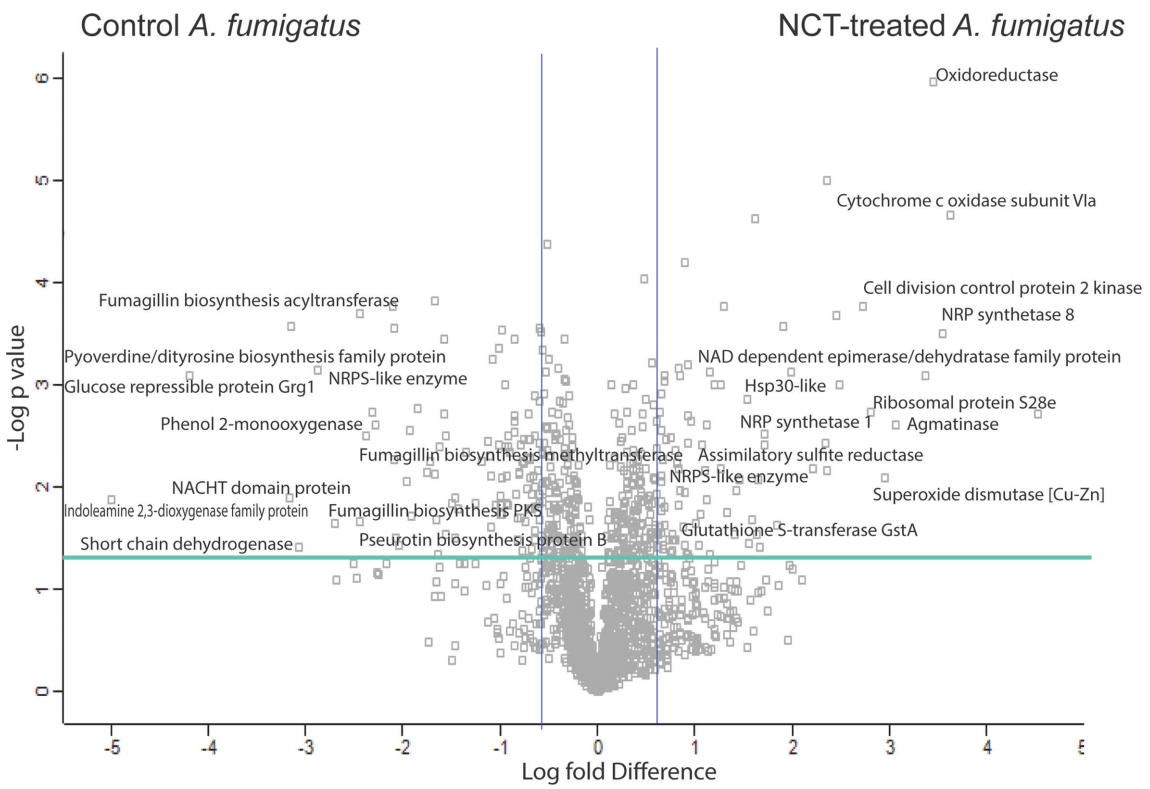

Fig. 6. Shotgun proteomics of 24 h preformed *A. fumigatus* ATCC 26933 mycelium exposed to N-chlorotaurine (6.8 mM) for 24 h. Volcano plots showing the distribution of quantified proteins according to *P*-value (−log10 *P*-value) and fold change (log2 mean LFQ intensity difference). Proteins above the 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. N-chlorotaurine increased the abundance of proteins associated with cellular response to drug, ribosomal biogenesis and secondary metabolism.

biosynthesis protein C (4.3-fold), NRP synthetase 14 (3.9-fold), fumagillin biosynthesis cluster P450 monooxygenase (3.6-fold), pseurotin biosynthesis protein F (3.0-fold)], Table S2.

DISCUSSION

In recent years it has become evident that environmental use of triazoles has selected for multiazole and panazole-resistant *A. fumigatus* isolates [42]. Mutations in the Cyp51A gene or its transcriptional enhancer in its promoter region is a common mechanism (50 to 80 %) related to altered or enhanced production of erg11 [43, 44]. *In vitro* data, animal models and some clinical data demonstrate that there may be a benefit of combination anti-fungal therapy in treating *Aspergillus* infections [45]. NCT shows broad spectrum antimicrobial activity, excellent tolerability *in vivo* and may be a potential anti-fungal therapy for superficial infections [21, 46, 47].

NCT significantly decreased the growth of *A. fumigatus* (ATCC 26933, ATCC46645, Af293) conidia in an *in vitro* susceptibility assay at concentrations ranging from 6.8 to 55 mM in sabouraud medium (Figs 1, S1-d,) and MEM supplemented with 5 % v/v FCS (Fig. S1) as compared to the relevant controls. The lower effect in MEM can be explained by reaction of NCT with reducing components of the FCS, which is connected with a loss of oxidative activity (chlorine consumption, [46, 48]). NCT reduced the biomass formation of *A. fumigatus* (ATCC 26933, ATCC46645, Af293) hyphae after 24 h at concentrations between 0.68 and 13.77 mM. Incubation of *A. fumigatus* conidia in NCT decreased their viability at 1, 4 and 24 h. The results are in agreement with the fungicidal activity of NCT against yeasts and moulds found in previous studies [29–31, 47, 49].

Within 1 min, NCT forms covalent chlorine covers (chlorination of the external protein matrix of the microbe) around *Staphylococcus aureus, Escherichia coli, P. aeruginosa* and *C. albicans* [50, 51], which is associated with a loss of virulence [29, 31, 32]. Longer incubation leads to cellular leakage and inactivation of pathogens [50]. In this work, incubation of 24 h preformed mycelium in NCT (13.77 mM) significantly increased extracellular amino acid levels indicating small molecule leakage from mycelium, which would damage the cells' ability to respire, grow and activate an oxidative stress response. Amino acid leakage is also observed with a variety of antimicrobials [52–55].

Interestingly, incubation of mycelium in 0.68 and 6.8 mM NCT decreased extracellular gliotoxin levels amongst *A. fumigatus* strains (**Figs 5, S3a and S3b).** This was observed previously where NCT diminished the activity of gliotoxin by reduction of the disulphide bridge, which is essential to its activity [30]. However, our results indicate that these NCT treatments also reduced intracellular gliotoxin levels (Fig. 5). Furthermore, NCT leads to decreased production of secreted aspartyl proteinases from *Candida* spp. [29]. Conversely, incubation of mycelium in the higher concentration of 13.77 mM NCT resulted in a significant increase in intracellular and extracellular gliotoxin concentration. At this concentration, reduced growth and increased gliotoxin production and secretion from *A. fumigatus* was observed. Therefore, we hypothesize that gliotoxin was liberated by active and/or passive mechanisms, through the damaged cell and that this effect overlays the reduced production of the toxin at low concentrations. Treatment of *A. fumigatus* with caspofungin and amphotericin resulted in a significant increase in intracellular and extracellular gliotoxin as a result of halted growth, which was observed in mycelium treated with 13.77 mM NCT [54, 56].

Shotgun proteomics was employed to determine the effect of NCT (6.8 mM) on *A. fumigatus* (ATCC 26933) after 24 h exposure. *A. fumigatus* responds to NCT by increasing the abundance of ribosomal proteins and proteins associated with transcription and translation. Shenton *et al.* 2006 found that a hydrogen peroxide treatment increased the number of ribosomal proteins, which may be utilized once the response to oxidative stress has subsided for transcriptional and translational remodelling [57].

There was also an increased abundance of proteins associated with cellular response to stress [e.g. oxidoreductase, superoxide dismutase (Cu-Zn), hsp30-like, hsp30/hsp42, nitroreductase family protein, glutathione s-transferase GstA, MFS multidrug transporter]. *A. fumigatus* responds to NCT by an increase in superoxide dismutase, which detoxifies superoxide anions, glutathione s-transferase, which quenches reactive hydroxyl free radicals, a range of heat shock proteins and nitroreductase family protein. This may be an attempt by the fungus to neutralize active chlorine compounds it would encounter within the phagosome.

There was an increase in the abundance of some secondary metabolism proteins which play a role in response to oxidative stress [e.g. nonribosomal peptide synthetase 1 (Pes1), non-ribosomal peptide synthetase 8 (Pes3)]. Pes1 confers protection against oxidative stress as the delta pes1 mutant showed attenuated virulence in *G. mellonella*, increased sensitivity to neutrophil mediated phagocytosis, which was mediated by changed conidial surface morphology and increased hydrophilicity [9]. Paradoxically, pes3 disruption augments *A. fumigatus* virulence (in *G. mellonella*) and increases fungal burden (murine pulmonary aspergillosis model), possibly due to defective innate immune recognition of *A. fumigatus in vivo* [10]. Pes3 deletion, on the other hand, increases susceptibility to voriconazole, produces shorter germlings and decreased surface beta glucan.

Following NCT treatment, there was a significant decrease in the abundance of a range of proteins involved in fumagillin and pseurotin biosynthesis (NRPS-like enzyme, fumagillin dodecapentaenoate synthase, pseurotin biosynthesis protein B, fumagillin biosynthesis methyltransferase, fumagillin biosynthesis acyltransferase, pseurotin biosynthesis protein C, NRP synthetase 14, fumagillin biosynthesis cluster P450 monooxygenase, pseurotin biosynthesis protein F). Fumagillin is a toxin produced during hyphal development in *A. fumigatus*. The toxin has well characterized amoebicidal activity, can retard the ciliary beat frequency of pulmonary epithelial cells, and can inhibit angiogenesis and microbicidal activity of human neutrophils and insect haemocytes [6, 58–60].

The results presented here indicate that NCT reduces the expression of a number of virulence factors of *A. fumigatus* similar to *Candida spp.* [29], before the killing of the fungus occurs. This may be caused by several pathways. Direct chlorination and oxidation reactions leading to abrogation of synthesis pathways are possible as well as consumption of most of the energy of the fungus by activation of anti-oxidative pathways. There was also a significant decrease in the azole target protein 14-alpha sterol demethylase (erg11, gene product of cyp51A), which is implicated amongst 50–80 % of *A. fumigatus* resistance isolates.

These results demonstrate that NCT is active against *A. fumigatus* and reduces growth, biomass formation and conidial viability. NCT reduces the gliotoxin levels from *A. fumigatus* at low, sublethal concentrations and causes increased intracellular and extracellular gliotoxin levels at higher ones. NCT triggers oxidative stress response in *A. fumigatus* and reduces the abundance of proteins associated with fumagillin and ergosterol biosynthesis. Understanding the mode of action of NCT is a good basis for both further elucidating its role in the human defense system and its potential clinical application for treatment of aspergillosis.

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

References

1. Dagenais TR, Keller NP. Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. *Clin Microbiol Rev* 2009;22:447–465.
2. Margalit A, Kavanagh K. The innate immune response to *Aspergillus fumigatus* at the alveolar surface. *FEMS Microbiol Rev* 2015;39:670–687.
3. Stevens DA, Moss RB, Kurup VP, Knutsen AP, Greenberger P *et al.* Allergic bronchopulmonary aspergillosis in cystic fibrosis-state of the art: Cystic Fibrosis Foundation Consensus Conference. *Clin Infect Dis* 2003;37:S225–S264.

4. Chotirmall SH, Al-Alawi M, Mirkovic B, Lavelle G, Logan PM *et al.* *Aspergillus*-associated airway disease, inflammation, and the innate immune response. *Biomed Res Int* 2013;2013:1–14.
5. Coméra C, André K, Laffitte J, Collet X, Galtier P *et al.* Gliotoxin from *Aspergillus fumigatus* affects phagocytosis and the organization of the actin cytoskeleton by distinct signalling pathways in human neutrophils. *Microbes Infect* 2007;9:47–54.
6. Fallon JP, Reeves EP, Kavanagh K. Inhibition of neutrophil function following exposure to the *Aspergillus fumigatus* toxin fumagillin. *J Med Microbiol* 2010;59:625–633.
7. Kwon-Chung KJ, Sugui JA. What do we know about the role of gliotoxin in the pathobiology of *Aspergillus fumigatus*? *Med Mycol* 2009;47:S97–S103.
8. Sugui JA, Pardo J, Chang YC, Zarember KA, Nardone G *et al.* Gliotoxin is a virulence factor of *Aspergillus fumigatus*: gliP deletion attenuates virulence in mice immunosuppressed with hydrocortisone. *Eukaryot Cell* 2007;6:1562–1569.
9. Reeves EP, Reiber K, Neville C, Scheibner O, Kavanagh K *et al.* A nonribosomal peptide synthetase (Pes1) confers protection against oxidative stress in *Aspergillus fumigatus*. *Febs J* 2006;273:3038–3053.
10. O'Hanlon KA, Cairns T, Stack D, Schrettl M, Bignell EM *et al.* Targeted disruption of nonribosomal peptide synthetase pes3 augments the virulence of *Aspergillus fumigatus*. *Infect Immun* 2011;79:3978–3992.
11. Schaffner A, Douglas H, Braude A. Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to Aspergillus. Observations on these two lines of defense in vivo and in vitro with human and mouse phagocytes. *J Clin Invest* 1982;69:617–631.
12. Mircescu MM, Lipuma L, van Rooijen N, Pamer EG, Hohl TM *et al.* Essential role for neutrophils but not alveolar macrophages at early time points following *Aspergillus fumigatus* infection. *J Infect Dis* 2009;200:647–656.
13. Gazendam RP, van Hamme JL, Tool AT, Hoogenboezem M, van den Berg JM *et al.* Human Neutrophils Use Different Mechanisms To Kill *Aspergillus fumigatus* Conidia and Hyphae: Evidence from Phagocyte Defects. *J Immunol* 2016;196:1272–1283.
14. Segal BH, Romani LR. Invasive aspergillosis in chronic granulomatous disease. *Med Mycol* 2009;47:S282–S290.
15. Dennis CG, Greco WR, Brun Y, Youn R, Slocum HK *et al.* Effect of amphotericin B and micafungin combination on survival, histopathology, and fungal burden in experimental aspergillosis in the p47phox-/- mouse model of chronic granulomatous disease. *Antimicrob Agents Chemother* 2006;50:422–427.
16. Klebanoff SJ. Myeloperoxidase: friend and foe. *J Leukoc Biol* 2005;77:598–625.
17. Test ST, Lampert MB, Ossanna PJ, Thoene JG, Weiss SJ *et al.* Generation of nitrogen-chlorine oxidants by human phagocytes. *J Clin Invest* 1984;74:1341–1349.
18. Zgliczyński JM, Stelmaszyńska T, Domański J, Ostrowski W. Chloramines as intermediates of oxidation reaction of amino acids by myeloperoxidase. *Biochim Biophys Acta* 1971;235:419–424.
19. Grisham MB, Jefferson MM, Melton DF, Thomas EL. Chlorination of endogenous amines by isolated neutrophils. Ammonia-dependent bactericidal, cytotoxic, and cytolytic activities of the chloramines. *J Biol Chem* 1984;259:10404–10413.
20. Weiss SJ, Klein R, Slivka A, Wei M *et al.* Chlorination of Taurine by Human Neutrophils. *J Clin Invest* 1982;70:598–607.
21. Gottardi W, Nagl M. N-chlorotaurine, a natural antiseptic with outstanding tolerability. *J Antimicrob Chemother* 2010;65:399–409.
22. Nagl M, Hess MW, Pfaller K, Hengster P, Gottardi W *et al.* Bactericidal activity of micromolar N-chlorotaurine: evidence for its antimicrobial function in the human defense system. *Antimicrob Agents Chemother* 2000;44:2507–2513.
23. Witko-Sarsat V, Delacourt C, Rabier D, Bardet J, Nguyen AT *et al.* Neutrophil-derived long-lived oxidants in cystic fibrosis sputum. *Am J Respir Crit Care Med* 1995;152:1910–1916.
24. Marcinkiewicz J, Kontny E. Taurine and inflammatory diseases. *Amino Acids* 2014;46:7–20.
25. Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med* 1989;320:365–376.
26. Gruber M, Moser I, Nagl M, Lackner M. Bactericidal and fungicidal activity of *n*-chlorotaurine is enhanced *in* cystic fibrosis sputum medium. *Antimicrob Agents Chemother* 2017;61:e02527–16.
27. Nagl M, Teuchner B, Pöttinger E, Ulmer H, Gottardi W *et al.* Tolerance of N-chlorotaurine, a new antimicrobial agent, in infectious conjunctivitis - a phase II pilot study. *Ophthalmologica* 2000;214:111–114.
28. Neher A, Nagl M, Appenroth E, Gstöttner M, Wischatta M *et al.* Acute otitis externa: efficacy and tolerability of N-chlorotaurine, a novel endogenous antiseptic agent. *Laryngoscope* 2004;114:850–854.
29. Nagl M, Gruber A, Fuchs A, Lell CP, Lemberger EM *et al.* Impact of N-chlorotaurine on viability and production of secreted aspartyl proteinases of Candida spp. *Antimicrob Agents Chemother* 2002;46:1996–1999.
30. Reeves EP, Nagl M, O'Keeffe J, Kelly J, Kavanagh K *et al.* Effect of N-chlorotaurine on *Aspergillus*, with particular reference to destruction of secreted gliotoxin. *J Med Microbiol* 2006;55:913–918.
31. Lackner M, Binder U, Reindl M, Gönül B, Fankhauser H *et al.* N-Chlorotaurine exhibits fungicidal activity against therapy-refractory scedosporium species and lomentospora prolificans. *Antimicrob Agents Chemother* 2015;59:6454–6462.
32. Nagl M, Hengster P, Semenitz E, Gottardi W. The postantibiotic effect of N-chlorotaurine on Staphylococcus aureus. Application in the mouse peritonitis model. *J Antimicrob Chemother* 1999;43:805–809.
33. Mustedanagic J, Ximenes VF, Nagl M. Microbicidal activity of N-chlorotaurine in combination with hydrogen peroxide. *AMB Express* 2017;7:102.
34. Williams D, Kadaria D, Sodhi A, Fox R, Williams G *et al.* Chronic granulomatous disease presenting as *Aspergillus* fumigatus pneumonia in a previously healthy young woman. *Am J Case Rep* 2017;18:351–354.
35. Levitz SM, Diamond RD. Mechanisms of resistance of *Aspergillus fumigatus* Conidia to killing by neutrophils in vitro. *J Infect Dis* 1985;152:33–42.
36. Gottardi W, Nagl M. Chemical properties of N-chlorotaurine sodium, a key compound in the human defence system. *Arch Pharm* 2002;335:411–421.
37. Sheehan G, Bergsson G, Mcelvaney NG, Reeves EP, Kavanagh K *et al.* The human cathelicidin antimicrobial peptide LL-37 promotes the growth of the pulmonary pathogen *Aspergillus fumigatus*. *Infect Immun* 2018:IAI.00097–18.
38. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV *et al.* Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res* 2011;10:1794–1805.
39. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS *et al.* Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* 2005;438:1151–1156.
40. Priebe S, Linde J, Albrecht D, Guthke R, Brakhage AA *et al.* FungiFun: a web-based application for functional categorization of fungal genes and proteins. *Fungal Genet Biol* 2011;48:353–358.
41. 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.
42. Snelders E, Huis In 't Veld RA, Rijs AJ, Kema GH, Melchers WJ *et al.* Possible environmental origin of resistance of *Aspergillus*

fumigatus to medical triazoles. *Appl Environ Microbiol* 2009;75:4053–4057.

43. **Fraczek MG, Bromley M, Bowyer P.** An improved model of the *Aspergillus fumigatus* CYP51A protein. *Antimicrob Agents Chemother* 2011;55:2483–2486.
44. **Lazzarini C, Esposto MC, Prigitano A, Cogliati M, de Lorenzis G *et al.*** Azole resistance in *Aspergillus fumigatus* clinical isolates from an italian culture collection. *Antimicrob Agents Chemother* 2016;60:682–685.
45. **Johnson MD, Perfect JR.** Use of antifungal combination therapy: agents, order, and timing. *Curr Fungal Infect Rep* 2010;4:87–95.
46. **Gottardi W, Debabov D, Nagl M.** N-chloramines, a promising class of well-tolerated topical anti-infectives. *Antimicrob Agents Chemother* 2013;57:1107–1114.
47. **Nagl M, Arnitz R, Lackner M.** N-Chlorotaurine, a Promising Future Candidate for Topical Therapy of Fungal Infections. *Mycopathologia* 2018;183:161–170.
48. **Gottardi W, Nagl M.** Active halogen compounds and proteinaceous material: loss of activity of topical anti-infectives by halogen consumption. *J Pharm Pharmacol* 2013;65:213–218.
49. **Nagl M, Lass-Flörl C, Neher A, Gunkel A, Gottardi W *et al.*** Enhanced fungicidal activity of N-chlorotaurine in nasal secretion. *J Antimicrob Chemother* 2001;47:871–874.
50. **Gottardi W, Nagl M.** Chlorine covers on living bacteria: the initial step in antimicrobial action of active chlorine compounds. *J Antimicrob Chemother* 2005;55:475–482.
51. **Marcinkiewicz J, Czajkowska B, Grabowska A, Kasprowicz A, Kociszewska B *et al.*** Differential effects of chlorination of bacteria on their capacity to generate NO, TNF-alpha and IL-6 in macrophages. *Immunology* 1994;83:611–616.
52. **Thati B, Noble A, Rowan R, Creaven BS, Walsh M *et al.*** Mechanism of action of coumarin and silver(I)-coumarin complexes against the pathogenic yeast Candida albicans. *Toxicol In Vitro* 2007;21:801–808.
53. **Kelly J, Kavanagh K.** Proteomic analysis of proteins released from growth-arrested Candida albicans following exposure to caspofungin. *Med Mycol* 2010;48:598–605.
54. **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.
55. **Johnston MD, Hanlon GW, Denyer SP, Lambert RJ.** Membrane damage to bacteria caused by single and combined biocides. *J Appl Microbiol* 2003;94:1015–1023.
56. **Eshwika A, Kelly J, Fallon JP, Kavanagh K.** Exposure of *Aspergillus fumigatus* to caspofungin results in the release, and de novo biosynthesis, of gliotoxin. *Med Mycol* 2013;51:121–127.
57. **Shenton D, Smirnova JB, Selley JN, Carroll K, Hubbard SJ *et al.*** Global translational responses to oxidative stress impact upon multiple levels of protein synthesis. *J Biol Chem* 2006;281:29011–29021.
58. **Mccowen MC, Callender ME, Lawlis JF.** Fumagillin (H-3), a New antibiotic with amebicidal properties. *Science* 1951;113:202–203.
59. **Amitani R, Murayama T, Nawada R, Lee WJ, Niimi A *et al.*** *Aspergillus* culture filtrates and sputum sols from patients with pulmonary aspergillosis cause damage to human respiratory ciliated epithelium in vitro. *Eur Respir J* 1995;8:1681–1687.
60. **Fallon JP, Reeves EP, Kavanagh K.** The *Aspergillus fumigatus* toxin fumagillin suppresses the immune response of Galleria mellonella larvae by inhibiting the action of haemocytes. *Microbiology* 2011;157:1481–1488.