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N-chlorotaurine, a long-lived endogenous oxidant, inactivates Shiga toxin 2 of enterohemorrhagic *Escherichia coli*.

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Background

N-chlorotaurine (NCT), a main representative of the long-lived oxidants produced by stimulated human granulocytes and monocytes, is known to exert broad-spectrum antimicrobial activity.
These findings encouraged the development of an analog with even higher solution stability, *N,N*-dichloro-dimethyltaurine (Cl_2-N-CH_2-$(CH_3)_2$-CH_2-SO_3-Na, NVC-422), a promising new antiinfective agent.
Loss of virulence of pathogens has been shown to be one of the first events that occur during their inactivation by NCT.
The aim of this study was to investigate the impact of NCT and NVC-422 on a virulence factor from the molecular process to the functional consequences.
Shiga toxin 2 (Stx2) was selected as a model since it is an important toxin secreted by enterohemorrhagic *Escherichia coli* (EHEC).

Methods

Stx production in relation to bacterial growth in the presence of NCT was tested by quantitative colony counts and Stx2 ELISA.
NCT- and NVC-422- treated supernatant of EHEC was incubated with Vero cells, and the toxin function was evaluated by cytopathic effect and by cell viability assays.
Purified and fluorochrome labeled Stx2 was incubated with NCT and added to human glomerular endothelial cells (GEnC).
Attachment and penetration of Stx2 into the cells was monitored by confocal microscopy.
In addition, surface-binding was investigated by FACS analysis.
SDS-PAGE was performed with purified NCT-treated Stx2. Shifted bands were subjected to mass spectrometry.

Results

Bacterial growth and Stx2 production were both inhibited at a threshold of 2 mM NCT.
Vero cell assays proved that 5.5 mM NCT or NVC-422 partially impaired the function of Stx while 55 mM of each compound was required to completely inactivate the toxin.
Confocal microscopy and FACS analyses showed that the binding of Stx2 to human glomerular endothelial cells (GEnC) was inhibited, and no NCT-treated Stx2 entered the cytosol.
Mass spectrometry clearly displayed oxidation of thio groups and aromatic amino acids.

Stx production in relation to bacterial growth in the presence of NCT

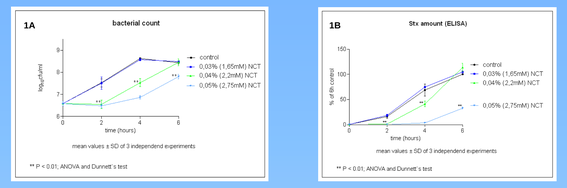


Figure 1. Inhibition of growth of EHEC 178 and of Stx2 production in the presence of sublethal concentrations of NCT.
(A) CFU counts of EHEC in Direct Medium, to which NCT was added to final concentrations of 0 mM (control), 1.65 mM, 2.2 mM and 2.75 mM at 37°C.
(B) Stx2 produced by EHEC under the same conditions as in (A), measured by ELISA, related to the 6h value of the control without NCT.

Vero cell assay (NCT and NVC-422 against supernatant of EHEC)

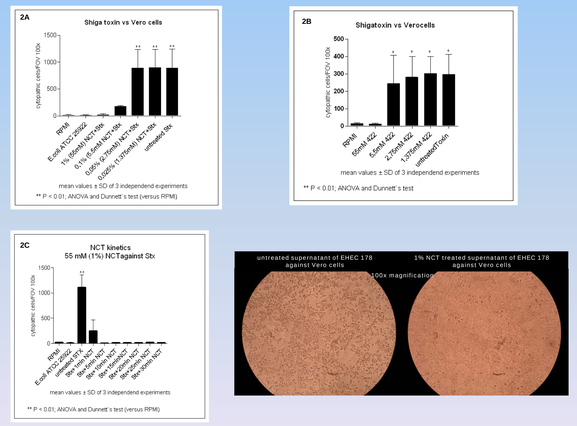


Figure 2. NCT and NVC-422 inhibit the cytopathic effect of Stx2 in Vero cells.
Supernatants of EHEC 178 cultures were treated with 1.38 – 55 mM of oxidants for 30 min (A-C), diluted 100-fold in RPMI + 10% v/v FCS, and added to Vero cell cultures, which were monitored for the typical cytopathic effect.
(A – C) Numbers of cytopathic cells per visual field at a 100x magnification after incubation with EHEC supernatant for 72 h. Comparison of RPMI+FCS without Stx (negative control), supernatant of the non-EHEC strain ATCC 11229 (shown in A), untreated supernatant (positive control), and supernatant treated with NCT **(A)** or NVC-422 **(B).**
(C) Supernatants of EHEC 178 cultures were treated with 55 mM NCT for 1 - 30 min, followed by Vero cell assay and evaluation as in A – C.
Mean values ± SD from three independent experiments are shown in (A-C).
* P < 0.05; ** P < 0.01

FACS analysis and Confocal laser scanning microscopy

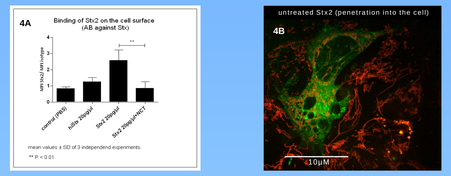
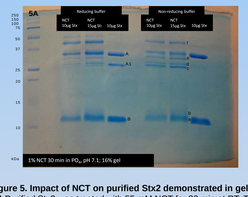


Figure 4. Binding to and penetration of purified Stx2 into human kidney glomerular endothelial cells (GEnC) was inhibited by NCT.
(A) FACS analysis of binding of Stx2 to GEnC cells. Purified Stx2 was incubated with 55 mM NCT (1% w/v) in PBS at 37°C for 30 min. Controls with Stx2 in PBS or without Stx2 were performed in parallel. Cells were incubated for 4 h at 37°C. Stx2 bound on the cell surface was detected using a mouse-anti-Stx2-antibody and a FITC-labeled polyclonal goat-anti-mouse secondary antibody. MFI mean fluorescence intensity. Mean values ± SD from three independent experiments are shown.
(B) Confocal laser scanning microscopy of penetration of Stx2 into GEnC cells. Labeling of Stx2 was done with Oyster®-488. Subsequently, labeled Stx2 was incubated in 55 mM NCT (1% w/v) or PBS (control) for 30 min at 37°C. The sample was 100-fold diluted in the cell culture, and the fluorescence was monitored. The figure shows a fluorescent cell treated with Stx2 in PBS after 1h (one representative of three independent experiments, 6000x magnification, scale bar 10 µm). By contrast, using NCT-treated Stx2, no fluorescence occurred, indicating the absence of penetration.

1D electrophoresis



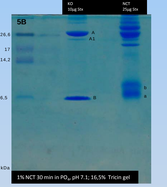


Figure 5. Impact of NCT on purified Stx2 demonstrated in gel electrophoresis.
(A) Purified Stx2 was treated with 55 mM NCT for 30 min at RT. Then, an aliquot was used for SDS-PAGE. Reducing buffer contained 4.5% v/v mercaptoethanol. A gel containing bands of aliquots containing 10 or 15 µg Stx2 is shown (one representative of three independent experiments). Bands a-f were subjected to in-gel digestion and mass spectrometry.
(B) Purified Stx2 was treated with 55 mM NCT for 30 min at RT and subjected to a tricine gel for improved separation of the low molecular weight bands. Note the separation of the B subunit in bands a and b, which were subjected to in-gel digestion and mass spectrometry.

Mass spectrometry

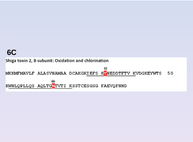
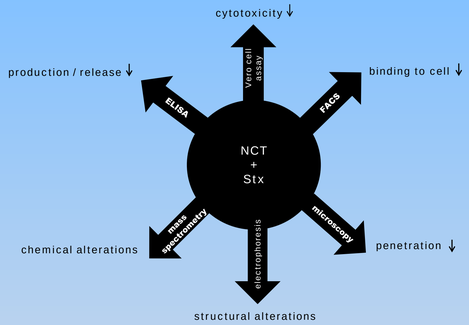


Figure 6. Oxidation of thio groups and aromatic groups of Stx2 detected with mass spectrometry.
Bands a-f from the gels, as depicted for one representative in Figure 5, were analyzed with mass spectrometry after in-gel digestion. Similar results gained from three independent experiments are shown. Positions and occurrence in bands are demonstrated. Amino acids 1-22 (subunit A) and 1-19 (subunit B), respectively, comprise the signal peptides.
(A) Oxidation of all methionines and cysteines of subunit A occurred. Positions of oxidized amino acids in the sequence are shown and their mono- to tri-oxidation in relation to the bands excised from the gels.
(B) Chlorination of phenylalanine, tyrosine, and histidine of subunit A.
(C) Oxidation of methionine and chlorination of tyrosine of subunit B.

Synopsis



Conclusions

NCT oxidizes and inactivates Stx2.
Therefore, long-lived oxidants may act as powerful tools of innate immunity against soluble virulence factors of pathogens.
Moreover, NCT and its novel analog, NVC-422, are in development as topical antiinfectives.
Inactivation of virulence factors may contribute to therapeutic success of this novel class of compounds.