

ORIGINAL ARTICLE

***N*-chlorotaurine, a potent weapon against multiresistant bacteria**C. Anich, D. Orth-Höller , M. Lackner  and M. Nagl 

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Abstract**Aims:** *N*-chlorotaurine (NCT) is a body-own mild oxidizing antiseptic that can be applied topically as a well-tolerated anti-infective at many body sites. The objective of this study was to demonstrate its activity against representative nosocomial multidrug-resistant bacteria.**Methods and Results:** The bactericidal activity of NCT was tested in quantitative killing assays against a panel of multiresistant Gram-positive and Gram-negative clinical isolates. *N*-chlorotaurine (1%, 55 mmol l⁻¹) reduced the number of CFU of strains of methicillin-resistant *Staphylococcus aureus*, linezolid-resistant *Staphylococcus epidermidis*, vancomycin-resistant, and linezolid- and vancomycin-resistant *Enterococcus faecium*, 3MRGN and 4MRGN *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* by at least 2 log₁₀ steps after 15 min and completely or nearly to the detection limit after 30 min at pH 7.1 and 37°C.**Conclusion:** The activity of NCT against these clinical isolates is similar to that against non-resistant ATCC strains and therefore not influenced by antibiotic resistance. This can be explained by the oxidizing and chlorinating mechanism of action of NCT, which leads to an attack of multiple targets in the microorganisms.**Significance and Impact of the Study:** The bactericidal spectrum of NCT is not restricted by resistance against antibiotics. Therefore, it can be used against resistant strains, too.**Introduction**

Antimicrobial resistance is an important cause of morbidity and mortality worldwide with an estimated 67 000 infections and 330 000 deaths in the European Union/European Economic Area (ECDC 2019) and more than 2.8 million infections and 35 000 deaths in the United States each year (CDC 2019). Emergence of antimicrobial resistance has been identified, in 2019, as one of the ten major global health threats by the World Health Organization. Overuse and misuse of antibiotics both in human medicine and agriculture are estimated as reasons (Gajdács and Albericio 2019). Carbapenem-resistant *Acinetobacter* and *Enterobacterales* are classified as urgent threats. Extended-spectrum β -lactamase (ESBL)-producing *Enterobacterales*, multidrug-resistant *Pseudomonas aeruginosa*, vancomycin-resistant enterococci (VRE) and

methicillin-resistant *Staphylococcus aureus* (MRSA) are defined as serious threats in the Antibiotic Resistance Threats report 2019 of the Centers for Disease Control and Prevention (CDC 2019). Due to the lack of effective antimicrobial agents, antibiotic resistant infections are often difficult, or sometimes even impossible to treat. Thus, there is a need for alternative strategies to combat infections with multidrug-resistant bacteria.

Measures of hygiene and antibiotic stewardship are attempts to keep resistance rates low or to avoid it. An additional strategy is to apply antiseptics as an alternative or supplement to antibiotics, whenever topical therapy of infections is possible. There are several advantages of antiseptics compared to antibiotics. Their unspecific mechanism of action is completely different so that in general resistance does not play a role (Dong *et al.* 2017). The microbicidal activity is broader and markedly

stronger (Dong *et al.* 2017). They have higher activity against biofilms, and they inactivate microbial toxins (Cole *et al.* 2008; Schultz *et al.* 2017). A problem restricting their application to non-sensitive body regions and to a short period of about one week in general, is the higher cytotoxicity of antiseptics due to the unspecific mechanism of action (Schultz *et al.* 2017).

However, this may be overcome by using body-own (endogenous) substances with mild, but sufficient microbicidal activity. A typical representative is *N*-chlorotaurine (Cl-HN-CH₂-CH₂-SO₃H) from the class of oxidants and group of chloramines (R-NHCl compounds) (Grisham *et al.* 1984), which can be synthesized as sodium salt (NCT, Cl-HN-CH₂-CH₂-SO₃Na) (Gottardi and Nagl 2010). It is part of innate immunity, produced from hypochlorite (HOCl) and taurine within activated human granulocytes and monocytes (Grisham *et al.* 1984), and it is involved in the defence against microbes as well as in termination of inflammation (Marcinkiewicz and Kontny 2014). Due to its oxidizing and chlorinating mechanism of action, it has microbicidal activity against all kinds of micro-organisms (bacteria, viruses, fungi, protozoa, worm larvae) (Gottardi and Nagl 2010). In parallel, it downregulates proinflammatory mediators, cytokines and chemokines (Marcinkiewicz and Kontny 2014). The mild reactivity of NCT is the reason for its high tolerability by human tissue so that it can be applied topically in sufficient concentration (usually 1%, 55 mmol l⁻¹) and repeatedly to regions all over the human body including sensitive ones, such as skin ulcers, mucous membranes, the eye, the ear, paranasal sinuses, the urinary bladder, organ abscesses, bone joints and others (Gottardi and Nagl 2010).

Due to the oxidizing and chlorinating mechanism of action of NCT, it can be expected that it is bactericidal against Gram-positive and Gram-negative bacteria irrespective of their resistance against antibiotics. It was the

aim of this study to prove the microbicidal activity of NCT against a panel of such microorganisms.

Materials and methods

NCT and other chemicals

N-chlorotaurine (NCT) (molecular weight 181.57 g mol⁻¹, lot 2018-11-06) was prepared as crystalline sodium salt (Cl-HN-CH₂-CH₂-SO₃⁻ Na⁺) from taurine and chloramine T in our laboratory at pharmaceutical grade, as reported (Gottardi and Nagl 2010), stored at minus 20°C, and freshly dissolved in sterile phosphate buffer (0.1 mol l⁻¹) to a concentration of 1% for each experiment. The chemical structure is shown in Fig. 1. Identity and purity were verified by spectrophotometric analysis (peak at 251.5 nm), infrared spectrometry (band at 3260 cm⁻¹), and mass spectrometry, and the content of active chlorine by iodometric titration (maximum chlorine content 19.52%) (Gottardi and Nagl 2002). The sodium salt can be stored at 20°C for about 2 weeks, at 2–4°C for 1 year, at –20°C or lower for at least 2 years, so that cooling is necessary for long-term storage. Solutions can be stored up to 1 year at 2–4°C and 2 weeks at room temperature protected from UV light. Phosphate buffer (0.1 mol l⁻¹), and tryptic soy broth were from Merck (Darmstadt, Germany), *L*-histidine from Serva (Heidelberg, Germany), and *DL*-methionine from Sigma-Aldrich (Steinheim, Germany).

Bacteria

Multiresistant clinical isolates deep frozen for storage at the Institute of Hygiene and Medical Microbiology were used. All strains were grown on Columbia blood agar at 37°C. For the tests, colonies from the agar were grown overnight for 16 h at 37°C in tryptic soy broth,

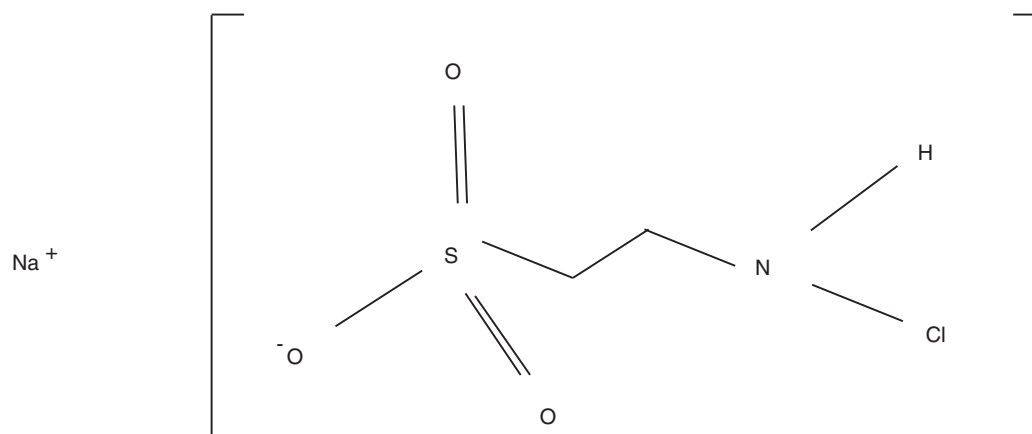


Figure 1 Chemical structure of *N*-chlorotaurine sodium salt.

centrifuged at 1800 g for 10 min and washed twice in 0.9% NaCl. The washed suspensions contained 5×10^8 to 1×10^9 colony forming units (CFU) per ml.

The following isolates were tested separately. Methicillin-resistant *S. aureus* (MRSA), three isolates; linezolid-resistant *Staphylococcus epidermidis* (LRS), three isolates; vancomycin-resistant *Enterococcus faecium* (VRE), three isolates; linezolid- and vancomycin-resistant *E. faecium* (LVRE), two isolates; three multidrug-resistant Gram-negative (MRGN) ESBL *Escherichia coli*, three isolates; 4MRGN *E. coli*, three isolates; 3MRGN *P. aeruginosa*, three isolates; 4MRGN *P. aeruginosa*, three isolates; 3MRGN *Acinetobacter baumannii*, two isolates; 4MRGN *A. baumannii*, three isolates; 3MRGN *Klebsiella pneumoniae*, two isolates; 4MRGN *K. pneumoniae*, three isolates. For the 3 and 4 MRGN classification, the KRINKO guidelines of the Robert Koch Institute were followed (KRINKO 2012).

The isolates originated from swabs from the skin or mucosa (14/33, 42.4%), from urine (13/33, 39.4%), from sputum (3/33, 9.1%), and from blood cultures (3/33, 9.1%).

Quantitative killing assays

To 3.96 ml prewarmed 1% NCT solution in phosphate buffer at pH 7.1, 40 μ l of the bacterial suspension (5×10^8 to 1×10^9 CFU per ml) was added and vortexed. Therefore, the final test suspensions contained 5×10^6 to 1×10^7 CFU per ml. After different incubation times at 37°C (5, 15, 30, 60 min), aliquots of 100 μ l were removed and mixed with 900 μ l of 1% methionine/1% histidine in distilled water to inactivate NCT. Aliquots of this suspension were spread onto Mueller-Hinton agar plates in duplicate (50 μ l each) using an automatic spiral plater (model WASP 2; Don Whitley, Shipley, UK). The detection limit was 100 CFU per ml, taking into account both plates and the previous 10-fold dilution in the inactivating solution. Plates were grown for 24 h at 37°C before colonies were counted. Plates with no growth or only a low CFU count were grown for up to 5 days to detect bacteria attenuated but not killed by the treatment. Controls in 0.1 mol l⁻¹ phosphate buffer were performed without NCT. Inactivation controls, where NCT was mixed with its inactivator 1% methionine and 1% histidine immediately before addition of pathogens at low CFU counts, showed full survival of bacteria as known from previous studies (Böttcher *et al.* 2017; Gruber *et al.* 2017).

Statistics

The data are presented as mean values and standard deviations (SD) of at least three independent experiments.

Student's unpaired *t* test in case of two groups, or one-way analysis of variance and Tukey's multiple-comparison test in case of more than two groups, were used to test for a difference between the test and control groups. A *P* value of <0.05 was considered significant for all tests. Calculations were done with the GraphPad Prism 7.00 software (GraphPad Inc., La Jolla, CA).

To gain an improved survey on the microbicidal activity of NCT against the different strains, the recently introduced Integral Method was used, which transforms the whole killing curve (log₁₀ CFU per ml vs time) into one value of 'Bactericidal Activity (BA, log₁₀ CFU per ml per min)' (Gottardi *et al.* 2015). The method is based on the area below the killing curve, which is calculated by addition of the areas of trapezoids between the single time points of incubation and transformed into an orthogonal triangle with the same area. Its hypotenuse forms with the abscissa the angle α , whose tangent, $\text{tg}\alpha = y/2x$, represents the sought average BA. The 'Specific Bactericidal Activity' as an intrinsic measure of the strength of activity was obtained by division by the concentration of NCT ($\text{SBA} = \text{BA}/0.055 \text{ mol l}^{-1}$) (Gottardi *et al.* 2015). The method allows an expanded statistical analysis, particularly between killing curves with small differences.

Results

N-chlorotaurine demonstrated its typical bactericidal activity against all multidrug-resistant test strains. In Fig. 2, four representative killing curves are depicted. The CFU per ml of all strains were reduced by at least 2 log₁₀ steps after 15 min and completely or nearly to the detection limit after 30 min.

An overview is provided by the BA and SBA values calculated with the Integral Method (Table 1). The higher the value, the higher the bactericidal activity. The advantage of the Integral Method is the possibility to reliably compare whole killing curves of the different strains. The descending order, starting with the highest susceptibility, was *K. pneumoniae*>*E. coli*, *P. aeruginosa*, *E. faecium* (VRE)>*S. aureus*, *S. epidermidis*, *A. baumannii* and *E. faecium* (LVRE). These differences between the species were statistically significant.

The variability of single strains within the same species was generally smaller and significant only between VRE and LVRE, between 3 and 4MRGN *E. coli*, and between 3 and 4MRGN *A. baumannii*.

Discussion

Resistance of bacteria against antibiotics is one of the emerging problems in the field of infectious diseases and chronic diseases where pathogens play a role, for instance

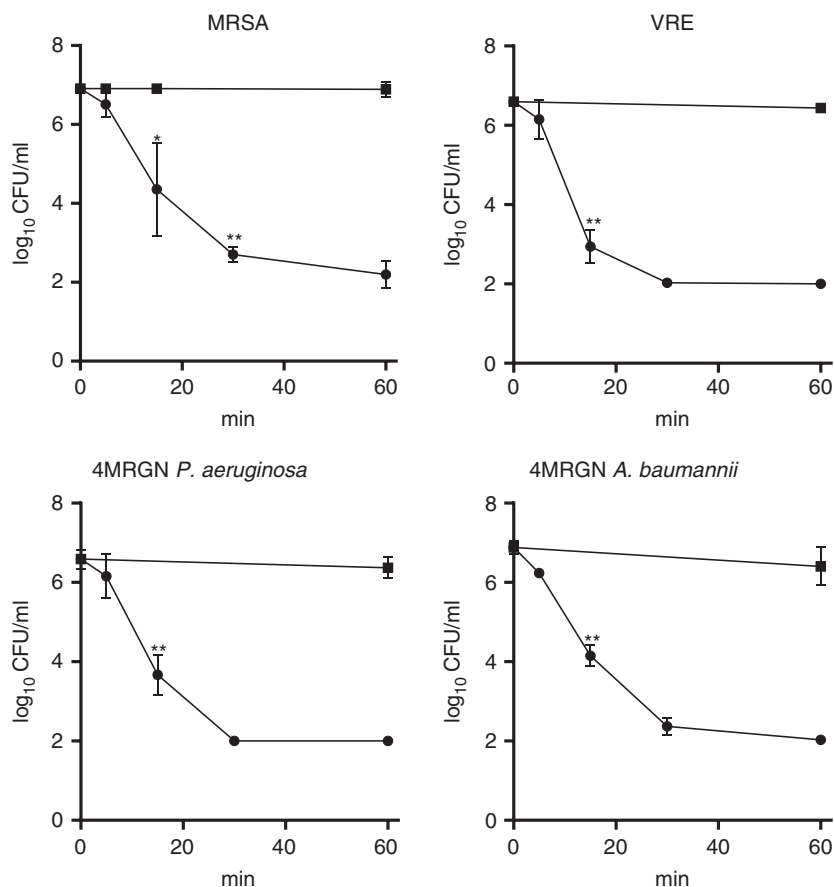


Figure 2 Bactericidal activity of 1% NCT against different multidrug-resistant clinical isolates at pH 7.1 and 37°C. ●- NCT; ■- phosphate buffer without NCT (control); mean values \pm SD of three independent experiments with three different strains. * $P < 0.05$, ** $P < 0.01$ vs control. The detection limit was 2 log₁₀ CFU per ml.

chronic obstructive pulmonary disease. Whenever topical therapy comes into question, antiseptics should be considered as an alternative. Our study clearly demonstrates the broad-spectrum bactericidal activity of a mild active chlorine compound irrespective of antibiotic resistance.

The absence of resistance against NCT can be explained easily by the oxidizing and chlorinating mechanism of action (Gottardi *et al.* 2013), which leads to multiple sites of attack within pathogens (Arnitz *et al.* 2006). Similarly, their virulence factors are chemically inactivated as shown with toxins and enzymes of *E. coli*, *S. aureus*, *Aspergillus fumigatus* and *Candida albicans* (Gottardi *et al.* 2013). Micro-organisms upregulate antioxidative and salvage mechanisms in the presence of NCT, such as heat-shock proteins, proteins associated with ribosomes, transcription and translation (Arnitz *et al.* 2006; Sheehan *et al.* 2019). These pathways may be effective against physiological micromolar concentrations of oxidants created by leukocytes during inflammation. However, because of its mild activity and high

tolerability, NCT can be applied therapeutically in 1000-fold higher concentration (55 mmol l⁻¹) than the natural one (about 20–50 μ mol l⁻¹, (Grisham *et al.* 1984)). In this range, even repeated exposure to NCT and other chloramines does not induce resistance (Nagl and Gottardi 1996; D’Lima *et al.* 2012).

Basically, the activity of NCT was in a similar range against all test bacteria in this study. When the susceptibility of defined, non-multiresistant test strains of *S. aureus* (ATCC 25923, ATCC 6538), *E. coli* (ATCC 11229), *P. aeruginosa* (ATCC 27853) to NCT is compared with that of the multiresistant clinical isolates of this study, similar killing curves and BA values can be found (Martini *et al.* 2012; Gottardi *et al.* 2015; Gruber *et al.* 2017). It is true that statistically significant differences can be seen, but they are small and represent a typical inter-strain and inter-species variability against NCT. The reason for this variability must be considered in differences in the speed of penetration of active chlorine through the covers of micro-organisms into the cytosolic

Table 1 Bactericidal activity of 1% NCT, calculated with the integral method

Bacterial strain	BA	SD	SBA	SD
LRS	0.1174	0.0075	2.13	0.14
MRSA	0.1276	0.0086	2.32	0.16
VRE	0.1916	0.0158	3.48	0.29
LVRE	0.1386	0.0087	2.52	0.16
3MRGN <i>Escherichia coli</i>	0.1780	0.0138	3.24	0.25
4MRGN <i>E. coli</i>	0.2082	0.0166	3.78	0.30
3MRGN <i>Pseudomonas aeruginosa</i>	0.1913	0.0162	3.48	0.29
4MRGN <i>P. aeruginosa</i>	0.1659	0.0154	3.02	0.28
3MRGN <i>Acinetobacter baumannii</i>	0.1092	0.0095	1.99	0.18
4MRGN <i>A. baumannii</i>	0.1498	0.0118	2.72	0.22
3MRGN <i>Klebsiella pneumoniae</i>	0.2840	0.0225	5.16	0.41
4MRGN <i>K. pneumoniae</i>	0.3126	0.0295	5.68	0.54

BA, bactericidal activity (\log_{10} CFU per min); SBA, specific bactericidal activity (BA mol^{-1}); SD, standard deviation; LRS, linezolid-resistant *S. epidermidis*; MRSA, methicillin-resistant *S. aureus*; VRE, vancomycin-resistant *E. faecium*; LVRE, linezolid- and vancomycin-resistant *E. faecium*; MRGN, multidrug-resistant Gram-negative.

Mean values of BA and SD of three to nine independent experiments.

compartment. Oxidation of the surface resulting in a chlorine cover removes virulence of bacteria and fungi, but does not kill them (Gottardi and Nagl 2005). Thereby, active chlorine from NCT is attached to the surface of pathogens within seconds to a minute. Such incubation of bacteria and fungi for sublethal time in NCT leads to a lag of their regrowth, a so-called postantibiotic (postantiseptic) effect. As a consequence, these attenuated micro-organisms have been shown to lose their potency to kill mice and moth larvae respectively (Nagl *et al.* 1999; Lackner *et al.* 2015). It is assumed that the postantiseptic effect contributes to therapeutic effects of NCT *in vivo*, too.

In previous studies, we throughout found a higher susceptibility of *E. coli* and *P. aeruginosa* to NCT than *S. aureus* (Martini *et al.* 2012; Gottardi *et al.* 2015; Gruber *et al.* 2017). Also in this study, the same order occurred with multiresistant strains. The difference between bacterial species seems to be higher than that between single strains within a species, irrespective of their resistance to antibiotics. The data of this study do not support any relevant influence of different mechanisms of antibiotic resistance on the susceptibility to NCT.

In any case, the extent of variability between bacterial species can be regarded as irrelevant for clinical efficacy of NCT, similar to stronger active chlorine compounds like chloramine T and hypochlorous acid (Gottardi *et al.* 2013). Although not being the main endpoint, an influence of the bacterial species on the efficacy of NCT was not evident in previous clinical studies, for instance in crural ulcers, conjunctivitis, and otitis (for review see

(Gottardi and Nagl 2010)). It has to be considered that the activity of NCT, in contrast to other active halogen compounds and other antiseptics, is not decreased but enhanced in the presence of organic matter (for instance body fluids, purulent exudation) (Gottardi and Nagl 2010; Gottardi *et al.* 2014; Gruber *et al.* 2017) due to transfer of the active chlorine to ammonium and formation of the more lipophilic and higher microbicidal monochloramine in equilibrium (Gottardi *et al.* 2007). This reaction outmatches chlorine consumption and formation of high molecular weight chloramines with lower activity (Gottardi and Nagl 2010; Gottardi *et al.* 2014; Gruber *et al.* 2017), both of which occur to a relatively low extent because of the low reactivity of NCT.

A further consequence of this is the high tolerability of NCT, allowing its application to sensitive body regions, for instance to the eye, the urinary bladder and organ abscesses (Gottardi and Nagl 2010). In the last few years, also inhalation became a topic of interest, whereby indications such as chronic bronchitis and cystic fibrosis are well conceivable (Arnitz *et al.* 2018). Early treatment of infective exacerbations in these diseases could attenuate these causative episodes of worsening and delay the progression of loss of lung function. Considering the resistance problem with antibiotics (Gajdacs and Urban 2019), topical therapy with a well-tolerated antiseptic appears to be an interesting alternative or additive. Several indications are near at hand in this regard. Cleansing of chronic, infected wounds and soft tissue infections is a significant, not satisfactorily solved problem. Decontamination of carriers of multiresistant bacteria with NCT on different body sites on skin and mucosa is conceivable. Further examples are chronic and recurrent urinary tract infections, lung infections and organ abscesses (Gottardi and Nagl 2010; Arnitz *et al.* 2018). The advantage of NCT as an active chlorine compound is the absence of resistance development because of its multiple targets, the absence of systemic distribution and systemic adverse effects, and the good topical tolerability as an endogenous, mild oxidant (Gottardi and Nagl 2010; Gottardi *et al.* 2013).

The present study was done at a neutral pH for standardized comparability. At sites of infection, the pH may decrease. This is no matter of concern since the bactericidal activity of NCT increases at acidic pH. This can be explained by formation of the protonated form of NCT, NCTH^+ , which more easily comes into reaction with nucleophilic agents such as amino groups (Gottardi and Nagl 2002).

To decrease the danger and burden of multiresistant pathogens is one of the great goals announced by international health organizations such as the World Health Organization, the Center for Disease Control, and the

European Center for Disease Control. Usage of antiseptics instead of antibiotics for topical treatment of localized infections is a logical strategy to fight antibiotic resistance and should be pursued in general. Our study clearly confirms that multiresistance against antibiotics does not even influence the bactericidal activity of a mild antiseptic from the class of active chlorine compounds with both high tissue tolerability and sufficient activity in exudates.

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Conflict of Interest

No conflict of interest declared.

Ethics approval

Not applicable.

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