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Activity of N-chlorotaurine against herpes simplex- and adenoviruses

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Abstract

N-chlorotaurine, an essential weak oxidant produced by stimulated human leukocytes, is known to have bactericidal, fungicidal and vermicidal properties. This study for the first time demonstrates its virucidal activity. By viral suspension tests at incubation times between 5 and 60 min, virus titers of both Herpes simplex virus type 1 and 2 were reduced about 1.3–2.9 $\log_{10}$ and 2.8–4.2 $\log_{10}$ by 0.1 and 1% (5.5 and 55 mM) N-chlorotaurine, respectively. Virus titer reduction of adenovirus type 5 between 15 and 60 min was 0.5–2.0 and 0.6–4.0 $\log_{10}$, respectively, by the same concentrations of N-chlorotaurine. These findings support a contribution of N-chlorotaurine in destruction of pathogens during inflammatory reactions and also the possibility of its application as an antiviral agent in human medicine.

Keywords: N-chlorotaurine; Antiviral agent; Herpes simplex virus; Adenovirus

1. Introduction

N-chlorotaurine (C1-HN-CH_2-CH_2-SO_3H, NCT), the N-chloro derivative of the amino acid taurine, is an essential weak oxidant produced by human granulocytes and monocytes during inflammatory reactions (Lampert and Weiss, 1983; Grisham et al., 1984; Test et al., 1984). The biological function of NCT is thought to play a role in destruction of pathogens (Thomas, 1979). Indeed, NCT demonstrated significant microbicidal efficacy against bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*), yeasts (*Candida albicans*) (Nagl and Gottardi, 1992, 1996) and verms (*Schistosoma mansoni*) (Yazdanbakhsh et al., 1987). The aim of this study was to investigate the activity of NCT against viruses causing human infections.

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2. Materials and methods

2.1. Reagents

Pure NCT as a crystalline sodium salt (MW = 181.52 g/mol) was dissolved in phosphate buffer at concentrations of 0.01–1% (0.55–55 mM). The concentration of the buffer was adjusted to ensure an osmolality of 290 mM and a pH of 6.8–7.2. Identity of NCT was proved by IR-spectrometry, purity by iodometric titration (calculated 19.53% Cl^+, found 19.3% Cl^+, which equals 99% purity). Cysteine and buffers (sodium *di*-hydrogen phosphate and *di*-sodium hydrogen phosphate) were purchased from Merck (reagent grade, Darmstadt, FRG).

2.2. Cells and viruses

MRC-5 cells (human embryo lung fibroblasts, American Type Culture Collection, ATCC CCL-171), Vero cells (kidney monkey cells, ATCC CCL-81) and HeLa cells (human epitheloid carcinoma of cervix, ATCC CCL-2) were grown in Eagle's minimal essential medium (MEM, Hyclone Europe, Cramlington, UK) containing 10% (vol/vol) fetal calf serum (FCS, Hyclone) and 2 mM glutamine and antibiotics. For viral susceptibility tests, MEM containing 2% FCS was used. Cells were plated out onto 96-well flat bottomed microplates (Costar, Cambridge, UK) at 180 μl per well ($n = 3$ for each single experiment).

Virus strains herpes simplex virus type 1 (WAL, HSV-1) and 2 (D316, HSV-2), both kindly provided by Dr D. Falke (Mainz, FRG), and adenovirus type 5 (ATCC 5-VR, AV-5) were used for the experiments. Cells and viruses from the ATCC were purchased directly from Rockville, MD.

2.3. In vitro antiviral assays

MRC-5 and Vero cells exposed to HSV-1 and HSV-2 as well as HeLa cells exposed to AV-5 were grown to a density of about 10^4 cells per well corresponding to approximately 80% confluency before infection. A total of 50 μl of the viral stock solution (5×10^8–5×10^9 PFU/ml) were added to 200 μl NCT-solution ($n = 3$ per experiment and incubation time). Subsequent to incubation for 5, 15, 20, 30, 45 and 60 min at room temperature, 250 μl of an aqueous 4% cysteine solution were added to inactivate NCT immediately to warrant exact incubation times. Sodiumthiosulfate commonly used to inactivate chloramines was not applied because of impairment of viral replication. The suspension was centrifuged at $500 \times g$ for 5 min to remove the produced crystalline cystine (oxidative product of cysteine). Subsequent to 10-fold serial dilutions of the supernatant in MEM, 20 μl were added to 180 μl MEM containing Vero, MRC-5 and HeLa cells, respectively. Subsequently, cells were incubated at 37°C. Virus titers used for inoculation of Vero and MRC-5 cells were identical because the experiments were done in parallel with the same stock solution of HSV.

The following control experiments were done: (i) the same procedure without NCT (ii) the same procedure without viruses to demonstrate lack of plaque formation and other visible morphological changes of the cells induced by the reagents (cystine was removed by centrifugation, see above) (iii) of the viral suspension 50 μl were added to 450 μl of a mixture of 3% NCT and 4% cysteine to demonstrate absence of antiviral activity of inactivated NCT and cysteine, respectively (iv) of the viral suspension 20 μl were added to the cells after dilution in MEM (without addition of chemicals) to demonstrate lack of viral reduction in the control experiments (i)–(iii).

Inactivation of NCT by cysteine was shown by addition of an excess of potassium iodide: Absence of increase of the absorption at 350 nm (λ_{max} of I_3^-) indicated complete inactivation of NCT.

2.4. Evaluation of the antiviral assays

2.4.1. HSV-1 and HSV-2

Plaque reduction assays (Crumpacker et al., 1979) were performed to determine logarithmic viral reduction: Dilutions resulting in 10–100 plaques per well were chosen for evaluation. Plaque forming units (PFU) of samples and controls were counted and the viral reduction factor (RF) was computed by the formula

Table 1
Viral reduction factors[a] of HSV-1 and 2 by NCT

Incubation time (min)	HSV-1				HSV-2			
	0.1% NCT		1% NCT		0.1% NCT		1% NCT	
	Vero cells	MRC-5 cells	Vero cells	MRC-5 cells	Vero cells	MRC-5 cells	Vero cells	MRC-5 cells
5	1.3 ± 0.2	1.7 ± 0.9	2.7 ± 0.3	2.8 ± 0.4	1.5 ± 0.2	1.4 ± 0.4	3.3 ± 0.7	1.9 ± 0.6
10	2.0 ± 0.3	2.5 ± 0.3	3.1 ± 0.2	2.8 ± 0.2	2.0 ± 0.3	1.4 ± 0.4	3.5 ± 0.5	1.9 ± 0.4
20	2.5 ± 0.7	2.3 ± 0.3	3.5 ± 0.1	3.4 ± 0.4	2.7 ± 0.1	1.5 ± 0.4	3.6 ± 0.6	2.2 ± 0.4
60	2.9 ± 0.4	2.7 ± 0.1	4.2 ± 1.0	3.6 ± 0.9	2.8 ± 0.4	1.9 ± 0.1	4.1 ± 0.9	2.3 ± 1.0

[a] Reduction factor = log PFU(control) − log PFU(NCT).
Results are presented as mean values ± S.D. of reduction factors ($n = 3$–6).

$$RF = \log (PFU)_{control} - \log (PFU)_{NCT}.$$

2.4.2. AV-5

Immune fluorescence and enzyme immuno assay were used.

2.4.2.1. Immune fluorescence. After incubation of the cells for 1 week at 37°C, fluorescein-conjugated monoclonal antibodies against the viruses were added (Biotrin, Dublin IR). The number of cells that showed fluorescence was determined by fluorescence microscopy (Axioplan, Zeiss) and compared to that of 10-fold serial dilutions of the control down to 10^{-7} which was the detection limit.

2.4.2.2. Enzyme immunoassay 'IDEIA™ Adenovirus' against a genus-specific epitope of the virus from DAKO Diagnostics (1993). After incubation of the cells for 1 week at 37°C, viruses were detected by monoclonal antibodies conjugated to horseradish peroxidase. After 60 min incubation at room temperature and washing steps to remove any unbound enzyme-labelled antibody, tetramethylbenzidine as a chromogen and hydrogen peroxide were added and incubated for 10 min. Subsequently, absorbance at 450 nm was determined by a spectrophotometer (Behring EL 311, Microplate Reader). For evaluation, a standard curve of the relation between absorbance and number of PFUs was assessed. Reduction factors were computed as described above.

A total of 3–6 independent experiments for each type of virus, each concentration of NCT and each incubation time were done. Mean log(-PFU) values of samples and controls of all experiments were compared by Student's paired t-test, and P values of less than 0.05 were considered significant.

3. Results

3.1. HSV-1 and HSV-2

Absolute logarithmic virus titers of NCT samples and controls are shown in Fig. 1 and reduction factors shown in Table 1. As expected, the efficacy of 0.1% NCT was at least 10-fold lower than that of 1% NCT.

The control experiments using phosphate buffer in place of NCT showed that the cell culture infective dose of the viruses in MRC-5 cells was $0.43 \pm 0.04 \log_{10}$ for HSV-1 ($P < 0.01$) and $1.65 \pm 0.08 \log_{10}$ for HSV-2 ($P < 0.01$) higher than the one in Vero cells. This phenomenon was observed at a smaller, not significant, extent for the parallel NCT samples ($0.23 \pm 0.29 \log_{10}$ for HSV-1 and $0.16 \pm 0.15 \log_{10}$ for HSV-2, $P > 0.05$) so that the reduction factors of HSV-2 were significantly lower when using MRC-5 cells ($P < 0.01$).

The limit of virucidal action was found to be at 0.025–0.05% NCT for HSV-1 and 0.01% NCT for HSV-2. HSV-2 seemed to be a little more

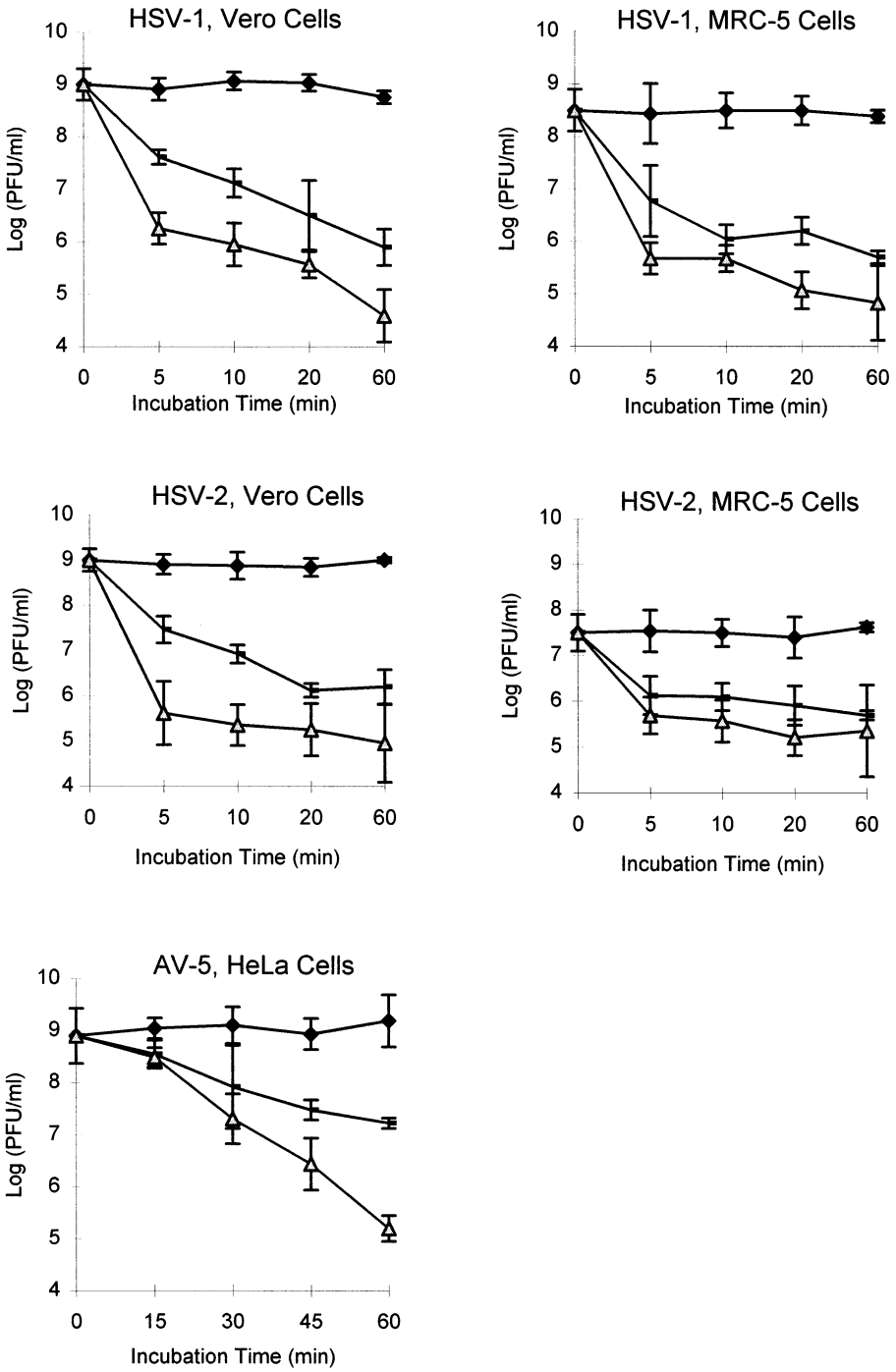

Fig. 1. Kinetics of viral titers ± S.D. of controls (-◆-), 0.1% NCT (-■-) and 1% NCT (-△-) samples; plaque reduction assays (HSV-1/2), enzyme immunoassay and immune fluorescence (AV-5) at room temperature and pH 7.0; $n = 3–6$; $P < 0.01$ at all values of all groups with one exception of $P < 0.05$ at 15 min in AV-5.

susceptible than HSV-1, but the difference was not significant ($P > 0.05$).

3.2. AV-5

Absolute logarithmic virus titers of NCT samples and controls are shown in Fig. 1 and reduction factors shown in Table 2. At short incubation times, the virucidal action against AV-5 was significantly lower than against HSV-1 and HSV-2. After 60 min, however, 1% NCT caused a reduction in AV-5 titer similar to that seen in HSV. At 0.1% NCT, inactivation of AV-5 occurred at a 10-fold lower rate than that of HSV.

3.3. Control experiments with HSV 1/2 and AV-5

No difference in the virus titer could be found in the control experiments (i), (iii) and (iv) ($P > 0.05$), indicating the absence of antiviral activity of the reaction products of NCT and cysteine. Also in the absence of virus (control (ii)), no visible cytotoxicity was observed.

4. Discussion

This study for the first time reveals a virucidal efficacy of NCT. Using HSV-1 and HSV-2, considerable viral inactivation by 1% NCT was observed within a few minutes, while AV-5 was more resistant at short incubation times. This difference is in keeping with the observation that enveloped viruses (like HSV) are usually more susceptible to disinfectants than non enveloped ones (Jülich et al., 1993). Nevertheless, after 60 min viral reduction of AV-5 by 1% NCT was similar to that occurring with HSV-1 and HSV-2 in Vero cells. Therefore, NCT exerts powerful virucidal action after incubation times similar to those necessary observed for bacteria and yeasts (Nagl and Gottardi, 1992, 1996).

Table 2
Viral reduction factors[a] of AV-5 by NCT

Incubation time (min)	0.1% NCT	1% NCT
15	0.5 ± 0.3	0.6 ± 0.2
30	1.2 ± 0.8	1.8 ± 0.5
45	1.5 ± 0.2	2.5 ± 0.5
60	2.0 ± 0.1	4.0 ± 0.3

[a] Reduction factor = log PFU(control) − log PFU(NCT). Results are presented as mean values ± S.D. of reduction factors ($n = 3–6$).

In our control experiments the cell culture infective dose in MRC-5 cells was higher than the one of Vero cells, particularly for HSV-2. The reason for this phenomenon might be found in differences in the distribution of cellular receptors on the host cell surfaces (Gerber et al., 1995). It is not known why the difference in cell culture infective dose between Vero and MRC-5 cells was not significant for the NCT-samples. Possibly, the viral surface was changed by reaction of viral proteins with NCT (see below), resulting in differences in the attachment of NCT-treated and non-treated viruses to the cells.

In concert to the unspecific mechanism of action of active N-chloro compounds, the spectrum of action of the weak endogenous oxidant NCT is similar to that of more reactive compounds like chloramine T and hypochlorite (Dychdala, 1991). NCT oxidizes S-H groups and N-H groups (Nagl and Gottardi, 1996), which may modify viral proteins of the envelope and of the capsid, resulting in viral inactivation.

As in-vitro PMA-stimulated human granulocytes and monocytes produce micromolar concentrations of N-chloramino derivatives, particularly NCT (Thomas, 1979; Lampert and Weiss, 1983; Grisham et al., 1984; Test et al., 1984), one may suppose its contribution in destruction of pathogens. In this study, the limit of virucidal action was 0.5–1 mM NCT at incubation times of 1 h, while the minimal bactericidal concentration is 0.1 mM NCT (Nagl and Gottardi, 1992). It is not known whether N-chloro derivatives could reach microbicidal concentrations in vivo. However, there might be a continuous production of chloramines by leukocytes at inflammatory sites. Moreover, 28 mM NCT can maintain 45–90% of the oxidation capacity for 2 h subsequent to its addition to inflammation samples (Nagl and Gottardi, 1996). Although the circumstances in vivo are not known, our findings may support the

assumption of contribution of NCT in destruction of pathogens during inflammatory reactions.

In human medicine, topical application of NCT could be of advantage for indications, that exclude usage of more powerful disinfectants because of toxic side effects. Indeed, cytotoxicity of NCT against leukocytes and erythrocytes (Grisham et al., 1984), and lung epithelial cells (Cantin, 1994; Schuller-Levis et al., 1994) proved to be low compared to that of $HOCl/OCl^-$. Moreover, in a recent clinical study 1% NCT solution was well tolerated in the rabbit and human eye (Nagl et al., 1998). These findings and the considerable microbicidal activity of NCT suggest its application for local treatment of viral and bacterial infections, e.g. conjunctivitis. Further studies on its tolerance and efficacy should be performed to evaluate its utility in medicine.

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