

Abstract

Background: *N,N*-dichloro-2,2-dimethyltaurine (NVC-422) and *N*-chloro-2,2-dimethyltaurine (NVC-612) are stable derivatives of the endogenous chlorotaurines. *N*-chlorotaurine (NCT) has been previously shown to have bactericidal activity in time-kill assays with potency dependent on the concentration of human plasma (Gottardi et al. 2001 JPP 53: 689-697). We studied the effects of human plasma, serum and albumin (HSA) on the Minimum Bactericidal Concentration (MBC) and time-kill kinetics of NVC-422, NCT, and NVC-612 against selected microorganisms. **Methods:** Activity against ATCC strains *E. coli* 25922, *S. aureus* 29213, and *C. albicans* 10231 was tested by MBC and time-kill assays using modified CLSI methods. The activity of NVC-422 against bacteria in biofilms was tested using a 96-well MBEC (Minimum Biofilm Eradication Concentration) assay. **Results:** MBCs of NVC-422 in low phosphate buffered saline pH 7 (PBS) alone compared with serum or HSA added; MBCs of NVC-612 or NCT were unaffected by addition of serum or HSA. All *N*-chlorotaurines at 1% concentration showed > 4 log₁₀ CFU reduction of bacteria by 2 h and yeast by 4 h with or without plasma, serum or HSA challenge. Supplementation of plasma, serum or HSA to NVC-422 in PBS slowed rate of kill against *S. aureus*, *E. coli* and *C. albicans* compared to NVC-422 in PBS alone. Conversely, addition of plasma, serum or HSA to NVC-612 or NCT in PBS maintained or accelerated time-kill against *E. coli*, *S. aureus* and *C. albicans*. 1% NVC-422 in 5 mM acetate saline pH 4 provided the most rapid biocidal activity against *S. aureus* and *E. coli* (complete kill in 15 min) and *C. albicans* (complete kill in 2 h) in the presence of 10% serum. NVC-422 was active against *E. coli* biofilms in the presence of serum or plasma. **Conclusions:** NVC-422 and related *N*-chlorotaurines demonstrate rapid bactericidal and antibiofilm activity in the presence of plasma, serum or albumin.

Introduction

N,N-dichloro-2,2-dimethyltaurine sodium salt (NVC-422) and *N*-chloro-2,2-dimethyltaurine sodium salt (NVC-612) represent chemically stabilized *N*-chlorotaurines that retain fast-killing and broad spectrum activity against a variety of microorganisms^{2,3}. *N*-Chlorotaurines are known to exercise significant bactericidal activity within the sequestered milieu of the neutrophil phagolysosome, but their effects may be modulated depending on the site of clinical application and exposure to physiological fluids such as plasma. *N*-chlorotaurines have been previously shown to oxidize thiol-containing plasma proteins resulting in inactivation of enzymes such as α₁-antitrypsin⁴. Nagl and Gottardi demonstrated augmented microbial activity of NCT via transchlorination of various amine compounds especially ammonium chloride⁵.

The focus of these studies was to compare the kinetics of time kill activity of specific *N*-chlorotaurines in the presence of human derived neutralizers (plasma, serum, and serum albumin) against relevant microorganisms over a pH range. Activity of *N*-chlorotaurines against bacterial biofilms was also examined.

Materials & Methods

CLSI Method Modifications. CLSI protocols for MBC and Time Kill testing were modified by substituting 0.1 M phosphate pH 7 for cation-adjusted Mueller Hinton broth (CAMHB) to compensate for the reactivity of *N*-chlorotaurines to hydrolyses of CAMHB⁶. Due to the rapid cidal nature of chlorotaurines, the MBC assay was shortened from 16-20 hours at 35°C to 60 minutes at room temperature, and time kill assays were conducted within 4 hours at room temperature. **Minimum Bactericidal Concentration (MBC).** Standard strains were purchased from the ATCC. The MBC is the lowest concentration of NVC-422, NCT and NVC-612 that gave >99.9% kill of the challenge organism. Microorganisms were grown to mid-log phase, resuspended in 0.1 M phosphate pH 7 and added to dilutions of test compounds to a final inoculum of 10⁸ - 10⁹ CFU/mL. After one hour, aliquots of the reaction mixture were transferred into 9 volumes of D/E neutralizing broth and drop plated for quantitation.

Time Kill (TK). Microorganisms were grown to mid-log phase, resuspended in saline, pH 4 or phosphate pH 7 and added to test compounds to a final inoculum of 10⁸ - 10⁹ CFU/mL. Aliquots were removed at specific intervals, neutralized as described above, and plated for quantitation. Time kill kinetics of 1% NVC-422, NCT or NVC-612 were tested against *S. aureus*, *E. coli*, and *C. albicans* in phosphate buffer pH 7 alone, and supplemented with either 10% (v/v) or 50% (v/v) serum (Fisher Cellcult). Time kill assays were also done with 1% NVC-422 in 0.9% NaCl pH 4 or 5mM acetate saline pH 4, each supplemented with 10% serum, against *S. aureus*, *E. coli*, and *C. albicans*.

Time kill kinetics of 1% (w/v) NVC-422, NCT or NVC-612 were tested against *S. aureus* and *E. coli* in phosphate buffer pH 7 alone, and supplemented with either 10% (v/v) or 50% (v/v) plasma [Alcalate]. As a major protein constituent of plasma [40 mg/L HSA (MP Biomedicals, low endotoxin)], was examined for interactions with *N*-chlorotaurines that might affect time kill efficacy. 0.4% (w/v) and 2% (w/v) HSA corresponding to 10% and 50% plasma diluted in phosphate pH 7, were tested in TK assays against *S. aureus* and *E. coli* with 1% (w/v) NVC-422, NCT or NVC-612. **Minimum Biofilm Eradication Concentration (MBEC).** The Calgary Biofilm Device (CBD) was used to determine the minimum biofilm eradication concentration (MBEC) of NVC-422. MBEC values were used to assess the concentration of an antimicrobial product required to kill bacterial biofilms. A standard culture of *E. coli* ATCC 25922 was grown and diluted to approximately 10⁷ CFU/mL for inoculation of the CBD assay. Biofilm was grown for 24 hr at 35°C prior to incubation with 0.8-4.0 mM NVC-422 for 60 min at room temperature, followed by neutralization in D/E broth and overnight incubation at 35°C in growth media. Biofilm quantitation was performed by measuring the absorbance at 650 nm the following day. Absorbance values of less than 0.1 were considered to be evidence of biofilm eradication.

Fig. 1. Comparative Time Kill of NVC-422, NCT and NVC-612 in various diluents with 10% (v/v) human serum

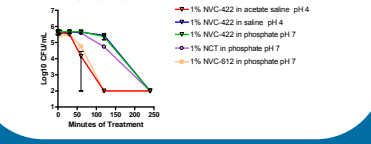
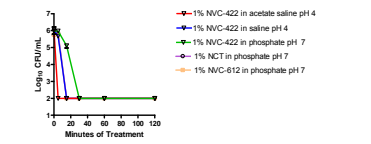
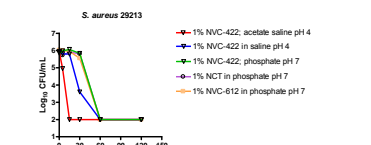


Table 1. MBC values of NVC-422, NCT and NVC-612 in 0.1 M Phosphate pH 7

Compound	Neutralizer	<i>E. coli</i> 25922	<i>S. aureus</i> 29213	<i>C. albicans</i> 10231
NVC-422	none	0.11	0.11	0.9
NCT	none	0.33	1.35	> 1.35
NVC-612	none	0.33	1.35	> 1.35
NVC-422	10% human serum	0.22	1.8	> 1.8
NCT	10% human serum	0.16	0.66	> 1.35
NVC-612	10% human serum	0.33	1.35	> 1.35
NVC-422	0.4% human albumin	0.45	1.8	> 1.8
NCT	0.4% human albumin	0.16	0.66	> 1.35
NVC-612	0.4% human albumin	0.67	1.35	> 1.35

Table 2. Time to 4 log Kill (min.): Select microorganisms in 0.1 M Phosphate pH 7 with human serum

Test Solution	0.1M Phosphate pH 7 only	+ 10% serum	+ 50% serum	Microorganism
1% NVC-422	15	60	60	<i>S. aureus</i> 29213
1% NCT	60	60	60	
1% NVC-612	60	60	30	
1% NVC-422	5	30	30	<i>E. coli</i> 25922
1% NCT	15	15	15	
1% NVC-612	15	15	30	
1% NVC-422	120	240	120	<i>C. albicans</i> 10231
1% NCT	> 240	240	120	
1% NVC-612	> 240	120	60	

Fig 2. Time Kill kinetics of NVC-422, NCT, and NVC-612 in 0.1 M Phosphate pH 7 in the presence of human plasma

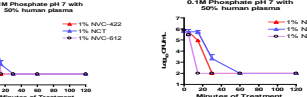
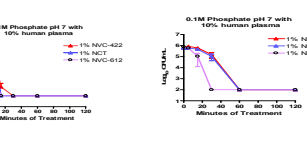
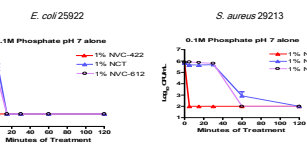


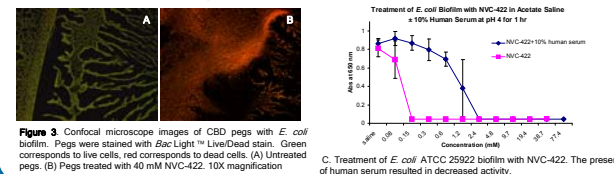
Table 3. Time to 4 log kill (min) in 0.1M Phosphate pH 7 with human serum albumin

Test Solution	0% HSA	0.4% HSA	2% HSA	Microorganism
1% NVC-422	15	60	60	<i>S. aureus</i> 29213
1% NCT	60	60	60	
1% NVC-612	60	60	60	
1% NVC-422	5	30	30	<i>E. coli</i> 25922
1% NCT	15	15	15	
1% NVC-612	15	15	15	

Table 4. pH shift with serum "challenge"

Assay Diluent	Diluent alone	10%	50%
0.1M Phosphate pH 7	7.00	6.98	7.03
0.6% Borate saline pH 8.2	8.23	8.22	8.03
0.9% NaCl pH 4	4.02	7.12	7.18
5mM acetate saline pH 4	3.97	4.95	6.76
Human serum	7.26		

Fig. 3. NVC-422 Activity Against *E. coli* Biofilm



Conclusions

The pH *N*-chlorotaurine solutions is critical for their efficacy as microbicides; NVC-422 showed the fastest kinetics of time kill in 5mM acetate saline pH 4 compared to all other diluents and chlorotaurines tested. In one hr MBC assays, NVC-422 gave lower MBCs than NCT or NVC-612 in phosphate pH 7 alone against three microorganisms; MBCs for NVC-422 were much higher in the presence of 10% serum or 0.4% HSA whereas MBCs of NCT and NVC-612 were not significantly changed. In 0.1 M phosphate pH 7 alone, NVC-422-NCTs gave >99.9% time kill activity, with 10% or 50% serum rate of time kill for NCT and NVC-612 are generally unchanged, whereas NVC-422 showed slower time kill activity against bacteria and similar TK activity against *C. albicans*. At equal percent concentrations of chlorotaurines in phosphate pH 7, NVC-612 showed a faster rate of kill in plasma compared to NVC-422 or NCT. Albumin, as a predominant plasma protein, may play a major role in modifying *N*-chlorotaurine activity. 4.8 mM NVC-422 (in the presence of 10% serum) or 0.3mM NVC-422 (in 5mM acetate saline pH 4 alone) was effective at eradicating *E. coli* biofilms after 1 hour exposure.

References

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