

N-Chlorotaurine Is an Effective Antiviral Agent against Adenovirus In Vitro and in the Ad5/NZW Rabbit Ocular Model

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PURPOSE. To determine whether *N*-chlorotaurine (NCT) demonstrates antiviral activity against adenovirus (Ad) in vitro and in the Ad5/NZW rabbit ocular model.

METHODS. The in vitro activity of NCT was evaluated by incubating different Ad serotypes with several concentrations of NCT for 1 hour and determining the reduction in Ad titers. In rabbit study 1, Ad5-infected eyes were treated with 2.5%, 2.0%, and 1.0% NCT; 0.5% cidofovir; or saline. NCT and saline groups were treated 10 times for 1 day and then 5 times daily for 6 days. In rabbit study 2, Ad5-infected eyes were treated with 1.0% NCT/0.1% ammonium chloride (NH₄Cl), 0.1% NCT/1.0% NH₄Cl, 0.1% NCT/0.1% NH₄Cl, and 0.5% cidofovir or saline. The NCT and saline groups were treated five times daily for 10 days. Cidofovir-treated eyes received the authors' standard cidofovir dose regimen: twice daily for 7 days.

RESULTS. In vitro, NCT demonstrated concentration-dependent direct inactivation of all ocular Ad serotypes tested. Rabbit study 1: 2.5%, 2.0%, 1.0% NCT, and cidofovir demonstrated significantly fewer positive cultures per total cultures during days 1 to 14, compared with saline. Rabbit study 2: 1.0% NCT/0.1% NH₄Cl, 0.1% NCT/1.0% NH₄Cl, 0.1% NCT/0.1% NH₄Cl, and cidofovir demonstrated significantly fewer positive cultures per total cultures, during days 1 to 14; shorter durations of shedding; and lower mean combined titers, during days 7 to 14, compared with saline. Cidofovir was significantly more effective than NCT in several outcome measures in both rabbit studies.

CONCLUSIONS. NCT demonstrated antiviral activity against adenovirus in vitro and in vivo. Further development of NCT as a topical antimicrobial is indicated. (*Invest Ophthalmol Vis Sci*. 2006;47:2021–2026) DOI:10.1167/iov.05-1270

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Adenovirus ocular infections (epidemic keratoconjunctivitis [EKC], follicular conjunctivitis, and pharyngeal conjunctival fever) are the most common ocular viral infections worldwide.¹ Although many of these infections are self-limiting, patients would benefit greatly from antiviral treatment, because it would reduce the significant morbidity that results in lost time from school and work and would limit the spread of these very contagious infections within households and communities.¹ At present, there is no U.S. Food and Drug Administration (FDA)-approved antiviral agent for the treatment of these infections. The antiviral agent cidofovir, a nucleoside analogue that inhibits adenovirus DNA polymerase,² was successfully tested through phase II clinical trials for this indication. However, toxicity (epiphora due to secondary lacrimal canalicular blockade) and marketing problems have led to the discontinuation of the development of topical cidofovir.² This has left the ophthalmic community without a potential antiviral agent to treat these infections.

N-chlorotaurine (NCT; Fig. 1) is the *N*-chloro derivative of the amino acid taurine. It is an essential, weak, long-lasting oxidant produced by human granulocytes and monocytes during inflammatory reactions.^{3–5} NCT has demonstrated significant in vitro microbicidal activity against bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and others), yeasts (*Candida spp.*), and molds.^{6,7} This activity can be markedly enhanced by addition of ammonium chloride whereby the more lipophilic and therefore stronger microbicidal monochloramine is formed via chlorine transfer.^{7,8} In addition, NCT has been shown to be virucidal in vitro against both adenovirus type 5 (Ad5) and HSV-1.⁹ Its mode of action is mainly the nonspecific oxidation of thio and amino groups on proteins, which results in the direct inactivation of the pathogens.^{10,11} The cytotoxicity of NCT is very low against human cells in vitro compared with powerful oxidants such as hypochlorite.¹² Furthermore, a 1% aqueous NCT solution has been shown to be nontoxic to rabbit and human eyes after topical instillation¹³ and also has been more effective than gentamicin for the treatment of viral conjunctivitis in patients.^{14,15}

Even with the evidence provided by the preliminary in vitro report⁹ and the clinical trials,^{14,15} more data must be generated about the antiviral activity of NCT against adenovirus to warrant further development as a topical antiviral agent. To date, there are no data demonstrating its antiviral activity against multiple ocular adenovirus serotypes in vitro, nor any data regarding the antiviral efficacy in an ocular animal model. This led to the present study in which the overall goal was to assess whether further clinical development of NCT as a topical anti-adenovirus agent should continue. Specifically, our experimental goals were to determine (1) whether NCT demonstrates antiviral activity in vitro against Ad serotypes that commonly cause ocular infections and (2) whether topical NCT formulated in water or with ammonium chloride, and using

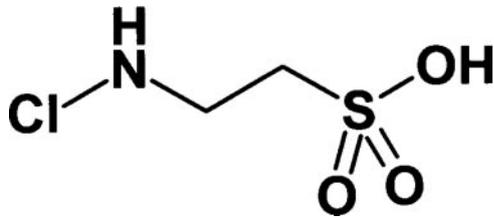


FIGURE 1. The chemical structure of *N*-chlorotaurine.

different treatment regimens, demonstrates antiadenovirus activity in the Ad5/NZW rabbit ocular model.

MATERIALS AND METHODS

Viruses and Cells

Clinical adenovirus isolates of serotypes 1, 2, 3, 4, 5, 7a, 8, and 19 were collected and subsequently coded by an uninvolved third person to protect patient identity and maintain confidentiality (University of Pittsburgh, Pittsburgh, PA, Institutional Review Board no. 000943) at the Charles T. Campbell Ophthalmic Microbiology Laboratory (UPMC Eye Center at the University of Pittsburgh). The protocol was in compliance with the Declaration of Helsinki for research involving human tissue. The serotypes of the isolates were determined by using serum neutralization. No clinical isolates of Ad37 were recovered; therefore, the American Type Culture Collection (ATCC, Rockville, MD) reference strain of Ad37 was used. The clinical isolates along with the ATCC Ad37 reference strain were grown in A549 monolayers and stocks were prepared, aliquotted, and frozen at -70°C .

A549 cells, an epithelial-like cell derived from a human lung carcinoma (CCL-185; ATCC), were grown and maintained in Eagle's minimum essential medium (MEM), supplemented with 10% fetal bovine serum, 2.5 $\mu\text{g}/\text{mL}$ amphotericin B, 100 units penicillin G, and 0.1 mg/mL streptomycin (Sigma Cell Culture Reagents, St. Louis, MO).

Experimental Drugs

NCT (Fig. 1) was prepared as the crystalline sodium salt (MW = 181.57 g/mol).⁶ Preparation of the NCT for the experimental studies is described in detail in the experimental design sections that follow. For the in vivo studies, cidofovir (0.5%) was prepared in intravascular (IV) saline from the 7.5% injectable form of cidofovir (Vistide; Gilead Sciences, Inc. Foster City, CA). IV saline (Baxter, Deerfield, IL) served as the control drug.

Animals

Two- to three-pound female New Zealand White (NZW) rabbits were obtained from Myrtle's Rabbitry (Thompson Station, TN). All animal studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) approval was obtained and institutional guidelines regarding animal experimentation were followed.

Determination of Viral Titers Using the Viral Plaque Assay

The samples to be assayed were diluted 1:10 for several dilutions. One-tenth milliliter of both the undiluted sample and the dilutions were inoculated onto duplicate wells of a 24-well multiplate containing A549 monolayers. The virus was adsorbed for 3 hours at 37°C in a 5% CO_2 -water vapor atmosphere without constant rocking. The plates were rocked intermittently to keep the cells from drying. After adsorption, 1 mL of media plus 0.5% methylcellulose was added to each well, and the plates were incubated at 37°C in a 5% CO_2 -water vapor atmosphere. After the appropriate incubation period, the cells were stained with 0.5% gentian violet, and the number of plaques counted.

The viral titers were then calculated, and expressed as plaque-forming units per milliliter (pfu/mL).

NCT In Vitro Antiviral Assay Experimental Design

The design of this experiment was based on the previous preliminary study.⁹ Briefly, NCT concentrations of 3.125%, 1.25%, 0.625%, 0.125%, and 0% (control) were prepared in PBS. Two hundred microliters of the NCT concentrations were aliquotted into sterile screw cap microfuge tubes. Fifty microliters of stock virus suspensions (Ad1, Ad2, Ad3, Ad4, Ad5, Ad7a, Ad8, Ad19, or Ad37) were added to each tube and gently mixed, yielding final NCT concentrations of 2.5%, 1.0%, 0.5%, and 0.1% once the virus was added. The tubes were then incubated at room temperature for 60 minutes. At the end of the incubation period, 250 μL of 4% cysteine was added to each of the tubes to crystallize the NCT and inactivate it. The tubes were then centrifuged at 500g for 5 minutes (Eppendorf, Fremont CA) to pellet the crystals. Viral plaque assays were performed on the supernatants to determine the titers of adenovirus present in each sample. This experiment was performed in duplicate.

Rabbit Study 1: NCT in Water

This study was performed in duplicate using a total of 50 rabbits (25 per trial). After appropriate systemic and topical anesthesia, NZW rabbits were inoculated with 50 μL (1.5×10^6 pfu/eye) of Ad5 in both eyes after 12 cross-hatched strokes of a no. 25 sterile needle were made in the corneal surface. Inoculation of both eyes of the rabbits allowed us to reduce the number of animals needed without jeopardizing statistical validity, in accordance with the Animal Welfare Act Policy no. 12 (Consideration of Alternatives to Painful/Distressful Procedures, June 21, 2000). Twenty-four hours later, rabbits were randomly assigned to one of five topical treatment groups: group I, 2.5% NCT (pH = 8.84; $n = 10$); group II, 2.0% NCT (pH = 8.78; $n = 10$); group III, 1.0% NCT (pH = 8.50; $n = 10$); group IV, 0.5% cidofovir ($n = 10$); and group V, control (saline; $n = 10$). In this study, the NCT solutions were prepared in Sterile Water for Injection, USP (Abbott Laboratories, North Chicago, IL). NCT and control rabbits were treated in both eyes 10 times daily for the first day and then 5 times daily for 6 days, for a total of 7 days of treatment. Cidofovir-treated rabbits received drug in both eyes twice daily for 7 days, according to our standard cidofovir treatment regimen established previously.^{2,16} All topical solutions (37- μL drops) were instilled using an electronic pipette set in the multidispensing mode (EDP; Rainin, Woburn, MA). Ocular swabbing to recover adenovirus from the tear film and corneal and conjunctival surfaces, after topical anesthesia with proparacaine, was performed on days 0, 1, 3, 4, 5, 7, 9, 11, and 14 after inoculation. The swabs from each eye were placed individually into tubes containing 1 mL of medium and were frozen at -70°C pending viral plaque assay.

Rabbit Study 2 : NCT plus NH_4Cl

This study differed from study 1, in that it sought to enhance the antiviral activity by combining NCT with NH_4Cl and increasing the duration of treatment from 7 to 10 days. The study was also performed in duplicate, using a total of 50 rabbits (25 per trial). The rabbits were inoculated the same as the animals in study 1. Twenty-four hours after inoculation, the rabbits were randomly assigned to one of five topical treatment groups: group I, 1.0% NCT+0.1% NH_4Cl (pH = 7.07; $n = 10$); group II, 0.1% NCT+1.0% NH_4Cl (pH = 6.108; $n = 10$); group III, 0.1% NCT+0.1% NH_4Cl (pH = 7.255; $n = 10$); group IV, 0.5% cidofovir ($n = 10$); and group V, control (saline; $n = 10$). In this study, the NCT solutions were prepared at the desired concentrations in Sterile Water for Injection (Abbott Laboratories). The appropriate amount of ammonium chloride (Sigma-Aldrich) was then added to the NCT solutions. NCT and control rabbits were treated in both eyes, using the same volume drops as in study 1, five times daily for 10 days, whereas cidofovir rabbits were treated in both eyes twice daily for 7 days. Ocular culturing was performed as in study 1.

Ammonium Chloride In Vitro Antiviral Assay Experimental Design

Based on the results of rabbit study 2, we wanted an explanation for our observed in vivo results—namely, how did the addition of ammonium chloride enhance the antiviral activity of NCT in the Ad5/NZW rabbit ocular model? Preliminary unpublished in vitro studies have suggested that the direct antiviral activity of NCT against Ad5 is not enhanced by the addition of ammonium chloride (Nagl M, unpublished results, 2004). However, those results did not determine whether ammonium chloride, in the concentrations used in rabbit study 2, had any direct in vitro antiviral effect on Ad5. To determine this, a study similar to the NCT in vitro antiviral study was designed. Ammonium chloride concentrations of 1.25%, 0.125%, and 0% (control) were prepared in PBS. Portions of the NH_4Cl concentrations (400 μL) were aliquotted into sterile screw cap microfuge tubes. A stock virus suspension (100 μL) of Ad5 was added to each tube and gently mixed, yielding final NH_4Cl concentrations of 1.0% and 0.1%, once the virus was added. The tubes were then incubated at room temperature for 60 minutes. At the end of the incubation period, viral plaque assays were performed on the samples, to determine the titers of Ad5 present in each sample. The experiment was performed in duplicate.

Penetration of Oxidation Capacity (Active Chlorine) through the Human Cornea

In addition, to explain our observed in vivo results, we hypothesized that ammonium chloride may enhance NCT antiviral activity by increasing its penetration through the cornea. To investigate this hypothesis, the following experiment was designed to compare the penetration of oxidation capacity (active chlorine) through corneas using NCT alone and NCT combined with ammonium chloride.

Human cornea samples were obtained from donors for corneal transplantation according to routine methods. Only corneas that were not suitable for transplantation were used in these experiments. Corneas were cultured in RPMI 1640 with 5% fetal calf serum (both from Biochrom, Berlin, Germany) at 31°C for 2 to 4 weeks. Small cylinders with diameters of 7 mm were filled with PBS. The corneas were then put over the open ends. The corneas were secured by screwing a second tube over the first tubes and corneas. The chambers above the corneas were filled with 0.5 mL of either 1% NCT or 1% NCT plus 1% ammonium chloride. After incubation for 2, 4, 6, or 24 hours at 37°C, the oxidation capacities in the fluids in both the upper and lower chambers were measured. Potassium iodide in excess was added and the absorbance at 350 nm (peak of the formed triiodide, $\epsilon = 22,900/\text{moles}/\text{cm}^{17}$) was determined using a spectrophotometer (model DU 7500; Beckman). The measures for the penetration of active chlorine (percentage) were determined by dividing the oxidation capacities in the lower chambers by those of the upper chambers.

Statistical Analysis of In Vivo Studies

After the completion of both trials from each study, the data from each trial were analyzed on computer (Minitab; Minitab, Inc., State College,

PA). Comparable results were obtained in both trials of each study, and the data were therefore pooled to obtain a larger number of subjects and tested with analysis of variance (ANOVA) and χ^2 analyses. Power calculations were performed with the same software. Significance was established at the $P \leq 0.05$ confidence level.

RESULTS

NCT In Vitro Antiviral Assay

The results of the duplicate trials of the in vitro antiviral assay (pfu/mL) were \log_{10} converted and are presented as \log_{10} means \pm SD in Table 1. All the ocular adenovirus serotypes demonstrated reduced titers after incubation with NCT in a dose dependent manner. NCT 2.5% reduced titers from 4.43 to 9.15 \log_{10} over the range of adenovirus serotypes. NCT 1.0% (2.99–5.80 \log_{10}) and 0.5% NCT (3.02–5.83 \log_{10}) demonstrated similar reductions in titers over the serotype range. NCT 0.1% demonstrated the smallest reductions of titers (0.99–3.19 \log_{10}) of the NCT concentrations tested. It appeared that Ad8 was the most resistant of the serotypes tested, in that it demonstrated the smallest reductions in titers at all concentrations. However, it must be noted that 2.5% NCT completely eliminated all adenovirus present in the sample. The mean starting titer of Ad8 ($4.43 \pm 0.81 \log_{10}$ pfu/mL) was the lowest of all the starting titers and therefore could not be reduced more than 4.43 logs. The remaining serotypes demonstrated similar in vitro susceptibilities to NCT.

Rabbit Study 1

The results of Rabbit study 1—NCT formulated in water and a 7-day treatment period—are presented in Table 2 and Figure 2. All the NCT concentrations, along with 0.5% cidofovir, demonstrated significant decreases in the number of Ad5-positive cultures per total over the entire course of the study (days 1–14; Table 2). When we broke the data down into the early (days 1–5) and late (days 7–14) phases of infection (Table 2), NCT demonstrated significant antiviral activity compared with the control during the early phase of infection, but not the late, whereas cidofovir demonstrated significant decreases during both phases of infection compared with the control. The daily reduction in the percentage of positive cultures for all treatment groups is presented graphically in Figure 2. The lack of antiviral activity of NCT during the late phase of infection is reinforced using the second outcome measure of mean Ad5 titer days 7 to 14 (Table 2). All three NCT groups showed no differences in titer compared with the saline control. On the other hand, the cidofovir group demonstrated significantly greater antiviral activity (i.e., fewer Ad5-positive eyes per total and mean Ad5 titer) during the late phase of infection compared with the NCT groups and control group. Only the cidofovir group demonstrated a significant decrease in the duration

TABLE 1. Adenovirus Titers after Incubation with NCT In Vitro

Group	PBS Control	2.5% NCT	1.0% NCT	0.5% NCT	0.1% NCT
Ad1	8.49 \pm 1.42	2.74 \pm 0.00	4.93 \pm 0.71	4.15 \pm 0.24	5.64 \pm 0.93
Ad2	9.81 \pm 0.13	2.72 \pm 3.84	4.14 \pm 1.78	6.03 \pm 0.72	6.48 \pm 0.07
Ad3	7.51 \pm 0.02	1.60 \pm 2.26	2.58 \pm 3.64	2.58 \pm 3.64	5.09 \pm 0.98
Ad4	10.01 \pm 0.04	5.04 \pm 0.48	5.56 \pm 0.91	5.40 \pm 0.91	7.44 \pm 0.11
Ad5	9.15 \pm 1.12	0.00 \pm 0.00	3.35 \pm 1.34	5.00 \pm 0.81	6.57 \pm 0.08
Ad7a	7.22 \pm 0.80	0.00 \pm 0.00	3.42 \pm 0.73	3.65 \pm 1.53	5.23 \pm 0.00
Ad8	4.43 \pm 0.81	0.00 \pm 0.00	1.44 \pm 2.04	1.41 \pm 1.99	3.44 \pm 0.37
Ad19	10.09 \pm 0.28	1.81 \pm 2.56	4.72 \pm 0.01	4.26 \pm 1.46	6.92 \pm 1.24
Ad37	7.48 \pm 0.07	1.68 \pm 2.38	1.72 \pm 2.43	3.23 \pm 1.52	4.78 \pm 0.11

Data are expressed as the mean \pm standard deviation \log_{10} PFU/mL. $n = 2$ experiments.

TABLE 2. Viral Outcome Measures of NCT in Water in the Ad5/NZW Rabbit Ocular Model

	2.5% NCT	2.0% NCT	1% NCT	0.5% Cidofovir	Saline Control
Ad5-Positive Cultures/Total					
Overall (days 1-14)	74/159 (47%)*	75/152 (49%)*	81/160 (51%)*†	67/160 (42%)*	115/160 (72%)
Early phase (days 1-5)	50/80 (62%)*	49/80 (61%)*	54/80 (68%)*	57/80 (71%)*	79/80 (99%)
Late phase (days 7-14)	24/79 (30%)*†	26/72 (36%)*†	28/72 (39%)*†	10/80 (13%)*	36/80 (45%)
Mean Combined Ad5 Titer (pfu/mL)					
Late Phase (Days 7-14) Mean \pm SD (Power = 0.9082-0.9377, depending on sample size)	4.2 \pm 11.0 \times 10 ¹ (n = 79)	7.6 \pm 14.3 \times 10 ¹ † (n = 72)	6.5 \pm 13.0 \times 10 ¹ † (n = 72)	4.2 \pm 2.2 \times 10 ⁰ * (n = 80)	7.0 \pm 16.6 \times 10 ¹ (n = 80)
Duration of Ad5 Shedding (Days)					
Mean \pm SD (n = 20) (Power = 0.7142)	6.5 \pm 4.8	6.7 \pm 5.1	7.2 \pm 4.6	4.9 \pm 2.7*	9.1 \pm 3.4

χ^2 was used for the analysis of Ad5-positive cultures/total. ANOVA was used for the analysis of mean combined Ad5 titer and duration of Ad5 shedding.

* $P \leq 0.05$ when compared with the control.

† $P \leq 0.05$ when compared with 0.5% cidofovir.

of Ad5 shedding compared with the control group. There were no significant differences among the NCT concentrations for any of the viral outcome measures.

Rabbit Study 2

The results of rabbit study 2 using NCT formulated with ammonium chloride and a 10-day treatment period are presented in Table 3 and Figure 3. As in the previous study, all the NCT formulations, along with 0.5% cidofovir, demonstrated significant decreases in the number of Ad5-positive cultures per total over the entire course of the study (Table 3). Similar to rabbit study 1, all NCT formulations demonstrated significant decreases in the number of Ad5-positive cultures per total during the early phase of infection (Table 3). However, in contrast to rabbit study 1, all NCT formulations also demonstrated significant decreases in the number of Ad5 positive cultures per total during the late phase of infection (Table 3). Figure 3 presents the daily percentage of Ad5-positive eyes per total over the entire course of the study. The increase in antiviral efficacy of NCT in this study during the late phase of infection was also

manifested by significantly lower mean combined Ad5 titers during the late phase of infection (Table 3) compared with the control, which was not the case in rabbit study 1. Overall, NCT demonstrated improved antiviral efficacy, compared with the previous study, by significantly reducing the mean duration of Ad5 shedding compared with the control (Table 3). As with the previous study, 0.5% cidofovir significantly decreased all viral outcome measures compared with the control group and demonstrated significant decreases in the number of Ad5-positive cultures per total during the late phase of infection and in the mean duration of Ad5 shedding compared with all NCT formulations. There were no significant differences among the three NCT formulations for any of the viral outcome measures.

The use of a 1% NCT concentration in both rabbit studies 1 and 2 allowed for a direct comparison, to evaluate any enhancement effect of NH_4Cl and a longer treatment period. The 1% NCT plus 0.1% NH_4Cl and 10-day treatment period significantly decreased the number of Ad5-positive cultures per total overall (days 1-14; $P = 0.025$, χ^2) compared with 1% NCT in water during a 7-day treatment period. The majority of fewer Ad5-positive cultures per total came during the late phase of infection (days 7-14) where the 1% NCT plus 0.1% NH_4Cl demonstrated significantly fewer positive cultures ($P = 0.002$, χ^2) compared with 1% NCT in water during the 7-day treatment period. There was no significant difference in the number of Ad5-positive eyes per total overall for the control between the two studies.

Ammonium Chloride In Vitro Antiviral Assay

The results of the duplicate trials of the ammonium chloride in vitro antiviral assay (pfu/mL) were \log_{10} converted and are presented as \log_{10} means \pm standard deviations. Neither 1.0% ammonium chloride ($7.95 \pm 0.08 \log_{10}$ pfu/mL) nor 0.1% ammonium chloride ($7.84 \pm 0.03 \log_{10}$ pfu/mL) demonstrated a reduction in Ad5 titers compared with the PBS control ($7.82 \pm 0.17 \log_{10}$ pfu/mL).

Penetration of Oxidation Capacity (Active Chlorine) Through the Human Cornea

When 1% NCT was applied in our in vitro corneal model, active chlorine penetrated through the cornea in a time-dependent manner: 2 hours, 0% to 1.3%; 4 hours, 1.1% to 4.8%; 6 hours, 20.7% to 25.4%; and 24 hours, 39% to 56%. When 1% ammonium chloride was added, there was a general increase in the active chlorine penetration as summarized: 2 hours, 17.2% to 18.6%; 4 hours, 28.8% to 30.1%; and 6 hours, 47.8% to 59.0%. The range reported for the percentage of active chlorine pen-

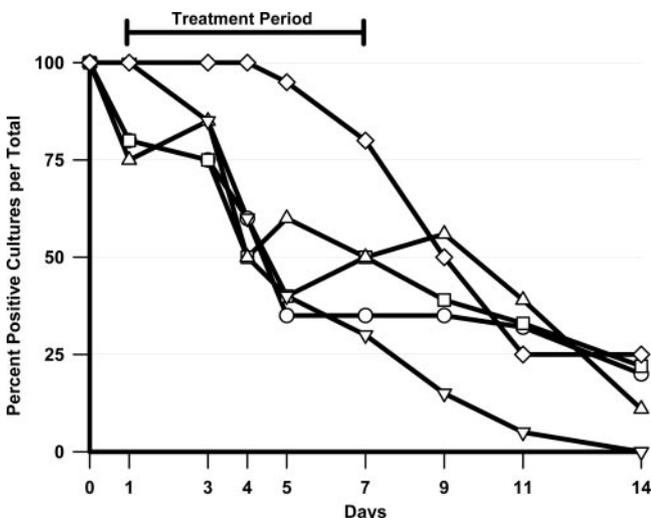


FIGURE 2. The number of positive cultures as a percentage of all cultures in the eyes on each day during rabbit study 1 (NCT in water). From days 1 through 7, 2.5% NCT (○; days 1, 4, 5, and 7), 2.0% NCT (□; days 1, 4, and 5), 1.0% NCT (△; days 1, 4, and 5), and 0.5% cidofovir (▽; days 4, 5, and 7) all demonstrated significantly fewer positive cultures compared with the control (◇). In addition, 2.5%, 2.0%, and 1.0% NCT demonstrated significantly fewer positive cultures compared with 0.5% cidofovir on day 1 ($P \leq 0.035$, χ^2 analysis).

TABLE 3. Viral Outcome Measures of NCT with Ammonium Chloride in the Ad5/NZW Rabbit Ocular Model

	1.0% NCT+ 0.1% NH ₄ Cl	0.1% NCT+ 1.0% NH ₄ Cl	0.1% NCT+ 0.1% NH ₄ Cl	0.5% Cidofovir	Saline Control
Ad5-Positive Cultures/Total					
Overall (days 1-14)	57/150 (38%)*†	53/143 (37%)*†	64/150 (43%)*†	36/141 (26%)*	115/150 (77%)
Early phase (days 1-5)	44/70 (63%)*	44/67 (66%)*	50/70 (71%)*	36/65 (55%)*	64/70 (91%)
Late phase (days 7-14)	13/80 (16%)*†	9/76 (12%)*†	14/80 (18%)*†	0/76 (0%)*	51/80 (64%)
Mean Combined Ad5 Titer (pfu/mL)					
Late Phase (Days 7-14) Mean ± SD (Power = 0.9610-0.9695, depending on sample size)	8.6 ± 41.0 × 10 ^{0*} (n = 80)	1.0 ± 3.9 × 10 ^{1*} (n = 76)	8.5 ± 30.5 × 10 ^{0*} (n = 80)	0 ± 0* (n = 76)	3.9 ± 11.0 × 10 ¹ (n = 80)
Duration of Ad5 Shedding (Days)					
Mean ± SD (Power = 1.0000)	5.5 ± 2.7*† (n = 20)	5.3 ± 2.3*† (n = 19)	6.4 ± 2.3*† (n = 20)	3.7 ± 1.1* (n = 19)	11.6 ± 2.7 (n = 20)

Probabilities and explanation of analyses are as in Table 2.

etration represents the results of two independent experiments.

DISCUSSION

There remains a pressing unfulfilled need for a topical antiadenoviral drug to treat sporadic cases and worldwide epidemics of ocular adenovirus infections. The Campbell Laboratory has been actively engaged in the evaluation of potential candidates since 1991, including the failed drug, cidofovir.² To warrant clinical development, we believe that three important criteria must be met: (1) the antiviral agent must show an in vitro antiviral effect against Ad serotypes (Ad3, Ad4, Ad7a, Ad8, Ad19, and Ad37) that commonly infect the eye, and its inhibitory activity against Species C serotypes (e.g., Ad 5) must be comparable to Species D (Ad8, Ad19, and Ad37). (2) The antiviral agent must demonstrate potent antiviral activity in the Ad5/NZW rabbit ocular model, a surrogate for clinical infec-

tions. For example, the results with cidofovir in the Ad5/NZW rabbit ocular model^{16,18-20} were predictive of those in the phase II clinical trial²¹. (3) The antiviral agent must be safe enough to treat everyone, including children and uninfected at-risk individuals (prophylaxis indication).

The goal of the present study was to determine whether NCT would meet these criteria for further development, and the experimental results were encouraging. The in vitro antiviral assays demonstrated significant antiviral activity against all serotypes in the ocular panel (Ad3, Ad4, Ad5, Ad7a, Ad8, Ad19, and Ad37) and the Species C serotypes (Ad1, Ad2, and Ad5) which suggested that the Ad5/NZW rabbit ocular model could be used with confidence to evaluate NCT.

The results of rabbit study 1 suggested that a 7-day treatment regimen with aqueous NCT was effective compared with a saline control, but not as effective as topical cidofovir, a previously proven antiviral^{16,18-20} in this model. These results are consistent with the mechanism of action of NCT⁹ (direct inactivation of adenovirus particles) and its short half-life in the eye (15 minutes),¹³ compared with cidofovir, a potent DNA polymerase inhibitor with a very long tissue half-life.²² Although NCT appeared to act only on the days of treatment, cidofovir continued to act (presumably from its tissue reservoir) long after the treatment period had ended. The 7-day treatment period, even with a loading dose on day 1, was presumably not long enough for NCT to eradicate the infection fully, as reflected in the lack of antiviral efficacy during the late phase of infection (Table 2).

Rabbit study 2 addressed these deficiencies by increasing the duration of treatment to 10 days and increasing the antiviral potency by combining NCT with ammonium chloride, which has been shown to enhance fungicidal activity of NCT.⁷ Because the direct in vitro antiviral activity against Ad5 was not significantly enhanced by the addition of ammonium chloride to NCT (Nagl M, unpublished results, 2004) and ammonium chloride had no direct antiviral activity on Ad5, the observed enhanced antiviral activity of the combination in the Ad5/NZW rabbit ocular model was presumably attributable to the improved penetration of active chlorine into the cornea through the more lipophilic monochloramine. However, we cannot rule out the possibility that ammonium chloride has an in vivo antiviral effect on adenovirus. Previous studies involving the study of the mechanism of adenovirus entry into cells demonstrated that ammonium chloride can inhibit the entry of adenovirus into cells by raising the pH, thereby inhibiting endosomal release of adenovirus into cells.²³⁻²⁵ Nevertheless, by whatever mechanism of action, our results from rabbit study 2 demonstrated that the antiviral efficacy of NCT combined with ammonium chloride was clearly enhanced, especially during

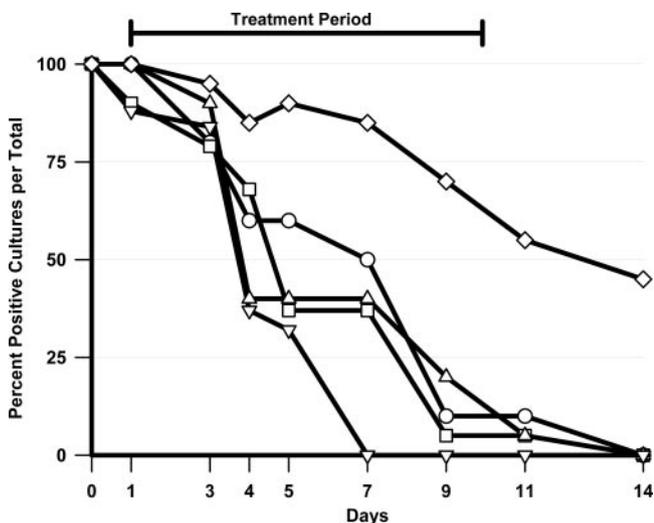


FIGURE 3. The number of positive cultures as a percentage of all cultures in the eyes on each day during rabbit study 2 (NCT+ammonium chloride). From days 1 through 14, 0.1% NCT+0.1% NH₄Cl (○; days 1, 3, 5, 7, 9, 11, and 14), 0.1% NCT+1.0% NH₄Cl (□; days 1, 3, 5, 7, 9, 11, and 14), 1.0% NCT+0.1% NH₄Cl (△; days 1, 3, 5, 7, 9, 11, and 14), and 0.5% cidofovir (▽; days 1, 3, 5, 7, 9, 11, and 14) all demonstrated significantly fewer positive cultures compared with the control (◇). In addition, 0.5% cidofovir demonstrated significantly fewer positive cultures per total compared with 0.1% NCT+0.1% NH₄Cl on day 7, 0.1% NCT+1.0% NH₄Cl on day 7, and 1.0% NCT+0.1% NH₄Cl on days 7 and 9 ($P \leq 0.04$, χ^2 analysis).

the late phase of infection compared with rabbit study 1. Overall, the combination significantly reduced the duration of shedding compared with the control, which was not the case in rabbit study 1 when NCT was used alone. This increase in antiviral efficacy was demonstrated using one tenth the concentration of the previous study, suggesting that 0.1% NCT, when formulated with either 0.1% NH₄Cl or 1.0% NH₄Cl, can be more effective than 1% aqueous NCT. It was not our intent in this study to determine whether ammonium chloride alone demonstrates antiadenovirus activity in vivo.

A comparison of the results obtained from the 1% NCT concentration in both studies demonstrated an increase in antiviral efficacy when the 1% NCT was combined with 0.1% NH₄Cl and the treatment regimen prolonged to 10 days. However, the design of this study cannot determine whether a single change or both were required to increase antiviral efficacy. At this stage, it was not our intent to determine which of the changes were responsible, but to determine whether a combination of both would result in increased antiviral efficacy.

Finally, the issue of safety must be considered, to support further development. The absence of ocular toxicity with NCT has been documented in previous studies. The cytotoxicity of NCT has been shown to be very low against human cells in vitro compared with powerful oxidants like hypochlorite.¹² A 1% aqueous NCT solution was shown to be nontoxic to rabbit and human eyes after topical instillation.¹³ Although NCT formulated with ammonium chloride was not formally evaluated for ocular toxicity in the present study, there were no overt signs of ocular toxicity (redness, discharge, swelling, lacrimation) during the treatment period. The only behavioral sign was that the rabbits immediately wiped their eyes on instillation of the NCT plus ammonium chloride formulations, suggesting that additional toxicity studies are indicated for the combination therapy.

In conclusion, the results of this study show that NCT possesses antiviral activity against a range of ocular and respiratory adenovirus serotypes in vitro and has potent antiviral activity in the Ad5/NZW rabbit ocular model. Based on these encouraging results, continued development of NCT as a topical antiviral agent for the treatment of adenovirus ocular infections is warranted.

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