Broad-Spectrum Virucidal Activity of (NVC-422) \(N,N\)-dichloro-2,2-dimethyltaurine against Viral Ocular Pathogens In Vitro

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**Purpose.** Viral conjunctivitis is a highly contagious infection often causing major epidemics. A safe broad-spectrum antiviral agent is needed to treat this unmet medical need. The purpose of this study is to demonstrate that in vitro NVC-422 is a safe, broad-spectrum topical virucidal agent with activity against ophthalmic viral pathogens.

**Methods.** The virucidal activity of NVC-422 against several serotypes of human adenovirus (HAdV), coxsackievirus A24, enterovirus 70, and herpes simplex-virus-1 (HSV-1) was tested in standard in vitro titer reduction assays with or without tears. An in vitro virucidal score for NVC-422 was determined using the MatTek EpiOcular tissue system.

**Results.** NVC-422 reduced the viral titer of HAdV-5, HAdV-8, HAdV-19, HAdV-37, and HSV-1 by at least 4 logs after 1 hour incubation at 250 μM. Incubation of coxsackievirus A24 and enterovirus 70 with 2.5 mM NVC-422 for 1 hour reduced the viral titer by 4 logs and 4.5 logs, respectively. The virucidal activity of NVC-422 is maintained in the presence of 10% synthetic tears. In the EpiOcular corneal tissue model, NVC-422 was nonirritating at concentrations up to 41 mM.


Infectious conjunctivitis is a highly contagious and wide spread infection of the eye causing approximately 1% to 2% of all family medicine consultations.2,3 It affects primarily young children with an incidence rate of over 80 episodes per person-years in infants less than 1 year old.2 The typical etiologic agents of infectious conjunctivitis are viruses and bacteria. Some forms of conjunctivitis can result in corneal etiologic agents of infectious conjunctivitis are viruses and bacteria, including multidrug resistant strains.15–17

Currently, there is no US Food and Drug Administration (FDA) approved drug available for the treatment of adenoviral conjunctivitis. NVC-422 is a stable analog of N-chlorotaurine (NCT), a mild oxidant produced by granulocytes and macrophages during the oxidative burst.18 NCT has been shown to have broad-spectrum in vitro activity against bacteria, viruses, fungi, and worms.19–23 In vivo, NCT has been shown to be safe and effective in a rabbit model of adenoviral conjunctivitis as well as in a keratocconjunctivitis phase 2 clinical trial. However, its clinical use is severely limited due to its low solution stability.23,24 NVC-422 is a new generation N-chlorotaurine-based molecule with excellent solution stability.25 In the present study, we will demonstrate that NVC-422 has broad-spectrum in vitro virucidal activity against major ophthalmic viral pathogens including adenovirus, coxsackievirus, enterovirus, and HSV-1.
**Materials and Methods**

**Materials**

All viral stocks (HAdV-5: VR-1516; HAdV-8, strain Trim: VR-1604; HAdV-19, strain AV-587: VR-254; HAdV-37, strain GW: VR-929; HSV-1 strain F: VR-733, enterovirus type 70 strain J670/71: VR-836; coxsackievirus type A24 strain DN-19: VR-1662) were obtained from ATCC (Manassas, VA). A549 (human epithelial carcinoma cells [ATCC CCL-185]), Chang C (human epithelial cells, HeLa contaminant [ATCC CCL-20.2]), and Hep-2 (ATCC CCL-25) were used for testing HAdV-5 and –8, HAdV-19, and coxsackievirus A24, respectively. WI-38 cells (human lung fibroblasts [ATCC CCL-75]) were used for testing HSV-1. Cell monolayers were sufficiently confluent and less than 48-hours-old before inoculating with each virus. The growth medium was F12K cell culture medium, 1X Minimal Essential Medium (MEM), or Dulbecco’s Modified Eagle Medium (DMEM; all from Mediatech, Manassas, VA). A549 (human epithelial carcinoma cells [ATCC CCL-185]), Chang C (human epithelial cells, HeLa contaminant [ATCC CCL-20.2]), and Hep-2 (ATCC CCL-25) were used for testing HAdV-5 and –8, HAdV-19, and coxsackievirus A24, respectively. WI-38 cells (human lung fibroblasts [ATCC CCL-75]) were used for testing HSV-1.

**Virucidal Activity of NVC-422**

It was recently demonstrated that NVC-422 has virucidal activity against Adenovirus 5 (HAdV-5), meaning that it very rapidly inactivates the free virus while not affecting viral replication. Based upon this information, we investigated in more detail the viral inactivation kinetics and pH-dependency. NVC-422 in 20 mM PBS solution at pH 7 inhibited HAdV-5 infectivity within 5 minutes with a 50% IC₅₀ of 83.27 µM (Fig. 1A, Table 1). Increasing the incubation time from 5 to 60 minutes reduced the IC₅₀ to 15.78 µM. A decrease from pH 7 to pH 4 enhanced the virucidal activity of NVC-422 approximately 15- to 20-fold depending on the incubation time (Fig. 1B, Table 1).

**RESULTS**

**Anti-Adenoviral Activity of NVC-422**

Viral stocks were incubated with NVC-422 at final concentrations of 2.5, 25, 250, and 2500 µM, respectively, at room temperature for 1 hour. Virus/NVC-422 mixtures were neutralized by adding an equal amount of the 2% neutralization media and incubated for an additional 1 hour at room temperature before being serially diluted in cell culture media. Diluted virus/NVC-422 mixtures were added to A549 cells for HAdV-5, Chang C cells for HAdV-19, Hep-2 cells for coxsackievirus A24, WI-38 cells for enterovirus 70, or Vero cells for HSV-1, which were prepared by seeding 5000 cells/well in a flat-bottom 96-well plate on the day before the assay, and incubated for 1 to 2 hours at 37°C to allow viral adsorption to the cells. Unbound viruses were aspirated off, and the cell culture medium, without any compound, was added back to the cells. Cells were then incubated for 6 days at 37°C, 5% CO₂, and 90% relative humidity. Viral CPE was determined using Dojindo cell counting kit-8 (Dojindo Molecular Technologies, Inc.) and SpectraMax plate reader (Molecular Devices). Viral titer was calculated according to the Spearman-Kärber method.

**EpiOcular Tissue Irritancy Assay**

EpiOcular tissues, which consist of normal, human-derived epidermal keratinocytes and form a cornea-like three-dimensional (3D) tissue structure (MatTek Corporation, Ashland, MD), were placed in 900-µL of cell culture media and 100 µL of the test compound in buffer or synthetic tears was added to the apical side of the tissue for varying concentrations of synthetic tears with 1.25 mM NVC-422 for 5 to 60 minutes. Mucin, ascorbate, or glutathione were added to synthetic tears where indicated. At the indicated times, NVC-422 was neutralized by adding an equal volume of 2X neutralization medium and incubated for an additional 1 hour at room temperature before being serially diluted in cell culture media. Diluted virus/NVC-422 mixtures were added to A549 cells for HAdV-5 or Vero cells for HSV-1, which were prepared by seeding 5000 cells/well in a flat-bottom 96-well plate on the day before the assay. These were incubated for 1 to 2 hours at 37°C to allow viral adsorption on the cells. Unbound virus was aspirated off, and the cell culture medium, without any compound, was added back to the cells. Cells were then incubated for 6 days at 37°C, 5% CO₂, and 90% relative humidity. Viral CPE was determined using Dojindo cell counting kit-8 (Dojindo Molecular Technologies, Inc.) and SpectraMax plate reader (Molecular Devices). Viral titer was calculated according to the Spearman-Kärber method.

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The cytotoxic concentration (CC50) was more than 2 mM and largely independent of the pH or incubation time (Figs. 1C, 1D). The CC50 of NVC-422 diluted in synthetic tears with or without mucin and antioxidants glutathione and ascorbate at concentrations reported to be present in human tears, 27, 28 0.15 mg/mL, 3.7, and 24 lM, respectively, did not significantly change from the CC50 measured in 20 mM PBS, pH 7 (data not shown). An accurate CC50 at pH 4 at incubation times longer than 15 minutes could not be determined since the 5 mM acetate/Saline buffer pH 4 was cytotoxic to the cells even in the absence of NVC-422. At a neutral pH, the TI of NVC-422 based on its HAdV-5 virucidal activity and A549 cytotoxicity is more than 136 with an incubation of 60 minutes and decreased in parallel with its virucidal activity to 50 at a 5 minute incubation time (Table 1). At pH 4, the TI increased to more than 2000 due to NVC-422’s higher virucidal activity (Table 1).

In addition to the IC50 for NVC-422’s anti-Adenovirus virucidal activity, which is an indicator of the NVC-422 concentration required to reduce viral infectivity by 50% at a fixed viral inoculum, the reduction of the HAdV-5 titer at three NVC-422 concentrations was also determined. In this titer reduction assay, incubation of HAdV-5 stocks with 2.5, 25, and 250 lM NVC-422 in 20 mM PBS pH 7 decreased the viral titer in a dose-dependent fashion. 250 lM NVC-422 reduced the HAdV-5 titer by approximately four logs (Fig. 2). As can be seen above, the virucidal activity of NVC-422 is further enhanced in

<table>
<thead>
<tr>
<th>pH 4</th>
<th>Ad5 IC50, lM</th>
<th>CC50, lM</th>
<th>Therapeutic Index, CC50/IC50</th>
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<tr>
<td>5 min</td>
<td>4.21</td>
<td>2062</td>
<td>490</td>
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<tr>
<td>10 min</td>
<td>1.62</td>
<td>2093</td>
<td>1330</td>
</tr>
<tr>
<td>15 min</td>
<td>0.79</td>
<td>2088</td>
<td>2680</td>
</tr>
<tr>
<td>20 min</td>
<td>1.10</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>40 min</td>
<td>0.57</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>60 min</td>
<td>0.99</td>
<td>ND*</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* ND, not determined; the CC50 could not be determined since exposure of A549 cells to 5 mM acetate/saline pH 4 reduced the cell viability.
an acidic environment (5 mM acetate/saline pH 4), reducing the viral titer by more than 6 logs at 25 and 250 μM and by still 2.5 logs at 2.5 μM NVC-422 (Fig. 2, Table 2).

Having established the virucidal activity of NVC-422 against HAdV-5, which served as a model virus, we next investigated its activity against adenovirus serotypes 8, 19, and 37, which are most commonly associated with keratoconjunctivitis. Two hundred fifty micromolar NVC-422 formulated in 5 mM acetate/saline pH 4 reduced the viral titer of the HAdV-8 by nearly 5 logs, the titer of HAdV-19 by 3.8 logs, and that of HAdV-37 by more than 4 logs (Table 2), revealing the broad spectrum virucidal activity of NVC-422 against clinically important adenovirus serotypes.

Virucidal Activity of NVC-422 against HSV-1, Enterovirus 70, and Coxsackievirus Type A24

In addition to adenovirus, viral conjunctivitis can also be caused by HSV-1, enterovirus, and coxsackievirus. We, therefore, first tested the virucidal activity of NVC-422 against HSV-1 in vitro. Because HSV-1 is unstable at pH 4, we determined the HSV-1 virucidal activity of NVC-422 at pH 5, 6, and 7. As was seen with the HAdV-5, the HSV-1 virucidal activity of NVC-422 is pH-dependent, decreasing with an IC₅₀ of 0.48 ± 0.22 μM at pH 5 to 7.1 ± 1.2 μM at pH 6, and 30.5 ± 3.3 μM at pH 7 (Fig. 3A). The HSV-1 virucidal IC₅₀, as measured at pH 7, also increased with shorter incubation times from 30.5 ± 3.3 μM at 60 minutes to approximately 590 μM with 5 or 10 minute incubation times (Fig. 3B). In addition to the IC₅₀ of NVC-422 against HSV-1, we also determined activity against HSV-1, enterovirus 70, and coxsackievirus A24 in a titer reduction assay (Table 2). In a 5 mM acetate/saline buffer pH 5, 250 μM NVC-422 reduced the HSV-1 titer by 4.8 logs. Incubation of coxsackievirus A24 and enterovirus 70 with 2.5 mM NVC-422 in acetate saline pH 4 for 1 hour reduced the viral titer by 4.0 logs and 4.5 logs, respectively.

Virucidal Activity of NVC-422 in Tears

To be effective as an ophthalmic drug, it is important that NVC-422 retains its activity in tears. Therefore, we tested its virucidal activity against HAdV-5 in synthetic tears in a time-dependent manner. In the absence of tears, 1.23 mM NVC-422 (corresponding to approximately 1/10 of the expected clinical concentration) reduced HAdV-5 infectivity to the limit of detection within 15 minutes (Fig. 4A). At low concentrations of synthetic tears, the virucidal activity of NVC-422 was delayed. A sharp reduction of viral infectivity within the first five minutes was followed by a slower second phase, resulting in a complete inactivation of HAdV-5 after 30 minutes (Fig. 4B). Higher concentrations of synthetic tears had anti-adenoviral

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**Table 2. Virucidal Activity of NVC-422 against Several Viruses Commonly Causing Ocular Infections**

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Log Titer Reduction after 1 Hour of Incubation at Room Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM Acetate/Saline pH 4</td>
</tr>
<tr>
<td>Ad5</td>
<td>0.28</td>
</tr>
<tr>
<td>Ad8</td>
<td>0.13</td>
</tr>
<tr>
<td>Ad19</td>
<td>0.06</td>
</tr>
<tr>
<td>Ad37</td>
<td>0.06</td>
</tr>
<tr>
<td>HSV-1†</td>
<td>0.11</td>
</tr>
<tr>
<td>Coxsackievirus A24</td>
<td>0.06</td>
</tr>
<tr>
<td>Enterovirus 70</td>
<td>0.06</td>
</tr>
</tbody>
</table>

ND, not determined.

* NVC-422 was formulated in 5 mM Acetate/Saline pH 4.

† For experiments with HSV-1, NVC-422 was formulated in 5 mM Acetate/Saline pH 5, since HSV-1 is unstable at pH 4.
activity by itself, reducing the viral titer in the vehicle control to 2 to 3 logs, respectively (Fig. 4C, 4D). The decrease in viral infection is most likely due to the IgG component in synthetic tears. While tears from healthy subjects contain low levels of IgG (15–29 mg/L), tear IgG levels rise significantly to 230 mg/L (0.023%) in patients with acute viral conjunctivitis.30 Our synthetic tears contain 0.05% IgG, therefore, 50% synthetic tears closely mimics IgG concentration in conjunctivitis patients. Viral infectivity in a synthetic tear preparation without IgG was the same as in 20 mM PBS buffer (Fig. 4E). At tear concentration of 50 and 100%, NVC-422 completely inactivates the remaining HAdV-5 within 15 minutes. Addition of mucin or antioxidants glutathione and ascorbate to synthetic tears at physiologically relevant concentrations27,28 did not

![Figure 3. HSV-1 virucidal activity of NVC-422. (A) The pH- and concentration-dependent HSV-1 virucidal activity of NVC-422 after 1 hour incubation. (B) The time- and concentration-dependent HSV-1 virucidal activity of NVC-422 in 20 mM PBS pH 7. Shown is one representative experiment of two or more independents experiments carried out in quadruplicates.](image)

![Figure 4. The HAdV-5 virucidal activity of NVC-422 in 20 mM PBS pH 7 (A), 10% synthetic tears (B), 50% synthetic tears (C), 100% synthetic tears (D), and 100% synthetic tears without IgG (E). Shown are the average and SD of two or more independent experiments carried out in triplicates.](image)
further delay the virucidal activity of NVC-422 (data not shown). Overall, NVC-422 retains its HAdV-5 virucidal activity in the presence of different concentrations of synthetic tears.

**Virucidal Activity of NVC-422 in Comparison with Other Compounds**

Finally, the virucidal activity of NVC-422 in comparison to other antimicrobial compounds was tested. NCT, which was well tolerated and active in a phase II clinical trial,^23^ Povidone-iodine, which has been studied in combination with the anti-inflammatory steroid dexamethasone,^31^ and IgG antibodies, which reduced HAdV-5 viral titers in a rabbit viral conjunctivitis model were selected as comparators.^32^ 2,2-dimethyltaururine (DMT), the inactive precursor of NVC-422 was included as a negative control. NVC-422 exhibited equal or greater activity than NCT and IgG against HAdV-5 and slightly less activity against HSV-1 (Table 3). As expected, DMT had no virucidal activity against either virus.

**In Vitro Safety Profile of NVC-422**

As mentioned previously, the CC\textsubscript{50} of NVC-422 on A549 was greater than 2 mM, resulting in a high in vitro therapeutic index. The safety aspect of NVC-422 in an ocular tissue model was further analyzed. The EpiOcular tissue model has a cornea-like 3D architecture and provides a nonanimal alternative to the traditional Draize rabbit eye test with excellent in vitro–in vivo correlation.\textsuperscript{33} In this model, tissue viability was reduced to less than 50% of an untreated control sample after exposure to 41 mM NVC-422 for more than 270 minutes, translating into a sub-Draize irritancy score of “nonirritating.” Tissue viability was still greater than 80% after a 270 minute exposure to 12.3 mM NVC-422. The irritancy score of NVC-422 was not affected by the presence of synthetic tears prepared with or without mucin and antioxidants glutathione and ascorbate at concentrations reported to be present in human tears\textsuperscript{27,28} (data not shown). In contrast, the 0.1% Triton X-100 control decreased tissue viability to less 50% after 60 minutes, resulting in a sub-Draize score of “moderately irritating” (Fig. 5). This biologically relevant ocular tissue irritancy model suggests that NVC-422 at a concentration that effectively inactivates multiple bacterial and viral ocular pathogens will be safe for use in human eyes.

**DISCUSSION**

In this study, we show that NVC-422 is a novel topical virucidal agent, useful for the treatment of viral infections of the eye. It is effective against many major pathogens that cause viral conjunctivitis including several serotypes of adenovirus as well as HSV-1, coxsackievirus A24, and enterovirus 70. This confirms our earlier data showing that NVC-422 has virucidal activity against HAdV-5,\textsuperscript{26} and microbicidal activity against a panel of 17 bacteria and yeasts.\textsuperscript{34} In addition to its broad-spectrum microbicidal activity, NVC-422 is also nonirritating in the EpiOcular tissues, an in vitro irritancy test system with excellent correlation to the traditional in vivo Draize test.\textsuperscript{35} The safety of NVC-422 measured in A549 cell culture and EpiOcular tissues was not affected by the presence of synthetic tears with or without

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**TABLE 3.** Virucidal Activity of NVC-422, NCT, DMT, and Povidone/Iodine against Ad5 and HSV-1

<table>
<thead>
<tr>
<th></th>
<th>Ad5 pH 4</th>
<th>Ad5 pH 7</th>
<th>HSV-1 pH 7</th>
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<tbody>
<tr>
<td>NVC-422</td>
<td>0.27 ± 0.12</td>
<td>4.9 ± 2.7</td>
<td>7.7 ± 2.1</td>
</tr>
<tr>
<td>NCT</td>
<td>0.8 ± 0.0</td>
<td>57.9 ± 17.0</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>2,2-dimethyltaurine</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Povidone/iodeine</td>
<td>1.40 ± 0.09</td>
<td>3.8 ± 0.84</td>
<td>3.8 ± 2.5</td>
</tr>
<tr>
<td>IgG</td>
<td>22.100 ± 707</td>
<td>12.750 ± 1345</td>
<td>ND</td>
</tr>
</tbody>
</table>

Shown are mean deviation and SD of two or more experiments; pH 4 experiments were carried out in 5 mM acetate-buffered saline pH 4 and pH 7 experiments were carried out in 20 mM PBS, pH 7. Please note that the 50% inhibitory concentrations are given in microgram per milliliter, since a molecular weight for Povidone-Iodine is not available. ND, not determined.
mucin and antioxidants glutathione and ascorbate at concentrations reported to be present in human tears. Based upon these in vitro results, NVC-422 is a potential therapeutic candidate for the treatment of various ocular infections. It could provide a first line treatment option for infectious conjunctivitis with a single therapeutic agent, rendering the often difficult decision of differentiating viral from bacterial etiologies unnecessary. There is currently no approved treatment for viral conjunctivitis. IgG antibodies were shown to reduce HADV-5 viral titers in vitro and in a rabbit viral conjunctivitis model. Up to 50 mg/mL (200X the concentration present in the tears of conjunctivitis patients) was required to demonstrate a significant effect.

We recently showed in the case of HADV-5, that NVC-422 oxidizes several sulfur-containing amino acids in at least two viral proteins, namely the hexon and fiber. Oxidation of these amino acids leads to changes in the conformation of the proteins and the morphology of the virus particle, ultimately resulting in a rapid loss of viral stability and infectivity. Targeting multiple amino acids in two or possibly more proteins has the advantage of forcing the pathogen under attack to accumulate multiple mutations to acquire resistance to this agent. This is a very unlikely process that would come with major expenses on the infectivity and virulence of the pathogen. While we have formally demonstrated this multitargeted mechanism of action only for HADV-5, this is likely a more general phenomenon. We recently demonstrated and confirmed in the present study that NVC-422 has an activity optimum at pH 4. However, given the small volume of the tearfilm on the eye (approximately 7 µL) compared with the average drop volume dispensed from eye drop bottles (30–60 µL), we expect that the buffering capacity of 5 mM acetate-buffered saline is sufficient to maintain an acidic pH for several minutes, giving NVC-422 an optimal environment to act. This is supported by our time-kill experiments, in which NVC-422 in 10% synthetic tears reduced the adenoviral titer within 30 minutes. Addition of mucin or antioxidants glutathione and ascorbate to synthetic tears at concentrations reported to be present in human tears, did not affect the activity of NVC-422. In addition, only a minor loss of approximately 2% of the initial concentration was detected by HPLC analysis after a 2 hour incubation of NVC-422 in 10% artificial tears (data not shown), further corroborating that NVC-422 is stable and retains its antimicrobial activity in tears.

Teucher and colleagues reported that treatment of patients with conjunctivitis caused by HADV-8 with 1% NCT was more effective than the control group treated with the antibiotic gentamicin. Based upon the increased virucidal activity in vitro and its longer solution stability, we believe that the effectiveness of NVC-422 for the treatment of viral conjunctivitis should be further corroborated in clinical trials.

Acknowledgments

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References


