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Virucidal mechanism of action of NVC-422, a novel antimicrobial drug for the treatment of adenoviral conjunctivitis

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ABSTRACT

Human adenoviral conjunctivitis is a highly contagious eye infection affecting millions of people worldwide. If untreated, it can further develop into keratitis, corneal ulceration, scarring and possible blindness. Despite the significant patient morbidity and socio-economic costs, it is an unmet medical need with no FDA approved treatment. Here, we demonstrate the virucidal activity of NVC-422 (*N,N*-dichloro-2,2-dimethyltaurine) against adenovirus type 5 (Ad5) and investigated its mechanism of action of Ad5 inactivation. NVC-422 inhibits Ad5-induced loss of cell viability *in vitro* with 50% inhibitory concentration (IC_{50}) ranging from 9 to 23 μM. NVC-422 does not cause any cytotoxicity at concentrations as high as 250 μM. *Invitro*, NVC-422 inactivates Ad5 but does not interfere with viral replication, indicating that NVC-422 acts on the extracellular adenovirus as a virucidal agent. NVC-422 inactivates Ad5 by oxidative inactivation of key viral proteins such as fiber and hexon as evidenced by SDS–PAGE, Western blotting and reversed-phase HPLC. These data, combined with measurements of the kinetics of the NVC-422 reactivity with selected amino acids, indicate that the changes in the viral proteins are caused by the selective oxidation of sulfur-containing amino acids. The conformational changes of the viral proteins result in the destruction of the viral morphology as shown by transmission electron microscopy. In summary, NVC-422 exhibits virucidal activity against Ad5 by the oxidative inactivation of key viral proteins, leading to the loss of viral integrity and infectivity.

1. Introduction

Viral conjunctivitis, characterized by inflammation of the ocular surface, is the most common and a highly infectious ocular disease affecting millions of people world-wide. However if untreated, it can further develop into keratitis, corneal ulceration, scarring and possible blindness (Kinchington et al., 2005). The primary pathogen of viral conjunctivitis is adenovirus, a non-enveloped double-stranded DNA virus with a characteristic icosahedral-shaped capsid composed of three major proteins, namely, hexon, penton and fiber, along with other minor proteins (Burnett, 1985; Liu et al., 2010; Reddy et al., 2010; Stewart and Burnett, 1995; Stewart et al., 1993). Currently, 55 adenovirus serotypes with different tissue tropisms have been identified. Depending on the serotype and tropism, adenoviruses can cause infantile gastroenteritis (predominantly serotypes 40 and 41), respiratory tract infections (3, 4, 7, 11, 21 and others), genital ulcers and urethritis (2, 19, 37) (Collier and Oxford, 2006; Sester et al., 2002). Adenoviral infection of the eye can result in three distinct clinical syndromes: follicular conjunctivitis (FC, predominately serotypes 1–11 and 19) and pharyngoconjunctival fever (PCF, predominately serotypes 3, 4 and 7), which last usually less than 7 days and have relatively mild symptoms and the more severe epidemic keratoconjunctivitis (EKC), which can last several weeks (Kinchington et al., 2005). In EKC, which is most often caused by adenovirus serotypes 8, 19 and 37 (Aoki and Tagawa, 2002) both cornea and conjunctiva are affected. In contrast, in the usually milder FC and PCF only the conjunctiva is affected. Typical symptoms of EKC include photophobia, eye redness, foreign body sensation, excessive tearing, blurred vision, pre-auricular lumphoadenopathy, and in rare cases conjunctival hemorrhage (Kinchington et al., 2005). During EKC, the acute viral replication phase is followed by a subepithelial infiltration (SEI) of leukocytes that can be seen as small white dots on the affected cornea (Chintakuntlawar et al., 2010; Kinchington et al., 2005). The resulting loss of visual acuity and light sensitivity can last several months (Kinchington et al., 2005).

Despite its prevalence, patient morbidity and socio-economic cost, adenoviral conjunctivitis is an unmet medical need with no US Food and Drug Administration (FDA) approved treatment. Previous efforts in the application of antiviral drugs such as cidofo-

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vir (CDV) to these conditions have proven unsuccessful due to their toxicity (Romanowski et al., 2001a). The efficacy of a combination of the antiseptic povidone-iodine (PVP-I) and the anti-inflammatory steroid dexamethasone against conjunctivitis is currently being investigated in human clinical trials (Pelletier et al., 2009). However, a 2002 clinical trial report indicated PVP-I is only effective against bacterial conjunctivitis, but not against viral conjunctivitis (Isenberg et al., 2002). Promising results were recently reported on the natural broad-spectrum antimicrobial agent *N*-chlorotaurine (NCT) (Nagl and Gottardi, 1996; Nagl et al., 2002, 1998, 2001) against viral conjunctivitis in animal models and human clinical trials without any adverse effects to the ocular tissues (Nagl et al., 2000; Romanowski et al., 2006; Teuchner et al., 2005, 2008; Uchio et al., 2010). Unfortunately, NCT lacks long-term solution stability at room temperature, which prevents it from being developed into a marketable drug.

In this study, we report anti-adenoviral activity of NVC-422 (*N,N*-dichloro-2,2-dimethyltaurine), a novel analog of NCT that has been structurally modified to gain good solid-state and solution stability while retaining the broad-spectrum activity of NCT (Wang et al., 2008). NVC-422 has been found safe and well-tolerated in a large phase II clinical trial for adenoviral conjunctivitis (unpublished data). We have recently demonstrated NVC-422's activity against various Gram-positive and Gram-negative bacteria and fungi (Wang et al., 2011). We now demonstrate the activity of NVC-422 against adenovirus, using adenovirus type 5 (Ad5) as a prototype. Furthermore, we show that the mechanism of action of NVC-422 is based on the rapid oxidation of sulfur-containing amino acids in key viral capsid proteins, resulting in the loss of viral structural integrity and infectivity.

2. Materials and methods

2.1. Materials

Ad5 was purchased from ATCC (VR-1516) and used without further purification. The Ad5 stock solution was formulated in 20 mM Tris, 25 mM NaCl, 2.5% glycerol at pH 8.0 (buffer A) with 5.8×10^{11} viral particles (VP)/ml. A549 human lung epithelial cells were also obtained from ATCC (CCL-185) and cultured in F12K cell culture medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan UT) and 20 mM L-glutamine (Mediatech). CDV as well as all other chemicals were obtained from Sigma Aldrich (St. Louis, MO). The synthesis of NVC-422 (mol. wt. 244, sodium salt) was described previously (Wang et al., 2008). The purity of NVC-422 powder was measured using HPLC. The method utilizes an Agilent 1100 HPLC or equivalent system using UV detection. The NVC-422 sodium salt weight percentage was determined by comparison of the sample peak area count against the average peak area count of the current NVC-422 reference standard. The purity of NVC-422 powder was found to be 99.83%. The NVC-422 tested solutions were formulated in 5 mM Na-acetate, 150 mM NaCl at pH 4.0 (buffer B), unless otherwise stated.

2.2. Combined adenovirus protection assay (CAVPA)

Original Ad5 stock was diluted 1:100 in 20 mM Na-phosphate, 150 mM NaCl pH 7 buffer (=20 mM PBS) supplemented with 2.5% glycerol, aliquoted and stored at −80 °C. Starting with a 40 mM stock solution of NVC-422 in 20 mM PBS, pH 7 and a 20 mM stock solution of cidofovir (CDV) in 20 mM PBS, pH 7, threefold serial dilutions spanning 0.01–250 μM for NVC-422 and 0.03–500 μM for CDV were prepared in 20 mM PBS, pH7 in 96-well plates in quadruplicates. 80 μl freshly thawed Ad5 was diluted 1:20 in 20 mM PBS, pH 7, was added to 80 μl of the diluted compounds and incubated for 1 h at room temperature. NVC-422 as well as other compounds were inactivated by adding 160 μl volume of 2× neutralization media (2× D-MEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 20% FBS, 1.2 g/L $NaHCO_3$, 20 mM L-glutamine and 1000 IU/ml penicillin/100 μg/ml streptomycin (Mediatech)) for 60 min at room temperature. A stability testing on NVC-422 in the presence of FBS showed that NVC-422 was inactivated very quickly by FBS. For example, 0.1 mM NVC-422 was neutralized by FBS in 2 min at room temperature. Therefore, there should be no residual NVC-422 after one hour incubation. 200 μl of the neutralized virus/compound mixtures were added to A549 cells. A549 cells were prepared by seeding 5000 cells/well in a flat-bottom 96-well plate on the day before the assay. The cell culture medium was aspirated immediately before adding the virus/compound mixtures. After a 2 h incubation at 37 °C to allow viral uptake to the cells, unbound virus was removed by aspiration.

To capture the possibility if NVC-422 or CDV inhibit replication of viruses during cell culture, the compounds (0.01–250 μM for NVC-422 and 0.03–500 μM for CDV diluted in F12K medium supplemented with 10% FBS) were added back to the cells. Cells were then incubated for 6 d at 37 °C, 5% CO_2, 90% relative humidity. Cell viability was determined using Dojindo cell counting kit-8 (Rockville, MD) and SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). Cytotoxicity controls without the addition of Ad5 were carried out in parallel. The% inhibition of viral induced loss of cell viability was calculated as follows: (Compound-treated infected sample − untreated infected control)/(untreated, non-infected control − untreated, infected control) × 100. The 50% inhibitory concentration (IC_{50}) was calculated by plotting (% inhibition) versus (log compound concentration) in GraphPad Prism 4 (La Jolla, CA) using the sigmoidal dose–response equation with a top value constraint set to 100.

2.3. Virucidal assay

The virucidal assay was carried out the same way as the CAVPA assay with the exception that compounds were not added back to the cell culture medium at the beginning of the 6 d viral replication phase. In brief, the viruses were incubated in the test solutions (concentration range for NVC-422 was 0.05–1000 μM, for CDV 0.03–500 μM) in 20 mM PBS, pH7, followed by the inactivation step and by addition of the neutralized virus/compound mixtures to A549 cells. Cells were then incubated for 6 d and monitored for a viral induced loss of cell viability as described above.

2.4. Viral replication assay

During the viral replication assay, the cells were pre-infected with Ad5 diluted 1:20 (v/v) in the cell culture medium for 2 h at 37 °C in the absence of compounds. Unbound virus was removed by aspiration and the cell culture medium supplemented with 10% FBS with serially (threefold) diluted compounds (concentrations for NVC-422 ranging from 0.05 to 1000 μM and 0.02 to 360 μM for CDV) was added. Cells were incubated for 6 d, and viral induced loss of cell viability was determined as described above.

2.5. Viral entry assay

A549 cells were washed with PBS and pretreated with the indicated concentrations of NVC-422 or the anti-CAR antibody RmcB (Millipore, Billerica, MA) diluted in 20 mM PBS for 60 min at room temperature. NVC-422 or RmcB was removed by washing cells twice with 20 mM PBS, pH7. A549 cells were then infected with Ad5 for 4 h at room temperature. Unbound virus was removed by aspiration, cells were incubated for 6 d, and viral induced loss

of cell viability was determined as described above. Uninfected controls were run in parallel to determine cytotoxicity.

2.6. Time-dependent inactivation

Ad5 virus stocks were incubated with 1.35 mM NVC-422 diluted in 20 mM PBS, pH7 or with 20 mM PBS, pH7 as a control for the indicated time points at room temperature. After each time point, viral suspensions were serially diluted in tissue culture media (F12K supplemented with 10% FBS) and then used to infect A549 cells for 2 h. Unbound virus was removed by aspiration and the cell culture medium without any compounds was added back to the cells. Cells were incubated for 6 d, and viral induced loss of cell viability was determined as described above.

2.7. Gel electrophoresis

Non-reducing and reducing sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS–PAGE) of NVC-422-treated Ad5 were performed, where 100 mM dithiothreitol (DTT) was added in the latter to reduce disulfide bonds to thiols. Samples were prepared by adding 5 μl of NVC-422 5× stock solutions (0.2, 2.0 and 20 mM solutions) to 20 μl Ad5 stock (1.2×10^{10} VP). After 5 min incubation at room temperature, 5 μl of 6× loading dye solution was added and incubated at 75 °C for 5 min. Samples were then loaded on a 10–20% Tris–Glycine minigel (Invitrogen) and electrophoresis was performed at 200 V for 60 min. Gels were then Coomassie stained. SeeBlue pre-stained or Mark12 unstained standards (Invitrogen) were used as molecular weight markers.

2.8. Western immunoblotting

Ad5 hexon and fiber proteins were identified by Western immunoblotting. SDS–PAGE was first performed as described above, omitting the Coomassie staining step. The protein bands were electro-transferred to polyvinylidene difluoride (PVDF) membrane at 100 mA for 60 min. The transfer membrane was incubated overnight at room temperature in PBS containing 0.1% Tween-20 (PBS-T) and 3% dry milk. The membrane was incubated with either anti-hexon (rabbit polyclonal anti-Ad5 hexon, Abcam ab24240, 1:2000 dilution) or anti-fiber (mouse monoclonal anti-fiber [4D2], Abcam ab3233, 1:200 dilution) primary antibody for 1 h at room temperature. After several washes with PBS-T solution, the membrane was further incubated with horseradish peroxidase-conjugated secondary antibody (goat polyclonal anti-rabbit, Abcam ab6721, 1:10000 dilution for anti-hexon detection and rabbit polyclonal anti-mouse, Abcam ab6728, 1:5000 dilution for anti-fiber detection) in 3% dry milk PBS-T solution. After three washes with PBS-T, the blots were visualized by using a chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific) and images were captured with a Chem-iDoc XRS system equipped with charge-coupled device camera and Quantity One software (Bio-Rad, version 4.6.5).

2.9. Reversed-phase high performance liquid chromatography (RP-HPLC)

RP-HPLC experiments were performed to separate Ad5 proteins. HPLC methods were adapted from previous studies (Blanche et al., 2001; Chelius et al., 2002; Lehmberg et al., 1999). Samples were injected into a Jupiter C_4 column (150 × 2 mm ID, 5 μm nominal diameter, 300 Å pore size; Phenomenex) and eluted with 0.1% trifluoroacetic acid (TFA; Pierce) in water and 0.1% TFA in acetonitrile as mobile phases A and B, respectively. The column was equilibrated at 20% B. After sample injection, HPLC was run for 10 min at 20% B, followed by a linear gradient to 40% for 15 min, followed by another linear gradient to 60% B for 40 min. The flow rate was 0.2 ml/min and the temperature was maintained at 50 °C. Ad5 samples were prepared by mixing 20 μl Ad5 stock (1.2×10^{10} VP) with reactants, and after incubation at room temperature for 5 min the mobile phase (80% A and 20% B) was added up to 100 μl. 100 μl sample was then injected into the column on an Agilent 1200 HPLC system. Peaks were detected by UV absorbance at 214 nm using a diode-array detector.

2.10. Liquid chromatography–mass spectrometry (LC–MS)

LC–MS analysis was performed to identify chemical modifications induced by NVC-422. A model protein, bovine cytochrome c (Cytc, Sigma) was used for this purpose. Samples were prepared by incubating 0.1 mM Cytc with 1 mM NVC-422 in 100 mM Na-phosphate (pH 7.5) buffer for 30 min at room temperature, followed by the addition of 100 mM DTT to neutralize excess NVC-422. The protein sample was then treated with Trypsin-Gold (Promega) and incubated overnight at room temperature. Digested peptides were separated using a Jupiter C_4 column (150 × 2 mm ID, 5 μm nominal diameter, 300 Å pore size; Phenomenex) and mass-to-charge (m/z) ratios were detected on Agilent 6410 LC QQQ mass spectrometer.

2.11. Transmission electron microscopy (TEM)

TEM experiments were performed using a Philips/FEI Tecnai 12 microscope operating at 100 kV and an FEI Tecnai G2 S-Twin electron microscope operating at 200 kV (University of California at Berkeley). Ad5 samples were prepared by mixing Ad5 stock solution in buffer A (pH 8.0) with buffer B (pH 4.0) or NVC-422 stock solution (12 mM, 0.3%) in buffer B (v:v = 1:1); 1:1 mixture of buffer A and buffer B had final pH of ~7.6. After 5–30 min incubation at room temperature, samples were placed on 300-mesh formvar/carbon-coated copper grids by dipping the grids into Ad5 sample solutions. Each sample was then negatively stained by placing a drop of 2% phosphotungstic acid (pH 7, adjusted with NaOH) on the grid. The sample grid was then dried in air for approximately 10 min. Finally, various areas of the grid were examined and photographs were taken from representative areas.

2.12. Kinetic measurements using RP-HPLC

The reaction rate of NVC-422 with glycine (i.e. amine) was measured using RP-HPLC, by spectrometrically monitoring the reactant NVC-422 (λ_{max} = 304 nm) and the product chloro-glycine (λ_{max} = 252 nm) that were chromatographically separated. Reaction samples were prepared by reacting NVC-422 (4 mM) and glycine (40 mM) in 100 mM Na-phosphate buffer at pH 8.0, and were kept in the sample chamber maintained at 37 °C during the measurement. Each sample was individually prepared and injected immediately into a Eclipse XDB-C18 column (50 × 4.6 mm ID, 1.8 μm nominal diameter; Agilent) and eluted with 50 mM Na-phosphate + 7 mM tetrabutylammonium hydroxide (Sigma) in water (pH 8) as mobile phase A and acetonitrile as mobile phase B. The column was equilibrated at 10% B. After sample injection, the HPLC was run for 0.5 min at 10% B, followed by a linear gradient to 30% B for 1.5 min, followed by an isocratic flow at 30% B for 1.5 min, and a linear gradient to 10% B for 1.5 min. The flow rate was 0.9 ml/min and the column temperature was maintained at 40 °C. Peaks were detected by UV absorbance at 248 nm using a diode-array detector, in which *N*-chloro-glycine was detected at 0.9 min and NVC-422 at 4.7 min elution time.

2.13. Kinetic measurements using stopped-flow spectroscopy

The reaction rates of NVC-422 with sulfur-containing amino acids cysteine, methionine and the tripeptide glutathione were measured using stopped-flow spectroscopy. The kinetic measurements were performed using a Hi-Tech SF-61 DX2 stopped-flow spectrophotometer with Xe arc lamp and PMT detector. All experiments were carried out at $T = 291$ K. The monochromatic kinetic traces were fit with HI-TECH KinetAsyst 3.14 software. Reactions were run under either pseudo-first-order conditions with amino acids in large excess over [NVC-422] or second-order conditions.

3. Results

3.1. Anti-adenovirus activity of NVC-422

NVC-422 is a stable analog of the naturally occurring compound NCT with well established anti-adenoviral activity *in vitro* as well as in an ocular infection rabbit model (Nagl et al., 1998; Romanowski et al., 2006). We therefore tested whether NVC-422 could similarly inhibit infection or replication of Ad5 in A549 cells. To this end, we incubated Ad5 with different concentrations of NVC-422 for 60 min at room temperature. The virus was then added to cells and incubated for 1 h to allow binding and uptake. After removing unbound virus, fresh compounds were added back to the cells; cells were incubated for an additional 6 d after which the viral induced loss of cell viability was measured. In this combined adenoviral protection assay (CAVPA), NVC-422 or the control inhibitor CDV were present during the inactivation phase and the viral replication phase (Fig. 1A). Under these conditions, NVC-422 inhibited viral induced loss of cell viability in a dose-dependent manner with IC_{50} of 9.2 ± 3.1 μM and had a similar activity as the known antiviral drug CDV(Gordon et al., 1991) (Fig. 1B, Table 1). NVC-422 and CDV were not cytotoxic to A549 cells at concentrations up to 250 and 500 μM, respectively.

3.2. Virucidal activity of NVC-422

While the CAVPA assay demonstrated that NVC-422 has anti-adenoviral activity, it remained unclear during which phase it was acting. To determine whether NVC-422 directly affects the cell-free virus or the viral replication inside the cell, we investigated these two aspects separately. The virucidal assay is described in Fig 1A., NVC-422 was only present during the initial viral inactivation phase (Fig. 1A, from −2 to −1 h), but not the viral replication phase since NVC-422 was inactivated by adding neutralization media before the virus cell binding and uptake took place(Fig. 1A). A stability testing on NVC-422 in the presence of FBS showed that NVC-422 was inactivated very quickly by FBS. For example, 0.1 mM NVC-422 was neutralized by FBS in 2 min at room temperature. NVC-422 showed virucidal activity against Ad5 with IC_{50} of 22.9 ± 11.6 μM (Fig. 1C). CDV did not have any virucidal activity in this test as it inhibited viral replication (de Oliveira et al., 1996; Gordon et al., 1991; Romanowski and Gordon, 2000; Romanowski et al., 2001a). NVC-422 was not cytotoxic at concentrations up to 1 mM to A549 cells under these conditions. Time-dependency of the virucidal activity of NVC-422 was also tested, and as a result, 1.35 mM NVC-422 was found to result in a 2 log reduction of the Ad5 titer within 5 min and complete inactivation of Ad5 within 15 min (Fig. 2).

We next determined if NVC-422, in addition to its virucidal activity, also inhibited viral replication. In the viral replication assay, the test compounds were only present during the viral replication phase (Fig. 1A, bottom line). CDV, a viral polymerase-inhibitor, which was used as a positive control (de Oliveira et al., 1996; Gordon et al., 1991; Romanowski and Gordon, 2000; Romanowski et al., 2001b) inhibited Ad5 replication with IC_{50} of 32.7 ± 9.0 μM. In contrast, no interference with adenoviral replication by NVC-422 at concentration up to 1 mM was detected (Fig. 1D, Table 1).

Finally, we tested the possibility that NVC-422 protected cells from adenovirus-caused loss of viability by altering the cellular receptor, such as the coxsackie-adenovirus receptor (CAR) or proteoglycans such as heparan sulfate-glycosaminoglycans (Dechecchi et al., 2000; Tomko et al., 1997). Pre-incubation of the A549 cells with RmcB, an antibody against the CAR receptor, protected the cells from virus-induced loss of cell viability (Fig. 1E). In contrast, pre-incubation with NVC-422 concentrations up to 50 μg/ml (corresponding to 225 μM) that had virucidal activity without cytotoxicity did not have any effect on the virus-induced loss of cell viability.

We concluded from these experiments that at the concentrations used NVC-422 had rapidly virucidal activity *in vitro*, meaning that it quickly inactivates the cell-free virus rather than inhibiting viral replication inside the cells or by altering or blocking one of receptor molecules essential for Ad5 infection.

3.3. Chemical reactivity of NVC-422 towards S-containing residues

To more fully understand the mechanism of action of NVC-422 at a molecular level, we first obtained the rates of reactions of NVC-422 with thiols, thioethers and amines, important functional groups which are known to readily react with chloramines (Gottardi et al., 2005; Gottardi and Nagl, 2002; Peskin and Winterbourn, 2003; Test et al., 1984; Thomas et al., 1986). In Table 2, second-order rate constants (k_2) of NVC-422 with methionine, cysteine and glycine are presented, along with k_2 of NVC-422 with glutathione, a thiol containing antioxidant present in cells. Since the reactions of NVC-422 with methionine, cysteine and glutathione are very rapid, the reaction rates were measured by stopped-flow spectroscopy. The reaction between NVC-422 and methionine (i.e. a thioether) was determined to be relatively fast with k_2 of 1.22 $M^{-1}\,s^{-1}$ (in 100 mM phosphate buffer, pH 7.4 at room temperature), while those with thiols were extremely fast with k_2 of $3 \times 10^7\ M^{-1}\,s^{-1}$ with glutathione. Reactions with cysteine were too fast to be measured even with the stopped-flow instrumentation (Table 2). In contrast, the chlorinating reaction of NVC-422 with glycine (i.e. transhalogenation) was determined to be much slower, with k_2 of 0.032 $M^{-1}\,s^{-1}$ in 100 mM phosphate, pH 8.0 at 37 °C. This rate corresponds to reaction half-life ($t_{1/2}$) of ∼90 min with 4 mM NVC-422 and excess glycine (40 mM), which would be even slower at room temperature and with lower concentrations of NVC-422 and glycine present in biological systems. This suggested that the chlorination of amine groups in the viral proteins by NVC-422 was too slow to be the main cause of the Ad5 inactivation, considering the fact that Ad5was inactivated by NVC-422 in less than 15 min (see above, Fig. 2). Therefore, our data indicated that the oxidations of the sulfur-groups in cysteine and methionine were the primary reactions responsible for the rapid virucidal activity of NVC-422 against Ad5.

Furthermore, we identified NVC-422-induced chemical modifications in proteins in general. We performed an experiment on a model protein, bovine Cytc, in which NVC-422-treated Cytc was trypsin-digested and its peptide fragments monitored with LC–MS. Cytc was chosen as it is a relatively small protein (∼12 kDa) with two methionines, but with no free cysteines, which allows the fast cysteine oxidation to be omitted in the analysis (Cytc has two cysteines that are not available for oxidation as these are covalently linked to the heme cofactor through thioether bonds). Comparing the untreated and NVC-422-treated Cytc data, changes in peptides associated with Met80 (Fig. 3A) and Met65 (Fig. 3B) were observed, demonstrating that both methionines in Cytc are

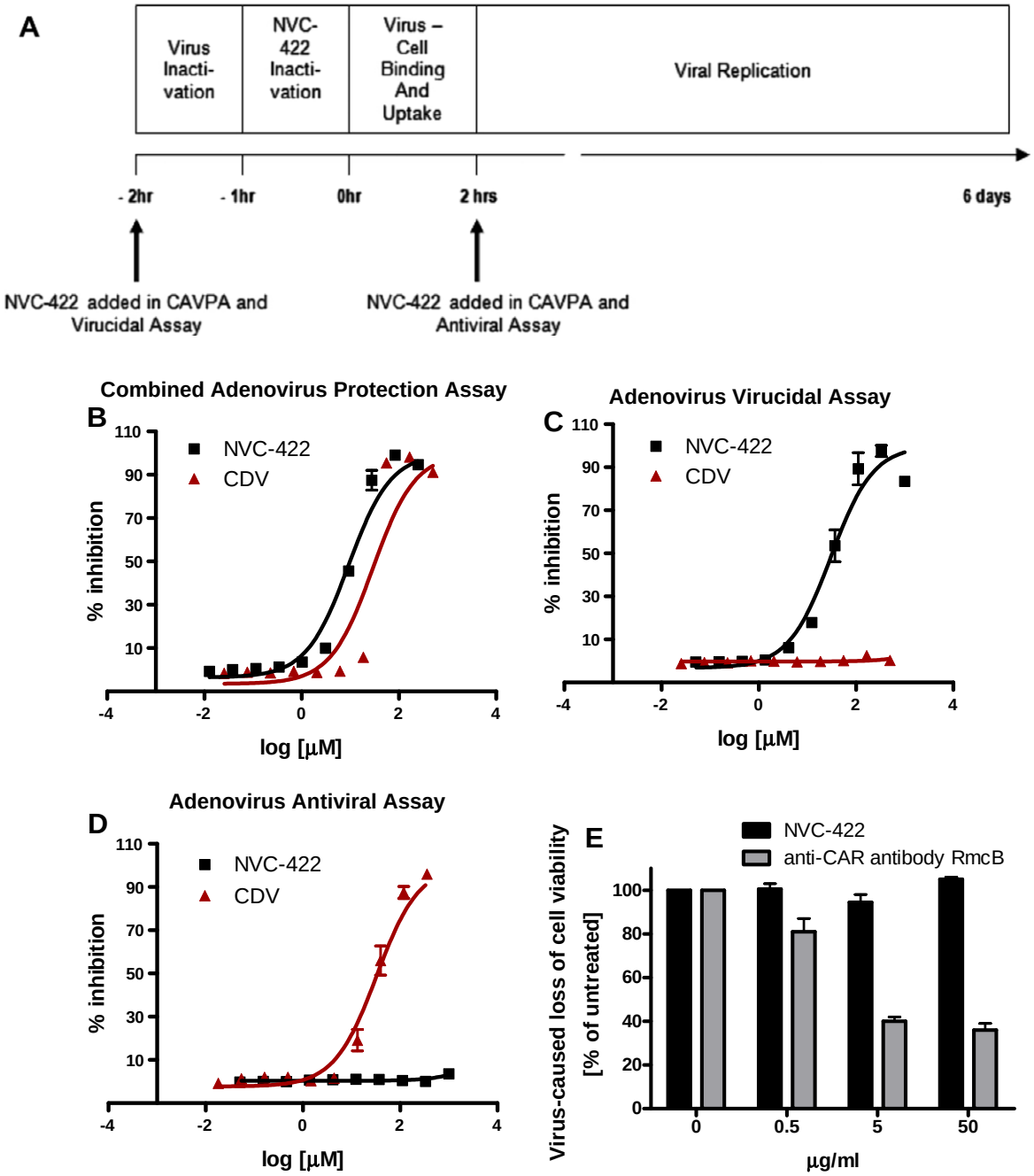

Fig. 1. NVC-422 was active as a virucidal compound during the viral inactivation phase, but not during the viral replication or cell-uptake phase. (A) Schematic overview of the time-of addition experiments. In the combined adenovirus protection assay (CAVPA), compounds were present during the initial 1 h virus inactivation phase and the 6 d viral replication phase. In the virucidal assay, compounds were only present during the viral inactivation phase. In the viral replication assay, compound was only active during the viral replication phase. Effect of various concentrations of NVC-422 or CDV in CAVPA (B), virucidal assay (C), viral replication assay (D) and of NVC-422 and the anti-CAR antibody RmcB in the entry assay (E). Shown are the results of one representative experiment carried out in quadruplicates of two or more independent experiments.

oxidized by NVC-422. No other changes were found; we also did not observe oxidation of the two cysteines in Cytc, which is consistent with the fact that these are covalently linked to the heme cofactor in Cytc (NVC-422 would still be highly reactive to free cysteines in other proteins).

3.4. Oxidation of cysteine residues in Ad5 proteins by NVC-422

The effects of NVC-422 on the proteins of Ad5 were determined by SDS–PAGE analysis. In particular, we performed non-reducing

Table 1
Effect of NVC-422 and cidofovir on Ad5-induced loss of cell viability in the combined adenoviral protection assay (CAVPA), virucidal assay and viral replication assay.

IC50 (μM)[a]	CAVPA	Virucidal assay	Viral replication assay
NVC-422	9.2 ± 3.1	22.9 ± 11.6	>1000[b]
Cidofovir	29.0 ± 6.5	>500[b]	32.7 ± 9.0

[a] Mean ± standard deviation of two or more experiments.
[b] No reduction of the viral CPE observed at the top-concentration of 500 or 1000 μM, respectively.

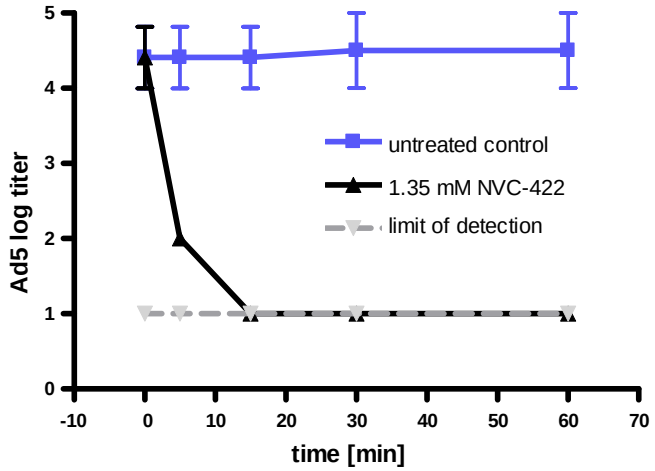

Fig. 2. Time-dependent inactivation of Ad5 by 1.35 mM NVC-422. Ad5 was incubated for various times with 1.35 mM NVC-422 or 20 mM PBS, pH 7. The reduction of the viral titer was determined by titration on A549 cells. Shown are the mean values ± SD of four independent experiments performed in triplicates.

Table 2
Second-order rate constants of reaction of NVC-422 with methionine, glycine, and thiols.[a].

	k_2 (M^{-1} s^{-1})	pH	Temp.
Methionine	1.22	7.4	r.t.
Glutathione	3×10^7	7.4	r.t.
Cysteine	$>3 \times 10^{7}$[b]	7.4	r.t.
Glycine	0.032	8.0	37 °C

[a] All measurements were performed in 100 mM Na phosphate buffers with indicated pH.
[b] Not available as the reaction was too fast to be measured with stopped-flow instrument.

and reducing SDS–PAGE in parallel to monitor the effects of cysteine oxidation (generating disulfide bonds) as suggested by the kinetics data (Table 2). First, non-reducing SDS–PAGE indicated significant changes in the protein profile with NVC-422 treatment (Fig. 4, left, lanes 1–4). Observed changes were detected for capsid proteins, II (hexon), III (penton), IIIa, IV (fiber) and V (penton) where the bands associated with these proteins disappeared or shifted with the appearance of various new bands. The highest molecular weight band at ~300 kDa, which is that of the hexon trimer, also disappeared after the NVC-422 treatment. In contrast, these changes are not present in the reducing SDS–PAGE (Fig. 4, left, lanes 5–8), in which excess amount (100 mM) of the reducing agent dithiothreitol (DTT) was added to reduce disulfide bonds back to thiols. The lack of any changes in the reducing SDS–PAGE indicates that the new bands found in the non-reducing SDS–PAGE were not peptide cleavage products, but rather, associated with disulfide bond formation. The appearance of new bands in the non-reducing SDS–PAGE (in particular, at apparently lower molecular weights than the original proteins) was likely due to the fact that the proteins in non-reducing conditions keep complex tertiary structures than those in the reduced environment in which the proteins were completely denatured. Therefore, SDS-protein complexes in non-reducing conditions, with smaller effective sizes, would migrate faster in the gel.

To verify that the proteins migrated to different positions in the non-reducing SDS–PAGE due to cysteine oxidation and disulfide bond formation, Western immunoblotting of the two major capsid proteins, namely, hexon (II) and fiber (IV), were performed (Fig. 4, middle and right). In the reference lanes 1 and 3 with untreated Ad5, bands consistent with the known size of the hexon and fiber proteins were detected, indicating the antibodies used were highly specific; the higher molecular weight bands in lane 1 of both blots are those associated with trimers of hexon and fiber, respectively. In contrast, lane 2 (non-reducing, without DTT) in each Western blot showed various new bands associated with hexon and fiber, respectively. These new bands were not found in lane 4 (reducing, with DTT), which was consistent with the fact that the changes found in lane 2 were mainly due to disulfide bond formation.

3.5. Oxidation of methionine residues in Ad5 proteins by NVC-422

Ad5 proteins were also analyzed using RP-HPLC. The HPLC chromatograms of Ad5 (1.16×10^{10} VP) treated with increasing amount of NVC-422 are shown in Fig. 5A. The chromatogram of the untreated Ad5 is highlighted by the most intense peak (~46 min, Fig. 5A, blue), which corresponds to the hexon protein (Lehmberg et al., 1999; Chelius et al., 2002). Several smaller peaks associated with other capsid and core proteins were also observed. With NVC-422 treatment, however, the sharp chromatographic features completely disappeared with the appearance of broad, unresolved features that are indicative of protein aggregation, which was consistent with the SDS–PAGE results presented above (Fig. 4, left, lanes 1–4).

The addition of excess DTT to NVC-422-treated Ad5 resulted in the recovery of most of the sharp and well-resolved chromatographic features of untreated Ad5, consistent with reducing SDS–PAGE (Fig. 4, left, lanes 5–8). Interestingly, however, the chromatogram did not fully revert to the original chromatogram of the untreated Ad5 (Fig. 5B). These irreversible changes were observed with several bands, including the most conspicuous change in the

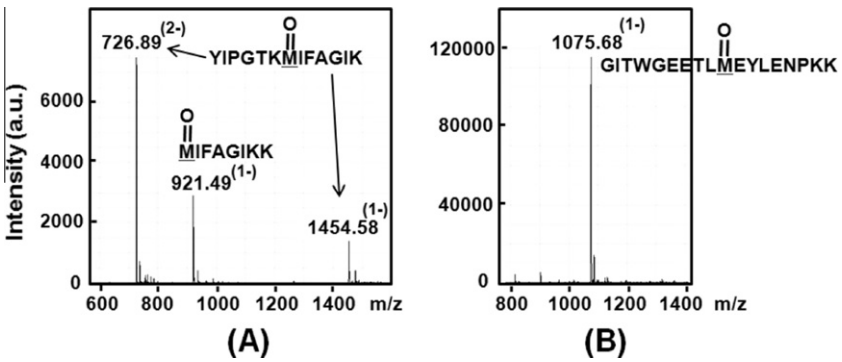

Fig. 3. Demonstration of Met oxidation in NVC-422-treated cytochrome c using LC–MS. Electrospray ionization mass spectrum of peptides was associated with the oxidation of (A) Met80 and (B) Met65. Bovine cytochrome c (0.1 mM) was treated with NVC-422 (1 mM) for 30 min at room temperature and then treated with 100 mM DTT before overnight tryptic digestion. Digested peptides were separated using a C_4 HPLC column and mass-to-charge ratios were detected using negative mode QQQ mass spectrometer.

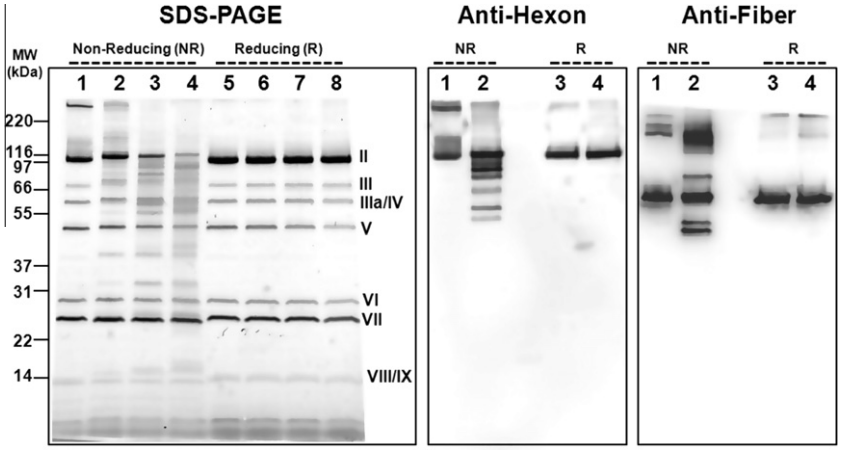

Fig. 4. SDS–PAGE and Western blotting analyses of NVC-422-treated Ad5. (Left) Non-reducing (lanes 1–4) and reducing (lanes 5–8, with 100 mM DTT) SDS–PAGE of NVC-422-treated Ad5. NVC-422 concentrations were as follows: Lanes 1 and 5: untreated Ad5, lanes 2 and 6: Ad5 + 0.04 mM NVC-422, lanes 3 and 7: Ad5 + 0.4 mM NVC-422, lanes 4 and 8: Ad5 + 4 mM NVC-422. Molecular markers (MARK12) were indicated on the left and viral proteins were labeled II–IX according to conventional nomenclature. (Middle and right) Western immunoblotting detection of hexon (II) and fiber (IV) proteins in Ad5 upon treatment with NVC-422. NVC-422 concentrations were as follows: Lane 1: untreated Ad5, lane 2: Ad5 + 2.4 mM NVC-422, lane 3: untreated Ad5 + 100 mM DTT, and lane 4: Ad5 + 2.4 mM NVC-422 + 100 mM DTT. For all experiments, Ad5 was treated with NVC-422 in pH 8.0 buffer at room temperature for 5 min before loading dye buffers (with and without DTT) were added.

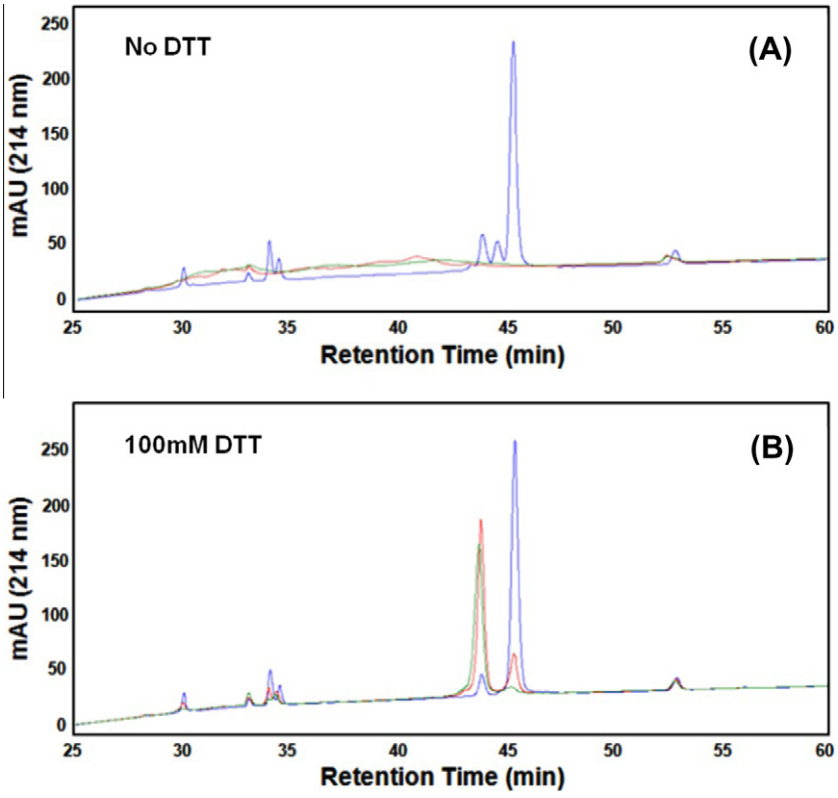

Fig. 5. RP–HPLC analysis of NVC-422-treated Ad5. (A) RP–HPLC chromatograms of Ad5 (1.16×10^{10} VP) treated with 0 mM (blue), 0.4 mM NVC-422 (red) and 4.0 mM NVC-422 (green). Ad5 was treated with NVC-422 in pH 8.0 buffer at room temperature for 5 min before injected into the HPLC column. (B) RP–HPLC chromatograms of NVC-422-treated Ad5 samples that were further treated with 100 mM DTT for 5 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hexon band, where the retention time was shifted from 46 to 44 min with reduction in band intensity. This was due to methionine oxidation generating methionine sulfoxide, based on the fact that methionine oxidation by NVC-422 is kinetically efficient compared with other reactions such as *N*-chlorination (Table 2). Methionine sulfoxide could not be reversed by DTT and the peaks shifted to shorter retention time with NVC-422 treatment, which was consistent with the increased hydrophilicity of the methionine sulfoxide product.

3.6. Effect of NVC-422 on Ad5 morphology

As described above, we demonstrated that NVC-422 was virucidal and reduced the viral infectivity. The loss of viral infectivity

was a consequence of the structural changes induced by the oxidation of proteinaceous cysteines and methionines by NVC-422. To confirm the loss of viral structural integrity upon NVC-422 treatment, we performed transmission electron microscopy (TEM) experiments to monitor the morphological changes in Ad5. In Fig. 6, the morphology of Ad5 virions (~70–80 nm) before and after the treatment with 6 mM NVC-422 are shown (incubated 5–30 min at room temperature). In the untreated Ad5 sample (Fig. 6, top left), the characteristic icosahedral shape as well as the fiber shafts were clearly observed. NVC-422 treatment caused marked changes in the Ad5 structure, including dissociation of proteins from the capsid (Fig. 6A), appearance of circular spots and cavities on the surface (Fig. 6B and E), deformation of the capsid structure (Fig. 6C), and aggregation with adjacent viruses (Fig. 6D). Moreover, fiber shafts, which were the key Ad5 components for infection, were no longer observed after NVC-422 treatment. The TEM data strongly supports the underlying mechanism of action of NVC-422 via oxidation of sulfur-containing amino acids that leads to structural changes in viral proteins and morphology and ultimately, the loss of viral infectivity.

4. Discussion

In this study, we provided biological and biochemical evidence that NVC-422, a novel analog of the natural antimicrobial oxidant NCT, effectively inactivated Ad5. Our time of addition experiments (Fig. 1) showed that NVC-422 was active as a virucidal compound by inactivating cell-free virus, while, at least under the experimental conditions chosen in this work, it did not alter the coxsackie-adenovirus receptor nor inhibit the viral replication inside the cell.

We further demonstrated that NVC-422 efficiently oxidized sulfur containing functional groups namely cysteine and methionine in viral proteins. At the low virucidal concentrations tested (0.04, 0.4 and 4.0 mM), these reactions resulted in non-specific disulfide bond formation, which could be reversed by the addition of DTT. The disulfide bridge formation as well as other protein conformational changes lead to a disruption of the viral capsid architecture that was seen in TEM pictures. In particular, we showed that NVC-422-induced chemical modifications occur in hexon and fiber proteins using Western blotting. This illustrated the loss of two essential functions of the capsid which were viral integrity (hex on) and infectivity (fiber).

In contrast to the well-described nucleoside inhibitor CDV, NVC-422 did not interfere with the viral replication process inside the cell. This was attributed to its inability to penetrate into mammalian cells. The virucidal mechanism of action of NVC-422 is also consistent with studies done on NCT where it was used to inactivate cell-free adenovirus and HSV-1 (Nagl et al., 1998; Romanowski et al., 2006). Shacter and coworkers have suggested that NCT is actively transported across mammalian cell membranes, inducing apoptosis (Klamt and Shacter, 2005; Klamt et al., 2009). The authors based these conclusions on measurements of intracellular NCT, whose taurine backbone was radioactively labeled (Park et al., 1993). However, the chlorinating function was readily lost (i.e. dechlorinated) prior to cellular uptake such that only the redox-inactive taurine penetrated the cell. Based on these results with NCT, we assumed that only trace amounts of NVC-422 may reach the cytoplasm, where it would be quickly deactivated, as it would be readily reduced by the high intracellular glutathione pool (Post et al., 1983) and lose its oxidizing ability. This assumption was supported by our observations that NVC-422 concentrations up to 250 μM were not cytotoxic and did not exhibit any measurable inhibition of viral replication inside the cell.

The absence of cytotoxicity induced by NVC-422 on A549 cells at concentrations as high as 250 μM and by exceeding the IC_{50} of 9–22 μM indicated a high therapeutic index *in vitro*. The low toxicity potential was confirmed using a well established EpiOcular® tissue irritancy model, in which 40 mM NVC-422 was shown to be a non-irritant (data not shown). In addition, in a phase II clinical trial for adenoviral conjunctivitis, administration of 0.3% NVC-422 (corresponding to 12 mM) 8-times per day for 10 days proved to be safe and well tolerated (data not shown).

The virucidal mechanism of action of NVC-422 on multiple amino acids in at least two viral proteins (hexon and fiber) promises a low risk of resistance development, as suggested for NCT (Nagl and Gottardi, 1996). Accumulation of multiple mutations was rare and often accompanied by the loss of viral infectivity.

In the experiments described herein, the well-characterized Ad5 was used as a representative surrogate for other adenovirus serotypes to elucidate the mechanism of inactivation of NVC-422. In virucidal assays, NVC-422 was also active against Ad8, Ad19 and Ad-37, which normally caused epidemic keratoconjunctivitis (data not shown). Due to its basic underlying mechanism of thiol or thioether oxidation, we expected that NVC-422 inactivated

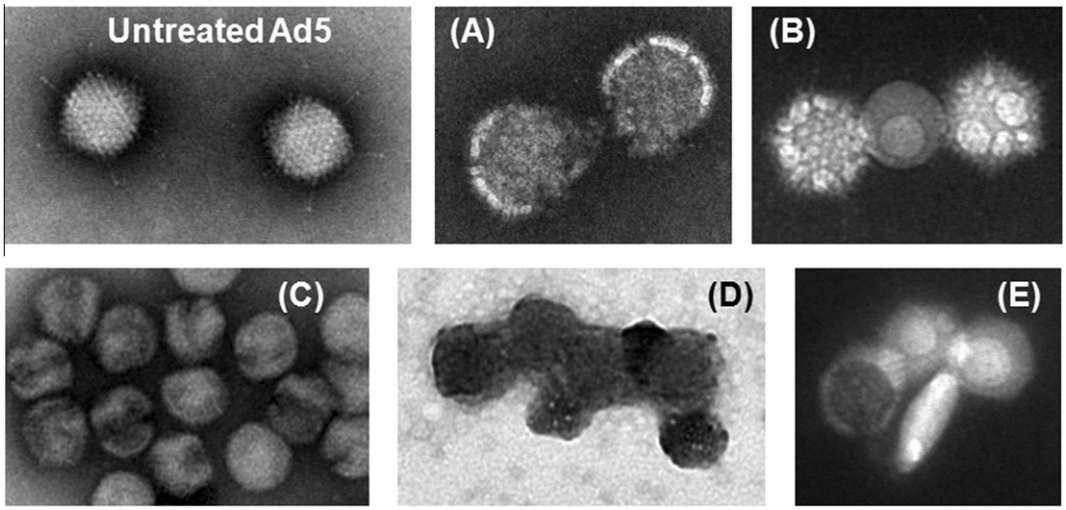

Fig. 6. TEM images of Ad5 before and after treatment with NVC-422. Comparisons were made between untreated Ad5 (top left) and NVC-422 treated Ad5 (A–E). Ad5 was treated with 12 mM NVC-422 (v:v = 1:1, final [NVC-422] = 6 mM) at room temperature for 5–30 min before negatively stained with 2% phosphotungstic acid. Pictures shown are taken from different areas of the sample grid.

these ocular serotypes and other ocular viral pathogens such as herpes simplex virus-1, enterovirus 70, and coxsackievirus A24 (Uchio et al., 2000; Wright et al., 1992). Such an extended virucidal activity was consistent with the broad-spectrum activity of NCT (Nagl et al., 1998; Romanowski et al., 2006). Furthermore, NVC-422 showed bactericidal activity against ocular pathogens such as *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Hemophilus influenzae*, *Pseudomonas aeruginosa* and *Serratia marcescens* (Wang et al., 2011). This data suggests that NVC-422 may have the potential for treating both viral and bacterial conjunctivitis.

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