

Enhanced fungicidal activity of *N*-chlorotaurine in nasal secretion

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The antifungal activity of *N*-chlorotaurine (NCT), a long-lived oxidant produced by stimulated human leucocytes, was investigated. Incubation of *Aspergillus* spp., *Candida* spp., *Fusarium* spp., *Penicillium* spp. and *Alternaria* spp. in 1% NCT (55 mM) for 1–4 h produced a log₁₀ reduction in cfu of between 1 and 4. In samples of nasal secretion, killing was significantly hastened (30 min), which may be explained by the formation of monochloramine by halogenation of ammonium, which was found at a concentration of 1 mM in these samples. For these reasons, NCT is of interest as a new agent for treatment of local inflammatory mycosis, e.g. eosinophilic fungal rhinosinusitis.

Introduction

N-chlorotaurine (NCT, Cl-HN-CH₂-CH₂-SO₃⁻), the *N*-chloro derivative of the amino acid taurine, is a long-lived oxidant produced by human granulocytes and monocytes from hypochlorite (HOCl) and taurine during the oxidative burst.¹ Because of its high stability and reduced reactivity compared with HOCl, NCT is thought to maintain oxidative capacity for many hours during inflammation.¹

Besides its immune controlling function exerted by downregulation of proinflammatory cytokines,² NCT is involved in the destruction of pathogens *in vivo* because of its bactericidal (Gram-positive and Gram-negative bacteria) and fungicidal (*Candida albicans*) activity down to physiological, micromolar concentrations.^{3–5}

As a result of these properties and in view of its low cytotoxicity against human cells,⁶ NCT has potential as an antimicrobial agent in local treatment of infections. NCT has recently become available as a crystalline sodium salt, which facilitates application.³ Initial clinical trials have revealed good tolerability, rapid *in vivo* bactericidal activity and signs of therapeutic effects in infectious conjunctivitis.⁷

Recently, chronic allergic rhinosinusitis has been reported to be associated with the presence of fungi, especially moulds, in more than 90% of cases; this is termed 'eosinophilic fungal rhinosinusitis'.⁸ The present study was designed to evaluate the activity of NCT in both aqueous solution and human nasal mucus against those fungal

species that are most frequently detected in chronic rhinosinusitis.

Materials and methods

Reagents

Pure NCT was synthesized as a crystalline sodium salt (mol. wt. 181.57 g/mol)³ and was dissolved in 0.01 M phosphate-buffered (pH 7.4) and citrate-buffered (pH 5.4) saline, unless stated otherwise. A final concentration of 1% was used in all experiments. Reagent grade phosphate, citrate, sodium chloride and sodium thiosulphate were purchased from Merck (Darmstadt, Germany).

Fungal strains, media and testing of fungicidal activity

One clinical isolate each of *Aspergillus flavus*, *Aspergillus fumigatus*, *C. albicans*, *Candida parapsilosis*, *Alternaria alternata*, *Fusarium moniliforme* and two isolates of *Penicillium commune* were grown on Sabouraud dextrose agar (Oxoid, Basingstoke, UK). Subcultures were performed in 10 mL of Eagle's minimal essential medium (EMEM) with alpha modification (Sigma, St Louis, MO, USA) for 24 h at 30°C. The fungi were centrifuged at 1800g for 10 min and washed in saline twice. This procedure yielded about 90% hyphae and 10% conidiophores and spores with the

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moulds, and for *Candida* spp. 60% pseudohyphae and 40% blastoconidia as observed by phase-contrast microscopy. Samples were diluted 10-fold in NCT solution for moulds and 100-fold for *Candida* spp. Cfus counts of between 0.6×10^3 and 2.3×10^5 were used in the test solution (see Results for concentrations of individual strains). At the start of the experiment and after incubation times of 0.5, 1 and 4 h at 37°C, NCT was inactivated by addition of sodium thiosulphate (3% to inactivate 1% NCT), and aliquots of 50 µL were spread on Sabouraud agar, supplemented with 80 mg/mL chloramphenicol, by means of an automatic spiral plater (WASP; Whitley, Shipley, UK). Cfus were counted after incubation at 37°C for 24, 48 and 72 h (10 days in the case of no growth) with a lower detection limit of 20 cfu/mL. In addition, the activity of 1% NCT in 0.1 M phosphate buffer at pH 7.0 was tested in the presence of 19 mM (0.1%) and 1 mM (0.005%) ammonium chloride (NH₄Cl). Corresponding controls in buffer solution without additives, in the presence of 0.1% NH₄Cl without NCT, and in the presence of NCT and NH₄Cl inactivated by thiosulphate before addition of fungi were performed in parallel. Multiple tests (three to seven) were conducted on each isolate under each test condition.

Fungicidal activity in nasal mucus

Samples of nasal mucus were obtained by inducing sneezing in healthy volunteers ($n = 7$, age range 32–64 years) and also from patients ($n = 3$, age range 30–57 years) suffering from rhinosinusitis. They were diluted 10-fold in 0.9% saline and vortexed vigorously. After removal of a sufficient volume for controls, pure NCT was dissolved in the samples to 1.1%. An aliquot of 0.1 mL of the washed fungal suspension was added to 0.9 mL of each sample and incubated at 37°C. *C. albicans*, *A. fumigatus*, *A. flavus* and *F. moniliforme* were chosen for these tests. Quantitative cultures were performed as above after incubation.

Determination of NH₄⁺ in human nasal mucus

Concentrations of NH₄⁺ in human nasal mucus were determined with the ammonia electrode NH 500/2 from WTW (Wissenschaftliche-Technische Werkstätten GmbH, Weilheim, Germany) and the reference electrode REF 401 from Radiometer (Lyon, France) in a stirred vessel containing 5 mL of a 1:30 aqueous dilution of nasal mucus maintained at 25°C.

After addition of 1 mL of 5 M NaOH the potential difference ΔE_1 was read. ΔE_2 and ΔE_3 , respectively, were measured after two consecutive additions of 50 µL of 0.01 M NH₄Cl. The concentration of NH₄⁺ in the original solution was calculated according to the double standard addition method.⁹

Statistical analysis

Student's paired *t*-test was used to compare cfu/mL of

samples and controls after different incubation times, and *P* values < 0.05 were considered significant.

Results

Fungicidal activity of NCT in aqueous solution

The aqueous solution of 1% NCT proved to be fungicidal at both pH 7.4 and 5.4 (Figure 1a–d). Killing of *Candida* spp. and *Aspergillus* spp. was independent of pH, while *F. moniliforme* and *P. commune* were slightly more susceptible at pH 7. In general, moulds were more resistant than yeasts and incubation times of 4 h were needed for a marked reduction in cfu. *A. alternata* revealed maximal cfu counts of only $2.8\text{--}4.2 \times 10^2$ /mL because of clumping, and cfus decreased to the detection limit after treatment with NCT for 1 h.

In contrast, when 0.1% NH₄Cl (19 mM) was added to the NCT solution, all test strains were reduced to <20 cfu/mL within 10 min (two independent experiments for each strain, data not shown). Also, in the presence of 0.005% NH₄Cl (1 mM), a concentration found in human nasal secretion (see below), killing was achieved within 10–30 min (Figure 1).

NCT inactivated with sodium thiosulphate before addition of fungi showed no antifungal effect in the presence or absence of ammonium. The same was true of NH₄Cl (data not shown).

Fungicidal activity of NCT in human nasal mucus

The pH of the samples after 10-fold dilution in saline ranged from 6.3 to 7.8 in patients and 6.0 to 7.5 in healthy volunteers. As depicted in Figure 2(a and b), the cfus of all fungi were reduced below the detection limit of 20 cfu/mL within 0.5–1 h (2 h for two of seven samples of *A. flavus*). Therefore, the activity of NCT in nasal mucus proved to be substantially higher than that in aqueous solution and was not influenced by the pH of the samples.

Concentration of ammonium in human nasal mucus

The concentration of NH₄⁺ detected in nasal mucus was 1.1 ± 0.15 mM (mean value \pm s.d., $n = 5$ mucus samples).

Discussion

In this study, NCT has been shown to have broad-spectrum fungicidal activity, which is in accordance with its non-specific oxidative mechanism of action. Longer incubation times were required for inactivation of fungi compared with bacteria, which are killed by 1% NCT within 30 min.^{3,4} In contrast to previous findings with bacteria,⁵ a decrease rather than an increase in killing of moulds has been demonstrated at acidic pH. The reason for variations in susceptibility, which have not yet been clarified, may be

Fungicidal activity of *N*-chlorotaurine

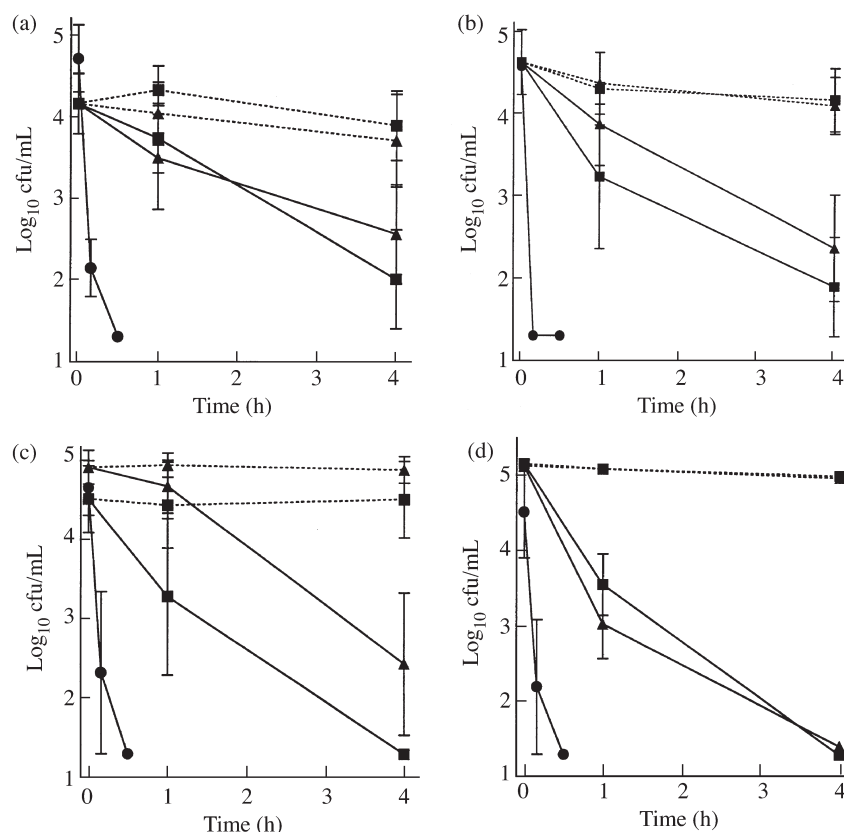
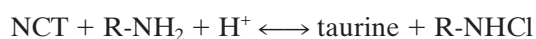
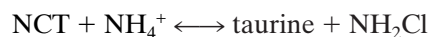


Figure 1. Fungicidal activity of 1% NCT at pH 7.4 (■), pH 5.4 (▲) and 1% NCT plus 0.005% NH₄Cl (●) in aqueous solution. The results are the mean ± S.E.M. of three to seven separate experiments ($n = 2$ for NCT + NH₄Cl). The differences between control (dotted lines) and test samples (solid lines) exceeding one log₁₀ step were significant ($P < 0.01$). (a) *A. fumigatus/A. flavus*, (b) *P. commune*, (c) *F. moniliforme*, (d) *C. albicans/C. parapsilosis*.

differences in cell wall and membrane composition and surface charges, resulting in differential penetration of NCT into the microbial cells.

The importance of the penetration of the oxidation capacity into the fungal cell is underlined by the enormous effect of ammonium when it is added to NCT, which leads to the formation of monochloramine by transhalogenation (see below). Because of its low bulk and lipophilic properties, NH₂Cl is able to penetrate cell membranes more easily than the hydrophilic NCT.³ By addition of 19 mM ammonium (0.1%) to 55 mM NCT (1%), 3.1 mM NH₂Cl was formed,¹⁰ which led to a 25-fold reduction in the incubation time required to kill $>10^4$ cfu/mL in the present study, compared with a 10-fold reduction against bacteria and a >100 -fold reduction against mycobacteria.^{3,10} This is in agreement with a study by Wagner *et al.*,¹¹ who demonstrated killing of *C. albicans* (10 cfu in microtitre wells) by monochloramine at 5 μM compared with NCT at 143 μM after an incubation time of 1 h.

The paradox of the finding of significantly more rapid killing of fungi in nasal mucus than in buffer solution is also explained by the transhalogenation mechanism. NCT equilibrates with NH₄⁺ and amino compounds to the corresponding *N*-chloro derivatives.¹



Some low molecular weight derivatives (mainly monochloramine, NH₂Cl, but also *N*-chloro alanine and glycine) are known to have stronger microbicidal activity than NCT.^{1,3} Since 1% NCT in the presence of 1 mM ammonium, the concentration found in our nasal mucus samples, showed markedly improved fungicidal activity, it follows that monochloramine will contribute significantly to the inactivation of fungi by NCT in these samples. This is in keeping with previous findings of enhanced activity of NCT against bacteria tested in samples of body fluids from inflammation sites.³

In conclusion, NCT was shown to possess powerful antifungal activity at concentrations applicable to human mucus membranes. The lower activity in aqueous solution compared with strong disinfectants like hypochlorite is compensated for by the marked enhancement of killing in mucus samples and the excellent tolerability to NCT. The reported role of yeasts and moulds in eosinophilic fungal rhinosinusitis suggests that treatment with antifungal agents may be beneficial. Application of NCT, an endogenous

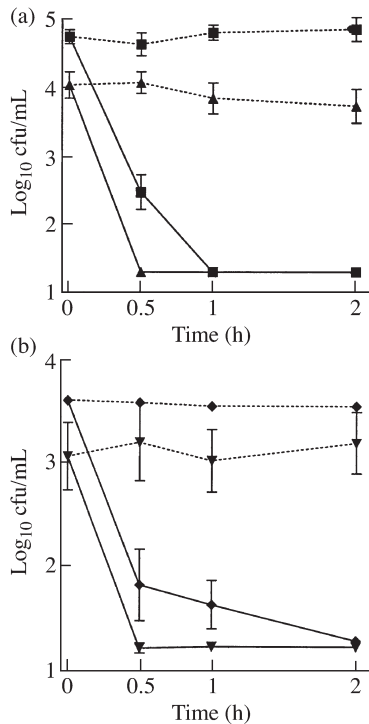


Figure 2. Fungicidal activity of 1% NCT in 10-fold diluted human nasal secretion. The results are the mean \pm S.E.M. of 10 mucus samples (seven for *A. flavus*). The differences between control (dotted lines) and test samples (solid lines) were significant ($P < 0.01$). (a) *C. albicans* (■), *F. moniliforme* (▲); (b) *A. flavus* (◆); *A. fumigatus* (▼).

agent the power of which is increased by components of the mucus surrounding these fungi, could therefore be advantageous.

Acknowledgements

We thank Professor Manfred P. Dierich, chairman of the Institute of Hygiene and Social Medicine, and Professor Ilse Jenewein for support. This study was supported by the Austrian Science Fund (grant P12298-MED) and by the Jubilee Research Fund of the Austrian National Bank (grants 6801/1 and 8366).

References

1. Grisham, M. B., Jefferson, M. M., Melton, D. F. & Thomas, E. L. (1984). Chlorination of endogenous amines by isolated neutrophils. Ammonia-dependent bactericidal, cytotoxic, and cytolytic activities of the chloramines. *Journal of Biological Chemistry* **259**, 10404–13.
2. Marcinkiewicz, J. (1997). Neutrophil chloramines: missing links between innate and acquired immunity. *Immunology Today* **18**, 577–80.
3. Nagl, M. & Gottardi, W. (1996). Enhancement of the bactericidal efficacy of *N*-chlorotaurine by inflammation samples and selected N-H compounds. *Hygiene und Medizin* **21**, 597–605.
4. Nagl, M., Hengster, P., Semenitz, E. & Gottardi, W. (1999). The postantibiotic effect of *N*-chlorotaurine on *Staphylococcus aureus*. Application in the mouse peritonitis model. *Journal of Antimicrobial Chemotherapy* **43**, 805–9.
5. Nagl, M., Hess, M., Pfaller, K., Hengster, P. & Gottardi, W. (2000). Bactericidal activity of micromolar *N*-chlorotaurine—evidence for its antimicrobial function in the human defense system. *Antimicrobial Agents and Chemotherapy* **44**, 2507–13.
6. Cantin, A. M. (1994). Taurine modulation of hypochlorous acid-induced lung epithelial cell injury *in vitro*. Role of anion transport. *Journal of Clinical Investigation* **93**, 606–14.
7. Nagl, M., Teuchner, B., Pöttinger, E., Ulmer, H. & Gottardi, W. (2000). Tolerance of *N*-chlorotaurine, a new antimicrobial agent, in infectious conjunctivitis—a phase II pilot study. *Ophthalmologica* **214**, 111–4.
8. Ponikau, J. U., Sherris, D. A., Kern, E. B., Homburger, H. A., Frigas, E., Gaffey, T. A. *et al.* (1999). The diagnosis and incidence of allergic fungal sinusitis. *Mayo Clinic Proceedings* **74**, 877–84.
9. Anonymous. (1988). Doppelte Standardaddition. In *ISE-Fibel zur ionenselektiven Messtechnik*, (Wissenschaftlich-Technische Werkstätten GmbH, Ed.), pp. 30–1. WTW, Weilheim, Germany.
10. Nagl, M. & Gottardi, W. (1998). Rapid killing of *Mycobacterium terrae* by *N*-chlorotaurine in presence of ammonium is caused by the reaction product monochloramine. *Journal of Pharmacy and Pharmacology* **50**, 1317–20.
11. Wagner, D. K., Collins-Lech, C. & Sohnle, P. G. (1986). Inhibition of neutrophil killing of *Candida albicans* pseudohyphae by substances which quench hypochlorous acid and chloramines. *Infection and Immunity* **51**, 731–5.

Received 30 October 2000; returned 2 January 2001; revised 26 February 2001; accepted 19 March 2001