

# Antiadenoviral effects of *N*-chlorotaurine in vitro confirmed by quantitative polymerase chain reaction methods

Eiichi Uchio<sup>1</sup>  
Hirotohi Inoue<sup>1</sup>  
Kazuaki Kadonosono<sup>2</sup>

<sup>1</sup>Department of Ophthalmology, Fukuoka University School of Medicine, Fukuoka, Japan;

<sup>2</sup>Department of Ophthalmology, Yokohama City University Medical Center, Yokohama, Japan

**Purpose:** Adenoviral keratoconjunctivitis is recognized as one of the major pathogens of ophthalmological nosocomial infection worldwide. *N*-Chlorotaurine (Cl—HN—CH<sub>2</sub>—CH<sub>2</sub>—SO<sub>3</sub>H, NCT) is the *N*-chloro derivative of the amino acid taurine, which is an oxidant produced by human granulocytes and monocytes during inflammatory reactions. Using conventional viral plaque assay, it was previously shown that NCT causes inactivation of several human adenovirus (HAdV) serotypes. In this study, we evaluated the antiadenoviral effect of NCT by quantitative polymerase chain reaction (PCR) methods.

**Methods:** A549 cells were used for viral cell culture, and HAdV serotypes 3, 4, 8, 19, and 37 were used. After calculating 50% cytotoxic concentration (CC<sub>50</sub>) of NCT by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) method, HAdV was cultured with NCT for 7 days, and extracted adenoviral DNA was quantitatively measured by real-time PCR.

**Results:** A statistically significant ( $P < 0.05$ ) dose-dependent inhibition was indicated for all serotypes except HAdV type 4 (HAdV4), which was maximally inhibited by only ~50%. Among the serotypes, NCT was particularly effective against HAdV8, HAdV19a, and HAdV37. The 50% effective concentration (EC<sub>50</sub>) obtained by real-time PCR of NCT ranged between 49 and 256 μM. EC<sub>50</sub> of NCT against HAdV3 was slightly higher than that against serotypes of species D. The selective index (CC<sub>50</sub>/EC<sub>50</sub>) ranged between 41 and 60 except for HAdV4 (11.5).

**Conclusions:** These results show that NCT has an antiviral effect against most serotypes of human HAdV inducing keratoconjunctivitis, indicating its possible therapeutic use.

**Keywords:** adenovirus, *N*-chlorotaurine, epidemic keratoconjunctivitis, antiviral agent

## Introduction

The human adenovirus (HAdV) family consists of 55 known types, which are divided into seven species, A—G.<sup>1,2</sup> The most common external ocular viral infections worldwide are caused by many HAdV serotypes.<sup>3,4</sup> Infectious diseases caused by HAdV include respiratory tract infection, keratoconjunctivitis, hemorrhagic cystitis, and arthritis, some of which may result in a lethal outcome in immunocompromised persons. The growing practice of transplantation accompanied by strong immunosuppressive therapy in recent decades has led to a gradual increase in the incidence of severe HAdV infections. Pediatric patients undergoing allogeneic stem cell transplantation are particularly prone to disseminated HAdV infections, with high associated morbidity and mortality.<sup>5</sup> Severe systemic HAdV infection can also occur in patients with acquired immune deficiency syndrome.<sup>6</sup> In particular, adenoviral conjunctivitis is known to be the major cause of acute contagious infections associated with community and nosocomial epidemics.

Correspondence: Eiichi Uchio  
Department of Ophthalmology,  
Fukuoka University School of Medicine,  
7-45-1 Nanakuma, Jonan-ku,  
Fukuoka 814-0180, Japan  
Tel +81 92 801 1011  
Fax +81 92 865 4445  
Email [euchio@fukuoka-u.ac.jp](mailto:euchio@fukuoka-u.ac.jp)

Thus, the development of effective anti-AdV drugs for the clinical treatment of adenoviral conjunctivitis and systemic HAdV infectious diseases is important. However, there is no approved antiviral therapy for HAdV infections at this time. In potentially lethal HAdV infections, medical treatment is currently carried out using antiviral agents, especially nucleotide analogs. Among nucleotide analogs, ribavirin,<sup>7</sup> trifluridine,<sup>8</sup> and cidofovir<sup>9–12</sup> are reported to be effective in vitro against HAdV. We have recently reported that nucleoside reverse transcriptase inhibitors, zalcitabine and stavudine, both show significant antiadenoviral activity.<sup>13</sup>

*N*-Chlorotaurine (Cl–HN–CH<sub>2</sub>–CH<sub>2</sub>–SO<sub>3</sub>H, NCT) is the *N*-chloro derivative of the amino acid taurine. NCT is the main representative of long-lived oxidants produced by stimulated granulocytes and monocytes during inflammatory reactions.<sup>14–16</sup> It has been demonstrated that NCT has significant in vitro microbicidal activity against bacteria, yeasts, and molds.<sup>17,18</sup> NCT has also been shown to be virucidal in vitro against HAdV serotype 5.<sup>19</sup> The cytotoxicity of NCT to human cells in vitro is very low compared with that of powerful oxidants such as hypochlorite.<sup>20</sup> Using a plaque assay, Romanowski et al previously reported that NCT demonstrated inhibitory effect against HAdV serotypes 1, 2, 3, 4, 5, 7a, 8, 19, and 37.<sup>21</sup> In a clinical trial of 1% NCT application in epidemic keratoconjunctivitis, safety and clinical usefulness were observed; however, the number of participants was limited and virological analysis was not carried out in that study.<sup>22</sup>

In the present study, using real-time polymerase chain reaction (PCR) method to directly quantify HAdV progeny in virus-infected cells, we investigated whether NCT displays potent and selective antiviral activity against HAdV serotypes causing keratoconjunctivitis, serotypes 3, 4, 8, 19a, and 37.

## Materials and methods

### Experimental compound

NCT, which was donated by Dr Nagl (Innsbruck, Austria), was prepared as the crystalline sodium salt (MW = 181.57 g/mol). It was dissolved in phosphate-buffered saline at various concentrations for in vitro antiviral assay as described later.

### Cells

A549 cells (alveolar epithelial cells; ATCC #CCL-185) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Eagle's minimum essential medium containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 7% fetal calf serum.

## Viruses

The viruses used were HAdV type 3 (HAdV3), HAdV4, HAdV8, HAdV19, and HAdV37. Four types, HAdV3, HAdV4, HAdV8, and HAdV37, were prototype strains and were provided by the ATCC. HAdV19a was a clinical strain<sup>23</sup> because HAdV19a is frequently associated with epidemic keratoconjunctivitis, whereas the prototype of HAdV19 (HAdV19p) is not responsible for outbreaks of keratoconjunctivitis.<sup>24</sup> These strains were propagated in A549 cells and stored at –80°C until use.

### Cytotoxicity assay

The cytotoxicity of NCT was evaluated in uninfected confluent A549 cells in 96-well plates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ). Dilutions of NCT were prepared in Eagle's minimum essential medium supplemented with 2% fetal calf serum. Seven concentrations (15, 30, 60, 120, 240, 480, and 960 µg/mL) of the test compound were used. After 7 days of incubation at 37°C with 5% CO<sub>2</sub>, the cells were subjected to a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based colorimetric viability assay according to the manufacturer's instructions (Promega, Leiden, The Netherlands). A490 values, corrected for the cytotoxicity exerted by NCT (as determined in mock-infected cultures), were used to calculate the percent cell viability. The 50% cytotoxic concentration (CC<sub>50</sub>) of NCT corresponds to a 50% reduction in cell viability.

### Antiviral analysis in A549 cells by real-time PCR

First, A549 cells were seeded in wells of 96-well plates at 10,000 cells/well and incubated for 4 or 5 days until confluency was reached. Then HAdV in 50 µL was added to 5 PFU/well. After 2 h at 37°C, the virus suspension was aspirated and replaced by serial dilutions of NCT (200 µL/well). The concentrations of NCT ranged between 1/80 and 1/10 of the CC<sub>50</sub> of NCT. Mock-infected cultures receiving only NCT were included in each plate. After 7 days of incubation at 37°C, microscopy was performed to score the virus-induced cytopathic effect (CPE). After removal of the culture supernatant, cells and virus particles were lysed by the addition of 70 µL lysis buffer (10 mM Tris-HCl (pH 7.8), 0.5% sodium dodecyl sulfate, 5 mM Na<sub>2</sub>EDTA, and 80 µg proteinase K/mL) and incubated at 50°C for 1 h and then at 65°C for 20 min to inactivate proteinase K. After centrifugation (23,000 × *g*, 10 min), soluble cell extracts were stored at –20°C until real-time

PCR was performed. Extracts were diluted 100-fold in water. Then 2  $\mu$ L of diluted extract was added to each well in optical plates containing 23  $\mu$ L SYBR green PCR master mix (Applied Biosystems, Foster City, CA), and forward and reverse primers (300  $\mu$ M) were added to the wells. The primers, derived from GenBank sequences, were chosen to amplify a 137-bp fragment in the conserved HAdV hexon DNA sequence, allowing analysis of all known HAdV types (forward primer, 5'-CGCTGGACATGACTTTTGGAG-3'; reverse primer, 5'-GAACGGTGTGCGCAGGTA-3'). Real-time PCR analysis was performed in an ABI Prism 7000 apparatus (Applied Biosystems) and consisted of 10-min activation at 95°C, followed by 40 thermal cycles, each consisting of 15 s at 95°C and 90 s at 60°C. The dissociation profile was obtained at the end of each analysis to confirm the specificity of the PCR amplification. In each individual experiment, a standard curve ( $R^2 > 0.98$  within the range of  $10^3$ – $10^8$  copies/reaction mixture) was obtained by amplification of known amounts of a pGEM T-vector in which a 691-bp fragment of HAdV hexon DNA was inserted using common cloning procedures. These standard curves were used to convert the cycle threshold values for the A549 extracts into the absolute number of HAdV hexon DNA copies. The 50% effective concentration ( $EC_{50}$ ) was calculated from the dose response to NCT measured 7 days postinfection and corresponded to the NCT concentration reducing the number of viral DNA copies by 50%. All experiments were carried out in triplicate. The lower limit for the detection of HAdV in this study was 10 copies/reaction as reported elsewhere.<sup>25</sup>

## Statistics

Nonparametric analysis was conducted. Shirley–Williams test was used to detect the significance of the dose-dependency of tested agent in each serotype. A level of  $P < 0.05$  was accepted as statistically significant.

## Ethical consideration

This research adhered to the tenets of the Declaration of Helsinki.

## Results

### Cytotoxicity and antiviral activity of NCT and $EC_{50}$

In the cytotoxicity assay for NCT,  $CC_{50}$  of NCT was 530  $\mu$ g/mL (2.9 mM). The relationship of the concentration of NCT and decrease in virus copy is shown in Table 1.  $EC_{50}$  obtained by real-time PCR of NCT ranged between 48.9 and 257  $\mu$ M. Among the serotypes, NCT appeared to be particularly effective against HAdV37, and other serotypes belonging to species D, HAdV8 and HAdV19a, were similarly sensitive to NCT.  $EC_{50}$  of NCT against HAdV3 was similar to that against species D serotypes. In contrast,  $EC_{50}$  of NCT to HAdV4, species E, was greater than that for other serotypes, showing ~fourfold lower antiadenoviral activity compared to that against species D serotypes. A statistically significant dose-dependent inhibitory effect ( $P < 0.05$ ) was observed in all serotypes except for HAdV4.

### Selectivity index of NCT

Selective index ( $CC_{50}/EC_{50}$ ) of NCT for each HAdV serotype was calculated based on the values of  $CC_{50}$  and  $EC_{50}$  of NCT. The results are shown in Table 2. Selective index of NCT for each HAdV serotype ranged between 11.5 and 60.2.

## Discussion

NCT is a weak endogenous oxidative amine whose main mode of action appears to be oxidation of thio and amino groups of proteins, which provides broad-spectrum activity against pathogens<sup>26,27</sup> with low probability of occurrence of resistance.<sup>22</sup> No additives are considered to be necessary for storage and application of NCT, and allergic reactions

**Table 1** Antiviral activity of N-chlorotaurine against HAdV in A549 cells and  $EC_{50}$

N-Chlorotaurine conc ( $\mu$ g/mL)	No of HAdV copies/cell (log copies/mL)				
	HAdV type 3 (HAdV3)	HAdV4	HAdV8	HAdV19a	HAdV37
53	9.40 $\pm$ 0.17*	9.73 $\pm$ 0.06	7.41 $\pm$ 0.94*	9.89 $\pm$ 0.47*	9.34 $\pm$ 0.66*
27	9.86 $\pm$ 0.05*	9.90 $\pm$ 0.01	7.63 $\pm$ 0.76*	10.01 $\pm$ 0.06*	9.73 $\pm$ 0.55*
13.5	10.07 $\pm$ 0.05*	9.96 $\pm$ 0.03	8.18 $\pm$ 0.76	10.24 $\pm$ 0.07*	9.93 $\pm$ 0.09*
6.8	10.23 $\pm$ 0.02*	10.01 $\pm$ 0.07	8.57 $\pm$ 0.02	10.67 $\pm$ 0.15	10.33 $\pm$ 0.31
0	10.40 $\pm$ 0.07	10.05 $\pm$ 0.21	8.64 $\pm$ 0.15	10.80 $\pm$ 0.04	10.54 $\pm$ 0.09
$EC_{50}$ ( $\mu$ g/mL)	12.9 (71.7 $\mu$ M)	46.2 (257 $\mu$ M)	11.1 (61.7 $\mu$ M)	9.5 (52.8 $\mu$ M)	8.8 (48.9 $\mu$ M)

**Notes:** A statistically significant dose-dependent inhibitory effect compared with control was observed in all serotypes except for AdV4. \* $P < 0.05$ .

**Abbreviations:** HAdV, human adenovirus;  $EC_{50}$ , 50% effective concentration.

**Table 2** Selective index of *N*-chlorotaurine for HAdV serotypes

N-Chlorotaurine conc (µg/mL)	HAdV serotypes				
	HAdV type 3 (HAdV3)	HAdV4	HAdV8	HAdV19a	HAdV37
Selective index	41.1	11.5	47.7	55.8	60.2

**Abbreviation:** HAdV, human adenovirus.

seem to be rare.<sup>21</sup> NCT has the additional advantage of low cytotoxicity against human cells in vitro compared with powerful oxidants like hypochlorite.<sup>20</sup> In the present study, EC<sub>50</sub>s of NCT ranged between 49 and 256 µM, which would be 1/10–1/60 of its CC<sub>50</sub> (2.9 mM), indicating pharmacological safety as an antiviral agent.

From a survey of published studies, several kinds of in vitro methodology have been used for anti-HAdV testing, and three antiviral assays, 1) based on a classical CPE assay, 2) MTS-based cell viability staining, and 3) direct quantitation of virus progeny by real-time PCR, yielded similar and reproducible values of antiviral EC<sub>50</sub>.<sup>28</sup> The finding of significant inhibitory activity of NCT in the present study was similar to that reported by Romanowski et al.<sup>21</sup> However, viral plaque assay based on conventional CPE assay was used in the report by Romanowski et al, and EC<sub>50</sub> values were not determined in that report,<sup>21</sup> whereas real-time PCR assay can provide less labor-intensive, more rapid, and equally reliable way of testing antivirals against HAdV.

In our present study, the antiviral activity of NCT against HAdV4, species E, was weaker than that against other serotypes and significant dose-dependency was not found. In contrast, EC<sub>50</sub> of NCT against HAdV3, species B, and HAdV8, HAdV19a, and HAdV37, species D, showed similar values, and these serotypes showed a significant dose-dependent tendency. The reason for this is unclear, considering the nonspecific mode of action (oxidation of SH and NH groups) of NCT.<sup>22</sup> As for receptors, HAdV4 uses constitutive androstane receptor (CAR), in contrast to serotypes belonging to species B and D.<sup>29</sup> A possible explanation for the ineffectiveness of NCT peptide in HAdV4 may derive from the fact that HAdV4 manifests superior binding and infectivity in epithelial A549 cells compared to members of species B and D.<sup>30</sup> In contrast, adenoviruses from species B and D show higher infectivity against human hematopoietic cells than does HAdV4.<sup>31</sup> These biological properties of HAdV4 in various cells suggest that HAdV4 has superior CAR capacity or uses an unknown co-receptor in addition to CAR, leading to its lower susceptibility to NCT. Although statistically significant dose-dependent inhibitory effect of NCT against HAdV4 was not observed in this

study, dose-dependent inhibitory tendency was observed in HAdV4; thus this result might be due to restricted number of test samples, triplicate. In addition, there is a possibility that NCT might be effective against HAdV4 similar to other serotypes studied here.

In conclusion, NCT was shown to be a potent and selective agent against HAdV, and there is a possibility that NCT could be applied to the eye as eye drops or an ointment for adenoviral conjunctivitis. Further examination in both experimental and clinical contexts is necessary before its clinical use.

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## Disclosure

The authors report no conflicts of interest in this work.

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