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N-chlorotaurine and its analogues *N*,*N*-dichloro-2, 2-dimethyltaurine and *N*-monochloro-2,2-dimethyltaurine are safe and effective bactericidal agents in *ex vivo* corneal infection models

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ABSTRACT.

Purpose: **N-chlorotaurine (NCT) and its analogues *N*-monochloro-2,2-dimethyltaurine (NVC-612) and *N*-dichloro-2,2-dimethyltaurine (NVC-422) are new anti-infectives for topical treatment for conjunctivitis. The aim of this study was to show that these compounds are safe in an EpiOcular model and effective in corneas infected *ex vivo*.**

Methods: **Corneal buttons were excised from porcine eyes. In 183 of the 229 corneas, erosion and artificial superficial stromal incision were induced. They were bathed in suspensions of *Pseudomonas aeruginosa* or *Staphylococcus aureus* for 24 hr at 37°C and incubated in solutions of the test substances at 37°C and pH 7.1. Subsequently, they were subjected to histology ($n = 20$) or homogenized followed by quantitative bacterial cultures ($n = 209$). Ocular irritation was tested using the EpiOcular™ tissue system (MatTek Corporation).**

Results: **Bacterial accumulations were detected histologically both on the corneal surface and also in the anterior third of the stroma of incised corneal buttons. All three test compounds at a concentration of 55 mm (equals 1% NCT) reduced the bacterial counts of *P. aeruginosa* and *S. aureus* by approximately 5 log10 after 60- and 120-min incubation, respectively. Significant killing was observed as early as after 5-min incubation. Also intrastromal bacteria were inactivated. In the EpiOcular™ tissue model, NCT, NVC-422 and NVC-612 had no or very low potential to irritate corneal tissue.**

Conclusion: ***N*-chlorotaurine, NVC-422 and NVC-612 are non-irritating in cornea and kill *P. aeruginosa* and *S. aureus*, even following penetration into the deeper corneal stromal layers.**

Key words: active chlorine compounds – anti-infective – antimicrobial agents – antiseptic – EpiOcular model – eye infection – keratitis – pig cornea

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Introduction

Pseudomonas aeruginosa and *Staphylococcus aureus* are among the most common bacteria causing corneal infection. They are capable of causing rapidly progressive severe infectious keratitis and can cause vision impairment (Marquart 2011). Although *P. aeruginosa* can invade corneal epithelial cells (Ijiri et al. 1993; Alarcon et al. 2009a), it is known to penetrate into the corneal stroma only in case of a disrupted epithelial cell layer and basement membrane (Alarcon et al. 2009b). Therefore, corneal infection with *P. aeruginosa* is most commonly associated with contact lens wear or minimal ocular trauma (Alarcon et al. 2011). Once the bacteria reach the corneal stroma underlying the multilayered epithelium, ulcers with large, deep infiltrates develop. Once initiated, corneal infection caused by *P. aeruginosa* or *S. aureus* is difficult to treat, and therefore, adequate treatment is desir-

able as early as possible (Davis & Chandler 1975).

Recently, new broad-spectrum microbicidal agents have been developed, with the goal of being effective irrespective of the causative pathogen. *N*-chlorotaurine (NCT) is a persistent oxidant produced by activated human granulocytes and monocytes, which can be synthesized chemically as crystalline sodium salt (Cl-HN-CH_2-CH_2-SO_3-Na, NCT) (Gottardi & Nagl 2002). Its even more stable derivatives *N*-chloro-2,2-dimethyltaurine (Cl-HN-CH_2-$(CH_3)_2$-CH_2-SO_3-Na, NVC-612) and *N,N*-dichloro-2,2-dimethyltaurine (Cl_2-N-CH_2-$(CH_3)_2$-CH_2-SO_3-Na, NVC-422) (Wang et al. 2008; Francavilla et al. 2009; Low et al. 2009) are *N*-chloro amino acids that may be suited for the treatment for infectious keratitis. They exert broad-spectrum microbicidal activity against all classes of pathogens without the development of resistance because of their oxidative mechanism of action (Gottardi & Nagl 2010). On the other hand, they are well tolerated by human tissue because of their mild activity (Gottardi & Nagl 2010). This has been demonstrated in clinical studies among others in the human and rabbit eye for 1% NCT (55 mM) and recently for 0.3% NVC-422, too (Koyama et al. 1996; Nagl et al. 1998, 2000). Moreover, NCT and, recently, NVC-422 have been found to attenuate symptoms when used to treat viral conjunctivitis (Romanowski et al. 2006; Teuchner et al. 2005 and unpublished results).

N-chlorotaurine has been shown to react with ammonium chloride (NH_4Cl), following which monochloramine (NH_2Cl) is formed in equilibrium (Grisham et al. 1984; Thomas et al. 1986). The latter is more lipophilic and has a higher bactericidal and fungicidal activity than NCT because of increased penetration into tissue and bacteria and fungi (Gottardi et al. 2007; Gottardi & Nagl 2010). Recently, a combination of 0.1% NCT and 0.1% NH_4Cl demonstrated activity superior to that of 1% NCT in the rabbit model of epidemic keratoconjunctivitis (Romanowski et al. 2006), and the tolerability of this combination has been proven in humans (Teuchner et al. 2008). A specific advantage of NCT plus NH_4Cl is the non-formation of irritating trichloramines, which is not the case with other active chlorine compounds in the presence of NH_4Cl (Gottardi et al. 2007).

In human corneal grafts infected with smallpox vaccinia virus or herpes simplex virus type 1 *in vitro*, NCT significantly reduced the number of infected cells and therefore prevented the spread and release of viruses at a concentration of 0.01%, which was far below the toxic concentration for the grafts (Huemer et al. 2010). Following this study, the aims of the present study were to investigate the bactericidal efficacy of NCT, NVC-612, NVC-422 and NCT plus NH_4Cl in the *in vitro* pig cornea infection model and the safety of these compounds in the standardized EpiOcular tissue model (MatTek Corporation, Ashland, MA, USA). Because availability of human grafts is limited, pig corneas were a reasonable choice for a larger series of tests (Panjwani et al. 1997; Rieck et al. 2003; Yuan et al. 2010).

Material and Methods

Chemicals, test solutions and media

Pure NCT as a crystalline sodium salt (molecular weight 181.57) was prepared according to the previously published method (Gottardi & Nagl 2002). Purity was proved by spectrophotometry (Gottardi & Nagl 2002). Pure NVC-612 (molecular weight 209.63) and NVC-422 (molecular weight 244.07) were supplied by NovaBay Pharmaceuticals, Inc. (Emeryville, CA, USA) (Wang et al. 2008). Each of these test substances was dissolved in 0.1 M phosphate buffer at pH 7.1 to the desired concentration of 55 mM (equals 1% NCT) before use. Additionally, 5.5 mM (0.1%) NCT plus 18.7 mM (0.1%) ammonium chloride and 11 mM (0.2%) NCT plus 37.4 mM (0.2%) ammonium chloride were tested. Ammonium chloride (NH_4Cl, reagent grade, molecular weight 53.49) was from Merck (Darmstadt, Germany). BD balanced sterile saline solution from BD Ophthalmic Systems (Bidford on Avon, Warwickshire, UK), ethanol 96% from Brenntag CEE GmbH (Vienna, Austria), tryptic soy broth and Mueller-Hinton agar from Merck. The solution for dehydrating corneas consisted of GIBCO® Minimal Essential Medium (Invitrogen, Carlsbad, CA, USA) containing 2 mM glutamine (Biochrom AG, Berlin, Germany), 2% foetal calf serum (Invitrogen) and 5% dextrane (Carl Roth GmbH, Karlsruhe, Germany). This solution maintained the form and transparency of the corneal buttons.

Preparation of pig corneal buttons

To avoid corneal erosion, porcine eyes were enucleated immediately after death and before scalding in a local abattoir (Chinnery et al. 2005). The eyes were transported and stored at 4°C for a maximum of 3 days in BD balanced salt solution. Subsequently, the eyes were fixed on a metal stand. A trephine with a diameter of 5 mm was turned slightly on the middle of the cornea to mark the area. Within this circle, the epithelium was carefully and slightly abraded with a hockey-knife (Alcon, Fort Worth, TX, USA) creating a defined epithelial erosion of the cornea, and a superficial stromal incision was performed. This step was omitted in cases, where the epithelium was meant to be preserved. Following this, a central corneal button was cut out with a 10-mm trephine and small scissors. Corneal buttons were put into 12-well plates (1 button per well) containing 1.5 ml deswelling solution (see above) per well.

Bacterial strains and infection of corneal buttons

Pseudomonas aeruginosa ATCC 27853 and *Staphylococcus aureus* ATCC 25923 (deep frozen for storage) were grown on Mueller-Hinton agar and used separately for the experiments. Single colonies from the plates were grown in tryptic soy broth at 37°C for 24 hr overnight to $1–5 \times 10^9$ colony-forming units (cfu)/ml. Aliquots (100 μl) of these bacterial suspensions were pipetted to the corneal buttons stored in 1.5 ml deswelling solution. These corneas were then incubated at 37°C for 3, 6, 7, 24, 31 or 48 hr in pilot experiments. Because there was no significant difference in the number of cfu between times of 6–48 hr, we decided to apply 24 hr for the final tests for logistic reasons. Subsequently, the corneal buttons were transferred with tweezers to 5 ml

0.9% NaCl in 50-ml plastic tubes (Greiner, Kremsmünster, Austria). Tubes were stirred upside down (because stirring of the buttons was more effective near the flat lid side than near the pointed bottom side of the tube) for 2 min with a frequency of 400/min on an IKA KS 250 Basic Horizontal Stirrer (IKA Works Inc., Staufen, Germany). They were washed and stirred twice in saline. To confirm the infection of the corneal buttons, adherence of the bacteria to the buttons and their penetration into the buttons with artificial erosion were confirmed by histology (see below) also at this stage. In all experiments, for each time-point and each test substance, a separate button had to be used.

Incubation of corneal buttons in test solutions of NCT, NVC-612, NVC-422 and NCT plus NH_4Cl

Infected and washed corneal buttons were transferred to another 12-well plate containing 2 ml per well of the respective test solution in phosphate buffer. Additionally, 25-cm^2 cell culture flasks (Sarstedt, Wiener Neudorf, Austria) filled with 20 ml of the solutions were used for one button each. Controls in buffer without the test substances were performed in parallel. The standard incubation time at 20°C was 60 min under continuos agitation (100/min), but also 5, 10, 20, 30, 40 and 120 min were applied. At the end of the incubation time, the corneal buttons were washed twice in phosphate buffer.

To test the activity of the *N*-chloroamino acids against bacteria that had penetrated into the corneal stroma, in special experiments, the pathogens on the surface were killed by incubating in 70% ethanol for 30 seconds prior to incubation in the test substances. To achieve this, each well in a six-well plate was filled with 10 ml of 70% ethanol. Corneal buttons, infected with *P. aeruginosa* and washed afterwards, were transferred into these wells (1 button per well) and continuously agitated (75/min) at 20°C. Exactly after 30 seconds, the buttons were washed in 10 ml distilled water and then in 10 ml phosphate buffer. Following this, they were incubated in the test solutions or in buffer as described above.

To prove the rapid killing of *P. aeruginosa* by 70% alcohol, 100 μl of an overnight culture in tryptic soy broth was added to 4 ml 70% ethanol. After 30 seconds at 20°C, 50 μl of the suspension was plated on Mueller-Hinton agar. In addition, 50 μl of the suspension diluted 100-fold in 0.9% NaCl was plated.

Homogenization of corneal buttons and bacterial counts for quantitative analyses

Subsequent to incubation in the test solutions and washing in phosphate buffer, the buttons were placed in IKA BMT-20-S-M-gamma tubes filled with 5 ml 0.9% NaCl and homogenized using an IKA Ultra Turrax Tube Drive® (IKA Works Inc., Staufen, Germany) on level 9. Undiluted homogenate, as well as 50 μl aliquots of this fluid diluted 100-fold in 0.9% NaCl were spread in duplicate on Mueller-Hinton agar plates with an automated spiral plater (model WASP 2; Don Whitley Scientific Limited, Shipley, UK). The plates were incubated at 37°C, and the cfu were determined after 24 and 48 hr. For both plates, the detection limit was 10 cfu/ml.

Histological evaluation for qualitative analyses

Following incubation in the test solutions and washing in phosphate buffer, the corneal buttons were fixed in 5% formaldehyde for 24 hr. Paraffin sections were prepared and stained with haematoxylin–eosin, periodic acid Schiff and Gram.

EpiOcular™ tissue irritancy assay

EpiOcular tissues (MatTek Corporation) were placed in 900 μl cell culture media, and 100 μl of each compound was added to the apical side of the tissue; the tissues were exposed to the compound for 3, 15, 60 and 270 min. Tissues were rinsed with PBS and placed in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution for 3 hr followed by treatment overnight with 2 ml isopropanol to extract all MTT. 200 μl of the extract was used to measure the absorbance photometrically at 550 nm. Viability (%) was calculated by the absorbance of the sample/absorbance of the negative control not exposed to the test agents ×100. The incubation time needed to achieve 50% viability of the controls with defined concentrations of the test substances (Effective Time-50, ET-50) was correlated with the Draize-type score (Draize et al. 1944) according to MatTek's instructions for the classification of irritancy.

Statistics

Data are presented as mean values and standard deviations (SD) of log10 values of cfu counts of bacteria. Student's unpaired *t* test in case of two groups or one-way ANOVA and Bonferroni's and Dunnett's multiple comparison test in case of more than two groups were used to test for a difference between the test and control group. $p < 0.05$ was considered significant for all tests. Calculations were made with GRAPHPAD PRISM 5 software.

Results

Histology

Both *P. aeruginosa* and *S. aureus* could be detected frequently on the epithelium, and to a lesser extent, on the surface of the lateral cutting side of the stroma. In corneas with intact epithelial cell layer and basement membrane ($n = 5$ per bacterial strain), they were also found between epithelial cells, but not in the underlying stroma. However, in corneal buttons with artificially created erosions ($n = 5$ per bacterial strain), some accumulations of bacteria were seen in the superficial stromal layers (Fig. 1). This was observed for both strains.

Bactericidal activity of NCT, NVC-612, NVC-422 and NCT plus NH_4Cl against *Pseudomonas aeruginosa* in corneal buttons

The cfu counts of *P. aeruginosa* in homogenates of corneal buttons with erosion after incubation for 1 hr in 2 ml of test solutions are shown in Fig. 2. The results of the same experimental design using corneal buttons without erosion were similar (with no statistical difference) to those in buttons with erosion (data not shown). When using this low volume (2 ml) of test solutions, 55 mM (1%) NCT and 11 mM (0.2%) NCT plus 37.4 mM

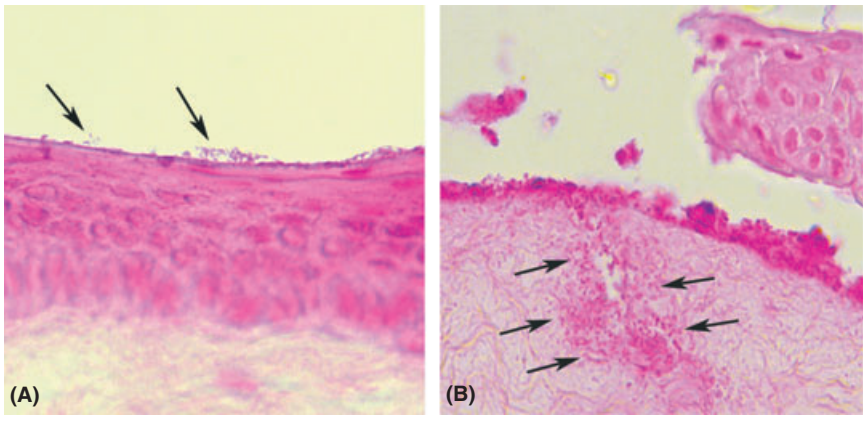

Fig. 1. Histological section of corneal buttons infected with *Pseudomonas aeruginosa*. Button without (A) and with artificial corneal wound (B) (one representative section of five independent experiments each). Note bacteria adhering to the epithelium (arrows in A) and within the stroma (arrows in B).

(0.2%) NH_4Cl caused a reduction in cfu counts by approximately 4 $\log_{10}$, which was significantly higher than the 1–2 $\log_{10}$ reduction by NVC-612, NVC-422 and 5.5 mM NCT plus 18.7 mM NH_4Cl (Fig. 2).

These differences between the test substances were assumed to originate in differences in chlorine consumption by reaction with thio groups and aromatic groups of corneal molecules, which leads to partial inactivation of the chlorine compounds (Gottardi & Nagl 2002, 2010). To compensate for this consumption, we augmented the volume of the test solutions to 20 ml per cornea in the subsequent experiments. Again, the results were found to be independent of erosion in the corneal samples (Fig. 3). However, all test substances reduced the cfu count similarly by 5 $\log_{10}$ at an average. This increase of activity by the higher volume shown in Fig. 3 was significant for NVC-612, NVC-422 and 5.5 mM NCT plus 18.7 mM NH_4Cl ($p < 0.01$).

Bactericidal activity of NCT, NVC-612, NVC-422 and NCT plus NH_4Cl against *Pseudomonas aeruginosa* in the corneal stroma

To investigate the impact of the active chlorine compounds against bacteria that had penetrated into the stroma, the corneal buttons were incubated for 30 seconds with 70% ethanol at first. Subsequently, the ethanol was washed off and the incubation with 2 ml chlorine compounds or buffer as a control started. As can be seen in Fig. 4, the alcohol killed approximately 83% (0.77 $\log_{10}$ reduction; range 54–90% or 0.34–0.99 $\log_{10}$) of the bacteria, in fact the accessible ones located on the surface of the corneal button. The following treatment with all chlorine compounds reduced the cfu nearly to the detection limit ($p < 0.01$ versus controls, $p > 0.05$ between single chloramines). When *P. aeruginosa* was suspended from an overnight culture in tryptic soy broth into 70% ethanol for 30 seconds, no more viable bacteria could be detected as expected.

Kinetics of the killing of *Pseudomonas aeruginosa* and *S. aureus* by NCT, NVC-612 and NVC-422 in corneal buttons

In experiments with *P. aeruginosa*, infected corneal buttons were treated for different incubation times with 2 ml of 55 mM NCT. The killing curve of *P. aeruginosa* is shown in Fig. 5. A significant reduction in cfu could be seen already after 5–10 min. As expected, all three test compounds killed *S. aureus* (Fig. 6). At an equal molar ratio, the dichloro compound NVC-422 demonstrated the highest bactericidal activity, followed by the

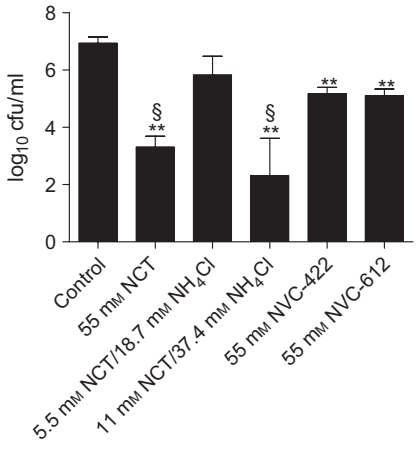

Fig. 2. *N*-chloroamino acids against *Pseudomonas aeruginosa*. Artificial erosion, 2 ml test solution per cornea; 60-min incubation time at RT and pH 7.1. Mean values ± standard deviation of three independent experiments. **$p < 0.01$ versus control; §$p < 0.05$ versus NVC-422, NVC-612, 0.1% (5.5 mM) *N*-chlorotaurine + 0.1% (18.7 mM) NH_4Cl.

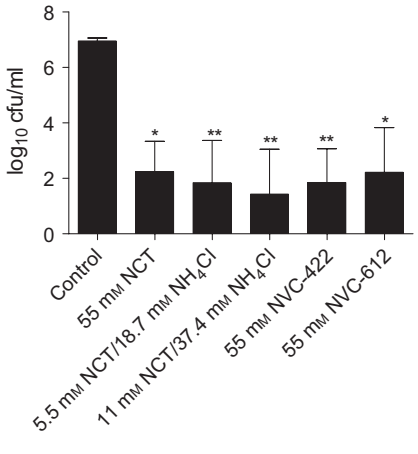

Fig. 3. *N*-chloroamino acids against *Pseudomonas aeruginosa*. Artificial erosion, 20 ml test solution per cornea; 60-min incubation time at RT and pH 7.1. Mean values ± standard deviation of three independent experiments. *$p < 0.05$ versus control; **$p < 0.01$ versus control.

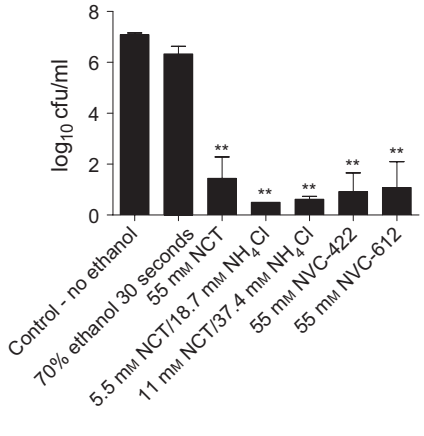

Fig. 4. 70% ethanol followed by *N*-chloroamino acids against *Pseudomonas aeruginosa*. Artificial erosion, 30-second incubation in 10 ml of 70% ethanol, two washing steps, followed by 60-min incubation time in *N*-chloroamino acids at RT and pH 7.1. Mean values ± standard deviation of three independent experiments. **$p < 0.01$ versus control.

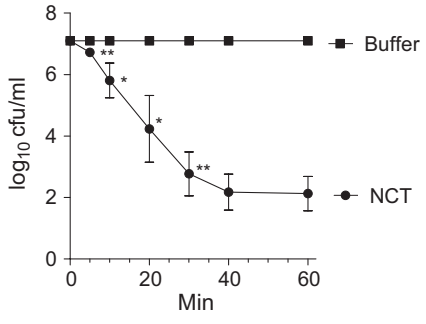

Fig. 5. Killing kinetics of 55 mM *N*-chlorotaurine against *Pseudomonas aeruginosa*. Artificial erosion, 2 ml test solution per cornea; 5-, 10-, 20-, 30-, 40-, 60- and 120-min incubation time at RT and pH 7.1. Mean values ± standard deviation of three independent experiments. *p < 0.05 versus control; **p < 0.01 versus control.

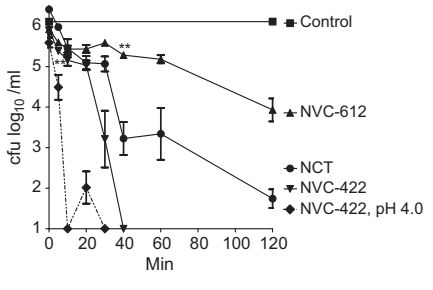

Fig. 6. Killing kinetics of 55 mM *N*-chlorotaurine (NCT), NVC-422 and NVC-612 against *Staphylococcus aureus*. Artificial erosion, 2 ml test solution per cornea; 5-, 10-, 20-, 30-, 40-, 60- and 120-min incubation time at RT and pH 7.1. NVC-422 was also tested at pH 4.0. Mean values ± standard deviation of 6–8 independent experiments. **Threshold to p < 0.01 between control (phosphate buffer) and NCT and NVC-422 (5 min each) and NVC-612 (40 min).

monochloro compounds NCT and NVC-612. Because NVC-422 is propagated in a use solution containing 5 mM acetate buffer (pH 4.0) (Wang et al. 2011), we tested this formulation in addition. Actually, this solution reduced the number of viable counts by 5 $\log_{10}$ steps already within 10 min (Fig. 6).

Irritancy of 1% NCT, 1.35% NVC-422 and 1.15% NVC-612 in the EpiOcular™ Tissue irritancy model

Using an EpiOcular™ tissue model developed for *in vitro* irritancy testing (Draize score), we showed that NCT, NVC-422 and NVC-612 have no or very low irritancy potential to corneal tissue (Table 1).

Table 1. Irritancy of 1% NCT, 1.35% NVC-422 and 1.15% NVC-612 in the EpiOcular™ Tissue model.

Compound	ET-50 (min)	Irritancy classification
1% (55 mM) NCT	106	Non/minimal
1.35% (55 mM) NVC-422	78	Non/minimal
1.15% (55 mM) NVC-612	125	Non/minimal
0.3% Triton X-100	20	Mild
20 mM PBS pH7	> 270	Non/minimal

NCT = *N*-chlorotaurine.

Discussion

We investigated the efficacy of the *N*-chloroamino acids NCT, NVC-612, NVC-422 and NCT plus NH_4Cl in porcine corneas infected with *P. aeruginosa* and *S. aureus* and the safety of these compounds in the EpiOcular tissue model. The multilayer epithelium and the basement membrane of the healthy cornea form a physical barrier against penetration of *P. aeruginosa* into the stroma (Alarcon et al. 2011). Consistent with that, we only found accumulations of bacteria in the stroma histologically if an artificial erosion had been created.

Corneal erosions may also allow antimicrobial agents to penetrate into the deeper corneal layers. We did not find any difference between corneas with or without erosion regarding the bactericidal activity of the test substances. It has to be taken into account that bacteria had access to the lateral cutting edge of the corneal buttons and that bacteria were detected in the stroma in histological sections at these sites, too. Therefore, the influence of erosion on the activity of the *N*-chloroamino acids could have been masked in our tests, and no reliable statement on this point can be made.

The dimethylated analogues NVC-422 and NVC-612 have a similar or even stronger killing activity towards bacteria suspended in buffer solutions than NCT, while demonstrating the same broad-spectrum activity as NCT (Gottardi & Nagl 2010; Wang et al. 2011). Therefore, it is not surprising that we found a similar inactivation rate of both test strains by NVC-422, NVC-612 and NCT in porcine corneas.

Killing kinetics resemble those found with NCT in buffer solution with the difference that reduction in cfu below the detection limit takes longer in cornea samples. This can be explained by the portion of bacteria that penetrated into the stroma and that obviously can be reached by the active chlorine compounds after a longer incubation time. Because 70% ethanol killed about 80% (0.77 $\log_{10}$) of the bacteria from the whole corneal button after 30 seconds, the remaining 20% of cfu were hidden in the stroma. The largest part of the latter was killed by the *N*-chloroamino acids within 1 hr, which indicates that these substances diffuse to some amount into the stroma and inactivate those intrastromal bacteria, too, with increasing incubation time (Figs 4–6). This is in accordance with former experiments, where we found slow penetration of NCT through human corneas (Romanowski et al. 2006). Furthermore, efficacy of 55 mM NCT (1%) in clinical studies, mainly on conjunctivitis (Nagl et al. 2000; Teuchner et al. 2005), purulently coated crural ulcerations (Nagl et al. 2003), and external otitis (Neher et al. 2004), and of 0.1–1.5% NVC-422 gel in impetigo (Iovino et al. 2011) confirms that *N*-chloroamino acids obviously reach and inactivate pathogens *in vivo*.

Such penetration into the stroma could be enhanced by the addition of ammonium chloride (NH_4Cl) to NCT, forming lipophilic monochloramine (NH_2Cl) (Romanowski et al. 2006; Gottardi et al. 2007). Accordingly, the bactericidal activity of NCT in the pig corneas in the present study could be increased by NH_4Cl. Both higher bactericidal activity and more rapid penetration of NH_2Cl compared to NCT will contribute to this effect (Dychdala 2001; Gottardi et al. 2007).

Interestingly, the bactericidal activity was not constantly high when the corneal buttons were incubated in small volumes of test substance (2 ml). This could be explained by the reaction of the active chlorine with organic residues of the corneas, during which oxidative activity is consumed in part (Dychdala 2001; Gottardi & Nagl 2002, 2010). On the other hand, we found only a loss of 11.2 ± 1.0% (mean value ± SD, $n = 4$) oxidation capacity when a corneal button was agitated for 1 hr in 2 ml of the test

substances. Therefore, we assume that the higher volume of 20 ml caused a more intensive movement of the buttons, which may have contributed to improved activity. Transferring these results and considerations to clinical application of eye drops, it seems to be important to apply a sufficient amount of active chlorine to the infected eye. Frequent dosing – at least every 15 min within the first hour of treatment – seems to be desirable to gain an optimal therapeutic effect and has been therapeutically effective with NCT in the rabbit and human adenoviral conjunctivitis (Teuchner et al. 2005; Romanowski et al. 2006).

Our *ex vivo* model has limitations compared to *in vivo* animal models, as the host immune response aspect is missing. It is believed that the release of extracellular toxic substances resulting from stimulation of the host immune response can exacerbate tissue damage. On the other hand, *in vivo* tear fluid protects against *P. aeruginosa* infection by enhancing the epithelial barrier function (Kwong et al. 2007). However, with our porcine corneal button infection model, we could demonstrate that active chlorine compounds can penetrate and reach bacteria in the stroma. Such aspects cannot be tested in an epithelial cell culture model.

The EpiOcular™ model developed for *in vitro* irritancy testing by MatTek Corporation provides a number of benefits compared to the traditional rabbit eye Draize model. It eliminates needless animal suffering, has objective, reproducible end-points, and it is not compromised by the species extrapolation error inherent in all animal studies. Using this model, we have shown that 55 mM NCT, NVC-422 and NVC-612 have ET-50 scores >60 min, which translates into Draize scores 1–15 (Draize et al. 1944), classifying them as minimal/non-irritating.

In summary, all three tested *N*-chloroamino acids, NCT, NVC-612 and NVC-422, have strong activity against *P. aeruginosa* and *S. aureus* in the pig cornea infection model. Their broad-spectrum microbicidal activity could enable immediate application irrespective of the causative pathogen. Safety and efficacy in corneal models warrant further clinical investigation of these novel agents in keratitis.

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