

Effect of *N*-chlorotaurine on *Aspergillus*, with particular reference to destruction of secreted gliotoxin

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The fungistatic and fungicidal activity of *N*-chlorotaurine (NCT), a long-lived oxidant produced by stimulated neutrophils, was investigated. Physiological concentrations (75–100 µM) of NCT showed clear fungicidal activity against a range of *Aspergillus* isolates. Moreover, killing by NCT was significantly increased in the presence of ammonium chloride, explained by the formation of monochloramine by halogenation of ammonium. One clinical isolate of *Aspergillus fumigatus* was characterized for the production of the immunosuppressive agent gliotoxin, and NCT was shown to cause destruction of gliotoxin, possibly via reduction of the disulphide bridge. Because of its endogenous nature and its high antifungal activity, NCT appears to be a good choice for topical treatment of *Aspergillus* infections, and the results of this study further substantiate its therapeutic efficacy.

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INTRODUCTION

The fungal pathogen *Aspergillus fumigatus* is capable of inducing a wide range of diseases, which can include saprophytic colonization and allergic reactions. Colonization may result in limited invasion of tissues in immunocompetent individuals, but may give rise to systemic diseases with high mortality rates in immunocompromised patients, particularly in cases of invasive aspergillosis (Daly & Kavanagh, 2001). Neutropenic and immunosuppressed patients are at particular risk of developing pulmonary and systemic aspergillosis, with associated high mortality rates (Brakhage & Langfelder, 2002).

A. fumigatus does not manifest a single virulence factor: its virulence is attributable to a range of factors, including the production of toxins and proteolytic enzymes, its mode of growth, and its ability to withstand the host immune system (Daly & Kavanagh, 2001). The hyphae of *A. fumigatus* produce a number of low-molecular-mass toxins, including helvolic acid, fumagillin and gliotoxin (Amitani *et al.*, 1995; Hogan *et al.*, 1996). The immunosuppressive potential of fungal toxins has been demonstrated using culture filtrates which contain a range of toxins secreted by the growing fungus (Watanabe *et al.*, 2003). Gliotoxin belongs to the group of epipolythiodioxopiperazine (ETP) metabolites (Waring & Beaver, 1996), characterized by an internal

disulphide bridge known to be essential for activity. Gliotoxin possesses a number of powerful bioactivities, including the induction of apoptotic cell death in macrophages (Waring, 1990) and the inhibition of activation of the NADPH oxidase of human neutrophils via targeting of the flavocytochrome *b*₅₅₈ subunit (Nishida *et al.*, 2005). Gliotoxin has been detected in tissue samples from animals (Richard *et al.*, 1996; Richard & DeBey, 1995) and humans (Shah *et al.*, 1995), in whom it may facilitate fungal persistence and colonization of tissue. In addition, gliotoxin has been implicated in the destruction of lung parenchyma in invasive aspergillosis (Sutton *et al.*, 1996) and the penetration of blood vessels in angio-invasive aspergillosis (Fraser, 1993).

The treatment of aspergillosis has traditionally relied upon the use of amphotericin B; however, in recent years, liposomal amphotericin B, new azole derivatives (e.g. itraconazole) and echinocandins (e.g. caspofungin) have been exploited for the control of this disease. The appearance of drug resistance to antifungal agents has prompted the search for novel antifungal agents that could complement the existing range of antifungal therapies. In fact, in a recent study of more than 18 000 strains of clinically important yeasts and moulds obtained over a period of 10 years, many *Candida* and *Aspergillus* isolates exhibited resistance to voriconazole, fluconazole and amphotericin B (Sabatelli *et al.*, 2004). In addition, amphotericin B has many undesirable side effects (Cohen, 1998), and it has recently been

Abbreviations: FCS, fetal calf serum; NCT, *N*-chlorotaurine.

shown that despite its antifungal effect, amphotericin B can induce the synthesis and release of gliotoxin from *A. fumigatus* (Reeves *et al.*, 2004), and therefore potentially exacerbate the effects of the toxin and facilitate fungal invasion.

The development of new antifungal drugs is of critical importance, and a new drug with great potential is *N*-chlorotaurine (NCT; Cl-HN-CH₂-CH₂-SO₃H), a long-lived oxidant produced by human neutrophils (Test *et al.*, 1984) and also available as a water-soluble crystalline sodium salt (Cl-HN-CH₂-CH₂-SO₃Na) (Gottardi & Nagl, 2002). Stimulation of neutrophils by bacteria or fungi leads to an increase in O₂ consumption, and the release of superoxide (O₂⁻) and related products of oxygen reduction (Klebanoff, 1980). There are many reports supporting a role for hydrogen peroxide (H₂O₂) and to a greater extent hypochlorous acid (HOCl) in the successful killing of invading organisms (Klebanoff, 1980). In turn, however, HOCl in the presence of taurine results in the formation of NCT (Test *et al.*, 1984). NCT at concentrations between 100 µM and 55 mM has demonstrated significant bactericidal, fungicidal, virucidal and vermucidal activities (Nagl *et al.*, 2000a, 2001, 2002; Nagl & Gottardi, 1996). In clinical trials, NCT has been successfully employed in the treatment of infections of skin ulcers (Nagl *et al.*, 2003) and the outer ear canal (Neher *et al.*, 2004), amongst other applications. NCT has also been shown to exert an immune regulatory role by down-regulating proinflammatory cytokines, such as tumour necrosis factor, nitric oxide and several interleukins (Marcinkiewicz, 2003; Park *et al.*, 1995). As a result of these properties, and in view of its low cytotoxicity against human cells (Nagl *et al.*, 2003), NCT may have potential as an antifungal agent in treatment of *Aspergillus* infections.

This study was undertaken to quantitatively evaluate the antifungal action of NCT against a range of *Aspergillus* isolates. Moreover, its bactericidal activity has been shown to increase in the presence of ammonium, resulting in the formation of monochloramine (NH₂Cl) (Nagl & Gottardi, 1996), and for that reason, experiments were repeated in the presence of ammonium chloride (NH₄Cl). In addition, the influence of NCT on the production and stability of gliotoxin, an important fungal virulence factor, was investigated.

METHODS

***Aspergillus* strains and culture conditions.** Clinical isolates of *A. fumigatus* used in this study included ATCC 26933, 16424, 13073 and 14109 (obtained from the American Type Culture Collection), and one strain of *Aspergillus niger* and one strain of *Aspergillus flavus* (environmental isolates) were also employed. *Aspergillus* cultures were grown in minimal essential medium (MEM) (Sigma-Aldrich) supplemented with 5% (v/v) fetal calf serum (FCS; Sigma-Aldrich) at 37 °C and 200 r.p.m. Stocks were maintained on malt extract agar (MEA; Oxoid) or Sabouraud dextrose agar (Oxoid).

Growth curve of *Aspergillus*. MEA plates containing sporulating *Aspergillus* colonies were washed with 10 ml 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 for 5 min in a Beckman GS-6 centrifuge) and enumerated. Flasks containing MEM (50 ml) plus 5% FCS were inoculated with 1×10^5 *Aspergillus* conidia and incubated at 37 °C and 200 r.p.m. A flask was removed every 24 h and the contents filtered through a Whatman No. 1 filter in a Buchner funnel and air-dried. A growth curve was constructed of dry fungal biomass versus incubation time.

Fungistatic and fungicidal effects of NCT. Pure NCT as a crystalline sodium salt ($M_r = 181.57 \text{ g mol}^{-1}$) was kindly donated by Waldemar Gottardi, Institute of Hygiene and Social Medicine, Innsbruck, Austria. Flat-bottomed 96-well plates (Sarstedt) containing MEM with 5% FCS (200 µl) were inoculated with 1×10^5 *Aspergillus* conidia. Cultures were supplemented with NCT at final concentrations ranging from 100 µM to 55 mM and grown at 37 °C for 24 h in a static incubator. Treatments were carried out a minimum of 24 times, and readings measured at 540 nm using a Bio-Tek Synergy HT spectrophotometer. The MIC₉₀ (concentration that inhibited growth by 90%) was determined.

To establish the fungicidal effects of NCT, conidia were exposed to increasing concentrations of NCT ranging from 100 µM to 55 mM. After incubation at 37 °C for 24 h, aliquots were removed and serial tenfold dilutions were made and plated in triplicate on MEA agar plates. Colony counts were performed in triplicate for each sample, and results were calculated as the mean (\pm SEM) from at least three experiments. The pH remained stable during assays to within 0.12 pH units of the starting pH.

Fungicidal activity of NCT in the presence of NH₄⁺. Conidia of *A. fumigatus* ATCC 26933 [4×10^5 (40 µl)⁻¹] were added to NCT (55 mM in 3.96 ml PBS) in the presence or absence of 18 mM NH₄Cl (Merck) and incubated at 37 °C. Aliquots (400 µl) were removed at times 0.02, 0.2, 2, 4 and 7 h and added to 3.6 ml 0.6% sodium thiosulfate to inactivate NCT in the presence or absence of NH₄Cl. Of these dilutions, 50 µl was spread onto Sabouraud agar in duplicate by means of an automatic spiral plater (WASP, Whitley). c.f.u. were counted after incubation at 37 °C for 24, 48 and 72 h. Since NCT was inactivated on the agar plates within 3 min, additional aliquots were plated without dilution and inactivation, allowing a detection limit of 10 c.f.u. ml⁻¹. The relevant controls included phosphate buffer alone, phosphate buffer plus 18 mM NH₄Cl, 0.6% sodium thiosulfate, and NCT (with or without NH₄Cl) inactivated by the addition of sodium thiosulfate prior to the addition of fungal spores.

Preparation of extracts for detection of gliotoxin by reversed-phase HPLC. Flasks containing MEM (50 ml) plus 5% FCS were inoculated with 1×10^5 *Aspergillus* conidia and incubated at 37 °C for 3 days. NCT (125 or 250 µM) was added and the cultures incubated at 37 °C for a further hour. Flasks were removed and the contents filtered through a Whatman No. 1 filter in a Buchner funnel. To the culture filtrate, an equal volume of chloroform (Hyper Solv, BDH) was added and shaken overnight at 4 °C. The chloroform fraction was collected and evaporated to dryness. For detection of gliotoxin, dried extracts were dissolved in 200 µl methanol (Hyper Solv, BDH) and subjected to reverse-phase HPLC (Spectra-Physics). The mobile phase was 34.9% (v/v) acetonitrile (Hyper Solv, BDH), 0.1% (v/v) trifluoroacetic acid. The gliotoxin extract (20 µl) was injected onto a C18 Hewlett Packard column and detected at 254 nm. A standard curve of peak area versus gliotoxin concentration was constructed using gliotoxin standards (25, 50, 100 and 200 ng ml⁻¹).

Statistical analysis. Quantification of gliotoxin was analysed by Student's two-tailed *t* test with values of *P* < 0.05 considered

Table 1. MIC₉₀ of *Aspergillus* strains grown in the presence of NCTMean values \pm SEM are shown.

Strain	MIC ₉₀ (μ M)
<i>A. fumigatus</i> ATCC 13073	35.4 \pm 0.6
<i>A. fumigatus</i> ATCC 16424	37.8 \pm 1.4
<i>A. fumigatus</i> ATCC 26933	16.0 \pm 0.2
<i>A. fumigatus</i> ATCC 14109	29.2 \pm 5.8
<i>A. flavus</i>	28.2 \pm 4.6
<i>A. niger</i>	65.2 \pm 4.5

statistically significant. The combined effect of NCT with NH₄Cl on fungal killing was analysed by one-way ANOVA and Dunnett's multiple comparison test (GraphPad Prism Software).

RESULTS AND DISCUSSION

Fungistatic and fungicidal activity of NCT

Neutrophils and alveolar macrophages are the main cellular components of the vertebrate immune system responsible for the defence against *Candida albicans* (Reeves *et al.*, 2002) and *A. fumigatus* (Washburn *et al.*, 1987). Myeloperoxidase plays a critical role in the antimicrobial armamentarium of neutrophils (Klebanoff, 1980) and aids in the formation of long-lived oxidants. As taurine is highly abundant in neutrophils, NCT is estimated to be the most concentrated representative of the chloramines *in vivo*. The synthesis of the pure crystalline sodium salt of NCT has opened up an area of clinical study whereby NCT has been successfully used in the treatment of infections such as conjunctivitis (Nagl *et al.*, 2000b; Teuchner *et al.*, 2005), eosinophilic fungal rhinosinusitis (Nagl *et al.*, 2001) and acute otitis externa (Neher *et al.*, 2004). A critical factor in the success of these trials is the fact that NCT is markedly less cytotoxic than highly reactive oxidants such as hypochlorite and oxygen radicals, and has been demonstrated not to be involved in tissue damage *in vivo* (Nagl *et al.*, 2003).

NCT is thought to be involved in the destruction of pathogens *in vivo* because of its bactericidal and fungicidal

activity (Nagl *et al.*, 2000a, 2002). Studies to quantify NCT production by stimulated neutrophils have measured concentrations of about 50 μ M (Grisham *et al.*, 1984; Weiss *et al.*, 1982). In the present study, the effect of NCT on conidial development and growth of a range of *Aspergillus* species and isolates was investigated, with results demonstrating NCT to have fungistatic and fungicidal effects, down to physiological concentrations. The MIC₉₀ of NCT was determined (Table 1). Exposure of *Aspergillus* conidia to NCT resulted in a reduction in growth, with MIC₉₀ values ranging from 16 \pm 0.2 μ M for *A. fumigatus* ATCC 26933 to 65 \pm 5 μ M for *A. niger*. The MIC₉₀ values for the four different isolates of *A. fumigatus* ranged from 16 \pm 0.2 μ M for *A. fumigatus* ATCC 26933 to 38 \pm 1.4 μ M for *A. fumigatus* ATCC 16424, but were found not to be significantly different ($P = 0.1$) from each other.

Fig. 1 shows the killing of *Aspergillus* isolates exposed to increasing concentrations of NCT at pH 7.2 for 24 h. The fungicidal effect was dose dependent, with micromolar concentrations of NCT demonstrating considerable fungicidal activity. As the dose was elevated, a concentration of 75 μ M reduced the survival of *A. flavus* and *A. fumigatus* ATCC 13073 by 75 % and was lethal to both *A. niger* and *A. fumigatus* ATCC 26933. Finally, the addition of 150 μ M NCT resulted in total killing of all *Aspergillus* species.

Enhanced fungicidal activity of NCT in the presence of NH₄⁺

The importance of cell wall and membrane permeability to the effect of oxidants is evident from the enormous effect of ammonium when it is included with NCT. Combining NCT and NH₄Cl leads to the formation of NH₂Cl by halogenation (Nagl *et al.*, 2001). Because of the smaller size and lipophilic properties of NH₂Cl, it is able to penetrate the cell membrane more easily than the hydrophilic NCT. In the present study, 1.4 mM NH₂Cl was produced from 55 mM NCT and 18 mM NH₄Cl ($K = [\text{NH}_2\text{Cl}] [\text{taurine}] / [\text{NCT}] / [\text{NH}_4^+] / f^2$, where $f = 0.0034$; W. Gottardi, Division of Hygiene and Medical Microbiology, Innsbruck Medical University, personal communication). The kinetics of fungal killing by NCT are illustrated in Fig. 2. Killing of *A. fumigatus* ATCC 26933 by 55 mM NCT occurred gradually, with over

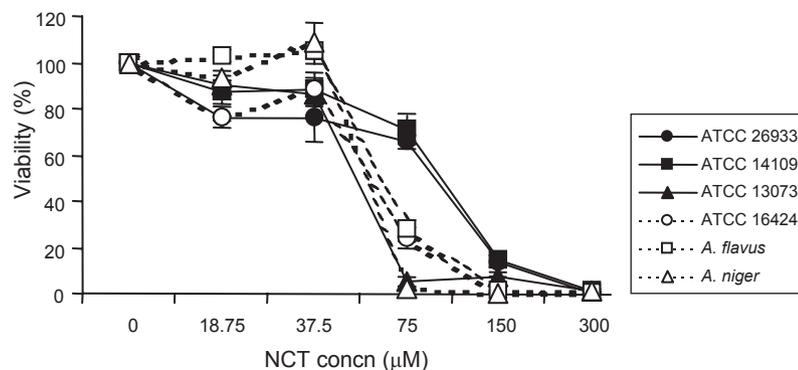


Fig. 1. Fungicidal activity of NCT against *Aspergillus* isolates. *Aspergillus* conidia [1×10^5 ($200 \mu\text{l}$)⁻¹] were suspended in MEM with 5% (v/v) FCS, pH 7.2, and the effect of increasing concentrations of NCT was tested after 24 h incubation at 37 °C. Two serial dilutions were made and reactions plated in triplicate on MEA. Mean \pm SEM of three separate experiments are shown.

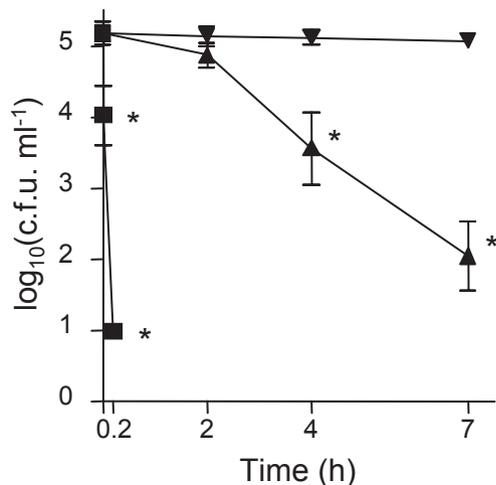


Fig. 2. Enhanced fungicidal effect of NCT in the presence of ammonium. The activity of 55 mM NCT (▲) and 55 mM NCT plus 18 mM NH_4Cl (■) against conidia of *A. fumigatus* ATCC 26933 is shown. Reactions were incubated at 37 °C and aliquots (400 μl) removed over time. Controls (▼) included phosphate buffer alone, phosphate buffer plus 18 mM NH_4Cl , 0.6% sodium thiosulfate, and NCT (with or without NH_4Cl) inactivated by the addition of sodium thiosulfate prior to the addition of fungal spores. Mean values \pm SD of three independent experiments are shown. * $P < 0.01$ versus control.

90% killed after just 4 h and almost 100% after 7 h. Addition of NH_4Cl enhanced this effect, and caused a tenfold reduction of c.f.u. within 1 min ($P < 0.01$). After 10 min, no viable conidia were detectable. In all controls, the c.f.u. remained constant and are summarized in Fig. 2. Similar results have been demonstrated for the killing of *C. albicans* by NH_2Cl at 5 μM compared with NCT at 143 μM (Wagner *et al.*, 1986). A development of this concept was observed *in vivo* upon treatment of local inflammatory mycosis (Nagl *et al.*, 2001). Enhanced killing by NCT via the formation of NH_2Cl was observed in human nasal mucus containing 1 mM ammonium.

Effect of NCT on secreted gliotoxin

Regarding the mechanism of action of NCT, the first impact of the drug on bacteria is chlorination of the cell surface (Gottardi & Nagl, 2005). By electron microscopy, membrane infolding and changes in cytosolic compartmentalization have been observed. In an *in vivo* situation, however, the first effect of the drug may not be directly against the micro-organism itself but against virulence factors (e.g. toxins and enzymes) that are released into the surrounding tissue to facilitate fungal invasion. Part of the ability of *A. fumigatus* to colonize the lung lies in its capacity to produce a range of toxins and enzymes, of which the ability of gliotoxin to facilitate colonization of lung tissue is well characterized (Sutton *et al.*, 1996). Gliotoxin production by *A. fumigatus* has been estimated to be in the range of

20–80 μg (ml culture)⁻¹ after 4–7 days (Müllbacher & Eichner, 1984). In this study, initial experiments sought to correlate hyphal growth with gliotoxin production. A growth curve of *A. fumigatus* ATCC 26933 (Fig. 3a) showed that the exponential growth phase began at 12 h and the stationary phase was reached at approximately 72 h [50 ± 4.8 mg (50 ml culture)⁻¹]. Gliotoxin production commenced after 24 h, with maximum gliotoxin produced after

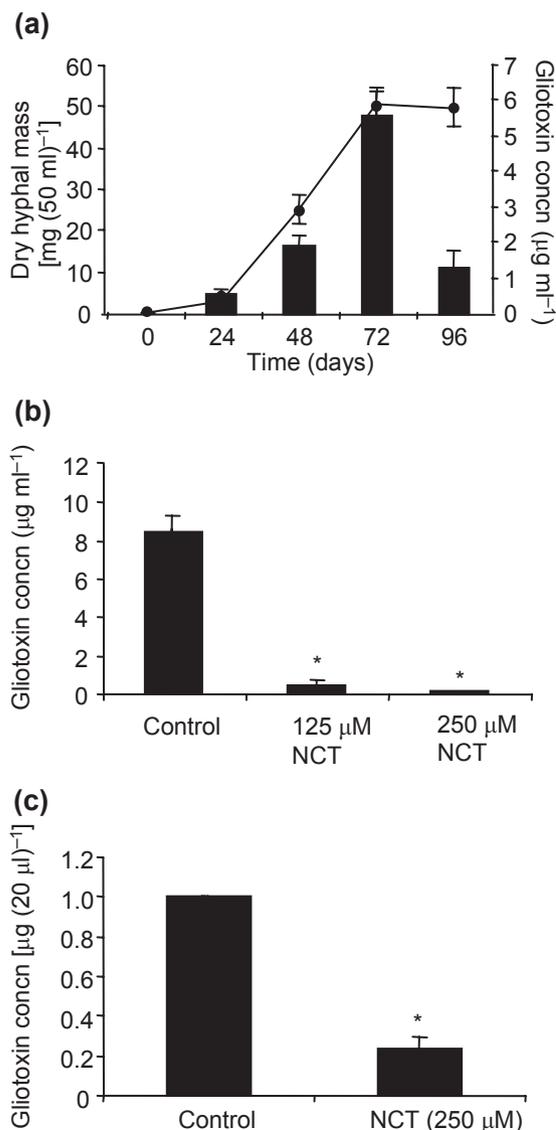


Fig. 3. Effect of NCT on gliotoxin. (a) Growth curve of *A. fumigatus* ATCC 26933 in MEM plus 5% (v/v) FCS at 37 °C, in conjunction with gliotoxin quantification (black bars), as a function of dry fungal biomass (●). All results are the means \pm SEM from three measurements. (b) Cultures of *A. fumigatus* ATCC 26933 were supplemented with NCT (125 or 250 μM) for 1 h, and the concentration of gliotoxin assessed by reversed-phase HPLC analysis. (c) Gliotoxin standard (50 μg ml⁻¹) was exposed to NCT (250 μM) and analysed by HPLC. *Statistically significant ($P < 0.05$) difference compared to the untreated control.

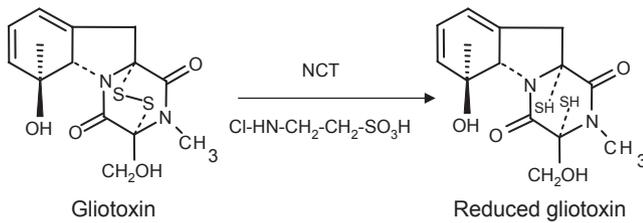


Fig. 4. Diagrammatic representation of gliotoxin and predicted oxidizing effect of NCT. The fungal toxin gliotoxin (left), indicating the bridged disulphide ring and (right) reduction of the disulphide bond by the oxidizing agent NCT are shown.

72 h incubation ($5.65 \pm 0.42 \mu\text{g ml}^{-1}$), followed by a subsequent decline in levels ($1.3 \pm 0.37 \mu\text{g ml}^{-1}$). Remarkably, a lower concentration of gliotoxin ($20\text{--}50 \text{ ng ml}^{-1}$) has been shown to have an inhibitory effect on phagocytosis of carbon particles by peritoneal exudates (Waring *et al.*, 1988).

As most gliotoxin was detected after culturing for 72 h, in ensuing experiments, 72-hour-old cultures of *A. fumigatus* were supplemented with NCT and gliotoxin levels determined (Fig. 3b). The gliotoxin level detected in control cultures was $8.25 \pm 0.6 \mu\text{g ml}^{-1}$. Exposure to NCT for a period of 1 h led to a significant decrease in gliotoxin, with concentrations of $0.2 \pm 0.04 \mu\text{g ml}^{-1}$ detected within the culture on application of $125 \mu\text{M}$ NCT and no detectable levels of the toxin on addition of $250 \mu\text{M}$ NCT. In a further experiment (Fig. 3c), an almost instantaneous effect of NCT on gliotoxin was observed. Gliotoxin standard ($50 \mu\text{g ml}^{-1}$) was exposed to $250 \mu\text{M}$ NCT for 30 s and immediately analysed by HPLC. Results showed levels of toxin to decrease significantly from 1 to $0.235 \mu\text{g (20 } \mu\text{l)}^{-1}$ ($P < 0.05$). The mechanism of action of NCT includes oxidation of thio groups to disulfides and sulphur-oxygen acids, and of amino groups to chloramines (Gottardi & Nagl, 2002). Therefore, a very plausible immediate site of attack could be the bridged disulphide region, which confers activity (Fig. 4). This result is of tremendous importance, as destruction of gliotoxin by NCT could abolish the ability of *A. fumigatus* to colonize tissue via its capacity to suppress the local immune response (Hogan *et al.*, 1996).

In conclusion, the work presented here demonstrates NCT to be effective against *A. fumigatus*, with clear advantages being the endogenous nature of this agent, and its excellent tissue tolerability and broad-spectrum activity. Part of the ability of *A. fumigatus* to colonize the lung is achieved through the action of mycotoxins such as gliotoxin, and separate from its direct effect against fungal cells, NCT eliminates this secondary metabolite of *A. fumigatus*. Our study has evaluated the use of NCT against infections of *A. fumigatus*, with positive results pointing to its therapeutic efficacy. Phase II clinical trials and case reports have demonstrated the successful use of NCT in topical treatment of diseases such as conjunctivitis (Nagl *et al.*, 2000b), acute

otitis externa (Neher *et al.*, 2004) and skin ulcerations (Nagl *et al.*, 2003). Prospective clinical trials on fungal infections have yet to be performed; nevertheless, the results outlined in this manuscript form a good foundation for such trials. In addition, the phenomenon of gliotoxin destruction by NCT has the potential to attenuate the level of tissue damage and immunosuppression associated with this fungal toxin.

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