N-Chlorotaurine shows high \textit{in vitro} activity against promastigotes and amastigotes of \textit{Leishmania} species

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Protozoan parasites of the genus \textit{Leishmania} are the causative agents of life-threatening visceral as well as cutaneous and mucocutaneous leishmaniasis. First-line drugs are antimonials, but toxicity and resistance in some endemic areas cause serious problems. In the current study, the antileishmanial activity of the weak oxidant \textit{N}-chlorotaurine (NCT) was investigated. NCT is a derivative of the amino acid taurine produced by granulocytes and monocytes during oxidative burst, but can also be synthesized chemically and used topically as an antiseptic at a concentration of 1\% (55 mM) \textit{in vivo}. NCT susceptibility tests were performed \textit{in vitro} with promastigotes and amastigotes of \textit{Leishmania infantum} and \textit{Leishmania donovani}. As NH\textsubscript{2}Cl is known to increase the activity of NCT by the formation of monochloramine (NH\textsubscript{2}Cl\textsubscript{2}), co-treatment assays were included in the study. Mean EC\textsubscript{50} values after 1 h of treatment were 5.94 mM for \textit{L. infantum} and 9.8 mM for \textit{L. donovani} promastigotes. Co-treatment with 5.5 mM NCT plus 19 mM NH\textsubscript{2}Cl led to complete killing of promastigotes of both strains within 15 min. Amastigotes were inactivated by treatment with 2 mM NCT alone. The results of this study indicate a high potential of NCT against \textit{Leishmania} species.

\textbf{INTRODUCTION}

\textit{Leishmania} species are the causative agents of visceral, cutaneous and mucocutaneous leishmaniasis. These vector-borne infections are endemic in more than 80 countries around the world, and according to the World Health Organization they have become the second highest cause of death worldwide after malaria among parasitic diseases, with a 42-fold increase within the last 15 years. Two million new cases – 1.5 million from cutaneous leishmaniasis and 500 000 from the visceral form – occur every year, but as registration of the diseases is compulsory in only 32 countries, a substantial number of cases are never recorded (Singh \textit{et al.}, 2006). Furthermore, co-infection with human immunodeficiency virus presents a serious problem concerning non-responsiveness to therapy and the fact that in AIDS patients almost every strain of \textit{Leishmania} species can cause any clinical picture mentioned above (Hofman \textit{et al.}, 2000; Mathur \textit{et al.}, 2006).

For therapy, pentavalent antimonials have been successfully administered since 1912, but although these drugs still are the first-line drugs in many countries, they require a prolonged series (up to 30 days) of daily intravenous infusions and they exhibit toxicity (Collin \textit{et al.}, 2004). Moreover, miscellaneous modes of resistance against antimonials are increasing (Ashutosh \textit{et al.}, 2007). A recent study has shown that, in Bihar (India), 60\% of patients currently suffering from leishmaniasis are infected with non-responding \textit{Leishmania} species (Croft \textit{et al.}, 2006); in 2000, this percentage was around 40\% (Eibl, 2000). Amphotericin B and its liposomal form, as well as miltefosine, are alternative drugs. However, these drugs also have shortcomings including toxicity, cost and possible resistance (Escobar \textit{et al.}, 2002; Sundar & Rai, 2005). The limited therapeutic options, the toxicity of
antileishmanial drugs and the increase in resistance thus necessitate a change in the treatment policy.

The aim of the present study was to evaluate the susceptibility of Leishmania infantum and Leishmania donovani promastigotes and amastigotes to N-chlorotaurine (NCT; synthesized chemically as the water-soluble crystalline sodium salt Cl-HN-CH₂-CH₂-SO₃Na, molecular mass 181.57 g; Gottardi & Nagl, 2002). In aqueous solution, NCT can be stored at 2–4 °C for 1 year (Gottardi & Nagl, 2002). As an antiseptic, it is possible that it could be used as a treatment for cutaneous forms of leishmaniasis. NCT is also a natural long-lived oxidant produced by human granulocytes and monocytes from hypochlorite (HOCl) and taurine during oxidative burst. In vivo, NCT is involved in the downregulation of pro-inflammatory cytokines, but its complete function has not yet been entirely elucidated. The ability of human tissues to tolerate synthetically produced NCT is exceptionally high. The human eye, a highly sensitive tissue, has been shown to tolerate 55 mM NCT in a clinical phase II study (Teuchner et al., 2005). Human skin and mucosa also tolerate 55 mM NCT without any noticeable adverse effects (Hofer et al., 2003; Nagl et al., 1998c, 2003). In vitro antimicrobial activity has been reported at micro- to millimolar concentrations against viruses (Nagl et al., 1998a), bacteria (Nagl et al., 2000), fungi (Reeves et al., 2006), heminths (Yazdanbakhsh et al., 1987) and recently against protozoa, i.e. Acanthamoeba species (Führkranz et al., 2008) and Trichomonas vaginalis (U. Führkranz, M. Nagl, W. Gottardi, M. Duchène, H. Aspöck and J. Walochnik, unpublished results). An enhancing effect of NH₄Cl on the efficacy of NCT, explained by the formation of the lipophilic and highly microbial monochloramine (NH₂Cl) in equilibrium (NCT + NH₄Cl→taurine + NH₂Cl+H⁺+Cl⁻), has been outlined previously (Gottardi et al., 2007; Nagl & Gottardi, 1996). Respective tests were included in this study.

**METHODS**

**Cultivation of promastigotes.** Experiments were carried out with L. infantum MCAN/ES/89/IPZ 229/1/89, zymodem MON 1 and L. donovani MHOM/ET/67/HU3. Promastigotes of L. infantum and L. donovani were cultivated as described elsewhere (Grimm et al., 1991) and subcultured every 72 h.

**Cultivation of monocytes and macrophages and subsequent culture of amastigotes.** Human leukaemia monocytes (THP-1) were grown at 37 °C with 5% CO₂ in 80 cm² culture flasks with filter caps in RPMI 1640 (Sigma), supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin and streptomycin, and were subcultured every 72 h (Gebre-Hiwot et al., 1992). Cells were harvested at exponential growth phase by centrifugation at 300 g for 5 min, counted in a haemocytometer and adjusted to 0.5 × 10⁶ cells ml⁻¹. For differentiation into macrophages, 12-well plates were seeded with 1 ml RPMI 1640 containing the adjusted concentration of cells, phorbol 12-myristate 13-acetate (20 ng ml⁻¹ final concentration; Sigma) was added and plates were incubated for 72 h. The medium was decanted and adherent and non-dividing macrophages were incubated for 24 h in fresh medium. Macrophages were infected with promastigotes that were 72 h old (‘metacyclic’: short slim cells with a long flagellum) at a ratio 1:10. After 24 h of co-incubation at 37 °C with 5% CO₂, extracellular promastigotes were removed by washing twice with pre-warmed PBS. In parallel, macrophage differentiation and infection with promastigotes was also carried out in chamber slides with 100 µl RPMI 1640 per well. Chamber slides were stained with Giemsa to confirm successful infection.

**Test procedure for promastigotes.** Experiments were carried out in six-well plates at 25 °C and were performed in culture medium and PBS in parallel. Each well was seeded with 10⁷ parasites ml⁻¹ in 2 ml medium or PBS. The promastigotes were treated with NCT at final concentrations of 2 mM (0.036%) to 55 mM (1%). Effectiveness was recorded after 1 and 3 h, as preliminary tests with 55 mM NCT in culture medium revealed a decrease in oxidative capacity by 85.5% within 30 min and by 93.8% within 2 h, due to chlorine consumption by reaction of NCT with ingredients of the culture medium. Viability was determined by counting mobile cells with a Bürker–Türk haemocytometer (filling both chambers and counting four 1 mm² squares each) by phase-contrast microscopy at a magnification of ×400. All experiments were carried out in triplicate and repeated twice. Mean EC₅₀ and EC₉₀ values were calculated from the respective data.

**Test procedure for monocytes, macrophages and amastigotes.** The efficacy of NCT against amastigotes was assessed with an in vitro model (Gebre-Hiwot et al., 1992) using the human leukaemia monocyte cell line THP-1, grown as described above. In brief, monocytes and macrophages were tested for their tolerability to NCT by adding NCT to the culture flasks containing monocytes and to the wells containing the differentiated macrophages to reach final concentrations of 0.02, 0.2 and 2 mM NCT. These concentrations were chosen with reference to previous studies revealing a tolerability of 1.5 mM NCT of RAW 264.7 macrophages (Park et al., 1995). Controls were incubated in RPMI 1640 without additives. The cells were incubated at 37 °C with 5% CO₂ for 3 h. Subsequently, the NCT was neutralized with sodium thiosulfate (3% to neutralize 1% NCT; Merck) and viability was assessed by trypan blue (0.4%; Sigma) exclusion directly in RPMI 1640. The results showed that monocytes and macrophages tolerated concentrations of 2 mM NCT for 3 h (data not shown). Therefore, this concentration was used for further experiments. The cytotoxicity of NCT against intracellular parasites was tested by adding NCT (2 mM final concentration) to the plates containing the macrophages infected with amastigotes (in the current study, cells without visible flagellum are referred to as amastigotes; cells were observed by light microscopy only). After incubation at 37 °C with 5% CO₂ for 3 h, the NCT was neutralized and the medium was decanted. The effectiveness of NCT against amastigotes was recorded microscopically by assessing amastigote viability and their ability to divide within the sessile macrophages. Killing of amastigotes was evaluated by microscopic observation of the plates for 1 week. The viability of treated and untreated amastigotes liberated from the macrophages was determined by transferring them from RPMI 1640 to promastigote culture medium (see above) and subsequent incubation at 25 °C instead of 37 °C. Transformation from amastigotes to promastigotes was checked daily for up to 7 days. Controls without NCT were treated the same way.

**Synergistic effects of NH₄Cl.** To investigate the possible synergistic effects of NH₄Cl (Sigma) on the effectiveness of NCT, promastigotes of both strains were co-treated with NH₄Cl in culture medium or PBS at a molar ratio of 1:3.4 (NCT:NH₄Cl). Experiments were carried out as described above and EC₅₀ and EC₉₀ values were compared. Promastigotes incubated with 20 mM NH₄Cl alone for 3 h showed no reduction in viability or motility.
RESULTS AND DISCUSSION

In the present study, NCT was shown to be highly effective against *Leishmania* species. Effective concentrations of NCT were in the millimolar range, which is relatively high compared with other substances used for antileishmanial treatment. However, NCT is an endogenous substance and even the highest concentrations tested in this study are well tolerated by human tissues after topical application (Nagl et al., 1998b, c, 2003; Teuchner et al., 2005). Promastigotes and amastigotes of both strains tested were susceptible to NCT, with *L. infantum* more susceptible than *L. donovani* and amastigotes more susceptible than promastigotes. The effectiveness of NCT was higher in PBS than in culture medium, and co-treatment with NH₄Cl enhanced the effectiveness of NCT.

Susceptibility of promastigotes

The lowest effective concentration against promastigotes was 2 mM NCT, resulting in growth inhibition in both *L. infantum* and *L. donovani*. Mean EC₅₀ values after 1 h of treatment were 5.94 ± 4.07 mM for *L. infantum* and 9.80 ± 1.30 mM for *L. donovani* (Table 1). Generally, at lower overall concentrations of NCT, *L. donovani* promastigotes were significantly less susceptible than the promastigotes of *L. infantum*. Such differences in drug susceptibility between relatively closely related species have been reported previously (Escobar et al., 2002; Yardley et al., 2005) and, in the genus *Leishmania*, have been explained by variations in sterol and lipid content in the cell membrane (Beach et al., 1979). This may also be the reason for the observed differences in susceptibility to NCT. As NCT possesses a rather non-specific mode of action, which is based on the oxidation of thio, amino and aromatic residues, oxidation of the surface of the parasites can be assumed to depend on the number of residues in the membrane. However, killing of microbes by NCT does not occur by simple oxidation of the surface; it also requires penetration of the oxidant (Gottardi & Nagl, 2005). Treatment with 55 mM NCT led to 100% lysis of promastigotes of both species investigated within 3 h (data not shown).

Susceptibility of amastigotes

Amastigotes were generally more susceptible to treatment than promastigotes. Treatment with 2 mM NCT for 3 h was highly effective, shown by the failure of treated cells to transform into promastigotes, or by reduced transformation in the case of *L. donovani* (Table 2). This phenomenon was also confirmed by a 1 week follow-up, thus also indicating a higher survival rate of amastigotes of *L. donovani* than those of *L. infantum*. Untreated controls transformed into promastigotes within 24 h after transfer to culture medium and proliferated within the next 72 h in both species. As THP-1 cells are able to tolerate 2 mM NCT for 24 h, as confirmed previously, we also treated the intracellular amastigotes for 24 h. The 24 h treatment with 2 mM NCT completely inhibited the later transformation into promastigotes, thus indicating complete inactivation of amastigotes in both strains. However, as described above, in culture medium the oxidative capacity of NCT is reduced by over 90% within 2 h, and thus complete killing of amastigotes cannot be assumed to be solely due to NCT. These late effects might be due to a destructive effect of NCT on factors essential for the parasites, to enhanced killing activity of the host cells or to toxic decay produced by dying or dead amastigotes.

Table 1. Mean EC₅₀ and EC₉₀ values (mM) of NCT in culture medium in experiments with promastigotes

<table>
<thead>
<tr>
<th>L. infantum</th>
<th>1 h</th>
<th>3 h</th>
<th>L. donovani</th>
<th>1 h</th>
<th>3 h</th>
</tr>
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<tbody>
<tr>
<td>EC₅₀</td>
<td>5.94 ± 4.07 (5.89 ± 3.05)</td>
<td>2.47 ± 1.31 (4.94 ± 1.23)</td>
<td>9.80 ± 1.30</td>
<td>5.76 ± 1.60</td>
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<tr>
<td>EC₉₀</td>
<td>20.01 ± 12.24 (10.69 ± 6.45)</td>
<td>8.56 ± 1.20 (8.81 ± 2.70)</td>
<td>17.34 ± 3.79</td>
<td>13.44 ± 5.22</td>
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EC₅₀ values were lower in *L. infantum* (*P* = 0.024 after 1 h of treatment; *P* = 0.03 after 3 h) than in *L. donovani*; EC₉₀ values were lower after 3 h of treatment (*P* = 0.017). In experiments with *L. infantum*, co-treatment with NCT plus NH₄Cl at a molar ratio of 1:3.4 (data in parentheses) led to lower EC₅₀ and EC₉₀ values after 1 h than without additives; however, these differences were not statistically significant. Data are given as means ± SD of data from nine independent experiments.
Synergistic effects of NH$_4$Cl

The addition of NH$_4$Cl led to enhancement of the efficacy of NCT. The highest level of effectiveness was achieved by co-treatment with 5.5 mM NCT plus 19 mM NH$_4$Cl (molar ratio 1:3.4) in PBS, which led to complete killing of promastigotes of both strains within 15 min (Fig. 1). These findings are in good accordance with previous studies, where the addition of NH$_4$Cl decreased the killing times of NCT markedly against bacteria and particularly fungi (Gottardi et al., 2007; Nagl & Gottardi, 1996; Nagl et al., 2001). The enhancing effects are explained by the transfer of active chlorine from NCT to NH$_4$Cl, whereby NH$_3$Cl is formed in equilibrium, which penetrates and kills pathogens more rapidly. Enhanced killing effects were higher in PBS than in culture medium. In culture medium, they were only observed for L. infantum, where co-treatment of promastigotes with NCT plus NH$_4$Cl led to a decrease in EC$_{50}$ and EC$_{90}$ values after 1 h compared with treatment with NCT alone (Table 1). These results are in keeping with the inhibitory effects of high-molecular-mass organic material, such as albumin, on the microbicidal potential of NCT (Nagl & Gottardi, 1996). Some ingredients of the culture medium, mainly thio groups and amino groups of high-molecular-mass proteins, for example from serum contents, partially inactivate NCT (Gottardi et al., 2001). This loss of activity in the medium used in the current study sufficiently explains the absence of significant killing activity of NCT at concentrations of 10 mM or lower. It also explains the lower effects of NH$_4$Cl on the effectiveness of NCT in culture medium compared with PBS. Nevertheless, 100% inactivation of promastigotes was achieved within 3 h with 55 mM NCT at 25 °C, indicating that the remaining oxidative capacity after 2 h is sufficient to complete the killing of Leishmania species in culture medium.

In conclusion, this study demonstrated the high in vitro activity of NCT against both L. infantum and L. donovani promastigotes and amastigotes, with co-treatment with NH$_4$Cl leading to a further enhancement of effectiveness. NCT is unlikely to provoke resistance, as the mechanism of action is non-specific (Arnitz et al., 2006; Gottardi & Nagl, 2002). Moreover, it is an endogenous amino acid derivative of low molecular mass; thus allergic reactions are unlikely to occur. Further studies to investigate the suitability of NCT for treatment of the cutaneous forms of leishmaniasis would appear to be justified.

ACKNOWLEDGEMENTS

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REFERENCES


Table 2. Conversion rate of treated and untreated Leishmania species amastigotes into promastigotes

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 7</th>
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<tbody>
<tr>
<td>L. donovani 2 mM NCT, 3 h</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. donovani control</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. infantum 2 mM NCT, 3 h</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>L. infantum control</td>
<td>+</td>
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