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Benzalkonium Chloride Demonstrates Concentration-Dependent Antiviral Activity Against Adenovirus *In Vitro*

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Abstract

Purpose: Adenoviral conjunctivitis is the most common cause of conjunctivitis worldwide with no approved antiviral treatment. Benzalkonium chloride (BAK) is a common preservative in ophthalmic medications and is the active ingredient in some skin disinfectants and hand sanitizers. BAK is known to be effective in killing bacteria and enveloped viruses; however, its activity against ocular types of nonenveloped adenoviruses (Ads) is unknown. The goal was to determine whether BAK is an effective antiviral agent against common human ocular types of adenovirus *in vitro*.
Methods: The direct inactivating activity of BAK was determined by incubating several human adenovirus types with BAK concentrations of 0.001%, 0.003%, 0.005%, 0.01%, 0.1%, and 0% for 1 h at 33°C. Resulting adenovirus titers were determined after treatment. Decreases in titers of ≥3 Log_{10} were considered virucidal, while decreases in titers of <1 Log_{10} were considered ineffective.
Results: BAK 0.1% was virucidal for Ad3, Ad5, Ad7a, Ad19/64, and Ad37, while it reduced titers >1 Log_{10}, but <3 Log_{10} for Ad4 and Ad8. Decreases in titers >1 Log_{10} were demonstrated for BAK 0.003%, 0.005%, and 0.01% for Ad5 only. Decreases in titers for the other adenovirus types for those concentrations were ≤0.53 Log_{10}. 0.001% BAK produced minimal decreases in titers for all types.
Conclusions: BAK, at 0.01% or less was not consistently effective as an antiviral against adenovirus, but higher concentrations, such as 0.1%, should be further investigated as a possible topical treatment for adenoviral ocular infections, providing ocular toxicity is not an issue.

Keywords: adenovirus, benzalkonium chloride, epidemic keratoconjunctivitis, antiviral, *in vitro*

Introduction

Adenoviral conjunctivitis is the most common cause of red eyes worldwide.[1] At present, there is no FDA or EMA approved treatment for adenoviral conjunctivitis, although several promising antivirals have been evaluated in preclinical trials in animal models.[2–8] Recently, antivirals that have direct inactivating mechanisms of killing of adenovirus (Ad) have been evaluated in human clinical trials of viral conjunctivitis.[9] Specifically, povidone-iodine and NVC-422 have been evaluated in clinical trials of adenoviral conjunctivitis,[10,11] while a combination of 0.6% povidone-iodine and 0.1% dexamethasone is currently under clinical evaluation (NCT02998554, NCT02998541).

This trend in evaluating direct inactivating antivirals for the treatment of adenoviral conjunctivitis led us to speculate whether another preservative/antiseptic used in ophthalmology could be used as an antiviral for this purpose. Benzalkonium chloride (BAK) is a preservative used in many ophthalmic medications up to concentrations of 0.01%. It is also used in alcohol-free hand sanitizers at concentrations of 0.1% or higher. BAK is known to be effective in killing bacteria and some viruses, fungi, yeasts, and protozoa.[12] It is a member of a family of compounds that are classified as quaternary ammonium compounds. They are commonly used as antiseptic agents because of their cationic amphiphilic property, having a distinct hydrophobic and hydrophilic region.[12] The mechanism of action of quaternary ammonium compounds against bacterial cells is thought to involve a general disruption of the bacterial membranes.[13] Such action leads to a generalized and progressive leakage of cytoplasmic contents out of bacterial cells resulting in cell death.[13]

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The antiviral activity of BAK, at concentrations contained in common ophthalmic medications, against common ocular types of adenovirus, a nonenveloped virus, remains unknown. Its antiviral activity against some enveloped and nonenveloped viruses, including the nonocular adenovirus types 25 (Ad25) and 20 (Ad20), has been evaluated previously.[14,15] The results from a study demonstrated that BAK possessed antiviral activity against enveloped viruses (HSV-1, HIV-1) but not against Ad25.[14] A second study demonstrated high antiviral activity against the enveloped viruses HSV-2 and CMV and demonstrated intermediate antiviral activity against Ad20.[15] No ocular adenovirus types were tested in either of these studies.

Since BAK is present in many ophthalmic medications, such as antibiotics, corticosteroids, and NSAIDs, could these medications, already being used by some to treat adenoviral conjunctivitis, be providing antiviral activity against these infections? This led us to the current study, in which the goal was to determine whether BAK, at concentrations contained in ophthalmic medications as well as skin disinfectants and hand sanitizers, was effective in reducing titers of common human ocular types of adenovirus *in vitro*.

Methods

Viruses and cells

Clinical ocular isolates of human adenovirus types Ad3, Ad4, Ad5 ($n=2$), Ad7a, Ad8 ($n=2$), and Ad19/64 (this type was formerly designated type 19, but now is designated type 64) were recovered from patients at the Department of Ophthalmology, University of Pittsburgh School of Medicine. The types of the Ad isolates were determined using serum neutralization. No clinical isolates of Ad37 were recovered, so the ATCC (American Type Culture Collection, Manassas, VA) reference strain of Ad37 was used. The rationale for the adenovirus types used was as follows: Ad8, Ad19/64, and Ad37 are the adenovirus types that cause epidemic keratoconjunctivitis; Ad3, Ad4, Ad5, and Ad7a are the adenovirus types that produce follicular conjunctivitis; and Ad5 is the adenovirus type that is used in the Ad5/NZW rabbit ocular model.[2–7,16,17]

A549 human lung carcinoma cells were used to prepare the stock adenoviruses and for the determination of adenoviral titers following treatment. The cells were grown in Eagle's MEM with Earle's salts, and 10% fetal bovine serum (FBS) from Sigma Cell Culture Reagents (St. Louis, MO).

Benzalkonium chloride

BAK (B6295) was obtained from Sigma-Aldrich (St. Louis, MO).

In vitro *log reduction assay*

The study design was based on 2 previous studies.[2,18] This assay was conducted in duplicate using $\sim 10^3$ to 10^6 PFU/mL (plaque-forming units per milliliter) of the adenoviral types. In brief, 50 μL of each of the test viruses were added to 450 μL of BAK concentrations of 0.11%, 0.011%, 0.0055%, 0.0033%, 0.0011%, and 0% prepared in sterile water. This produced final BAK concentrations of 0.1%, 0.01%, 0.005%, 0.003%, 0.001%, and 0% (negative control). The virus/BAK mixtures were incubated at 33°C to mimic the temperature of the ocular surface. After 60 min of incubation, 500 μL of tissue culture media was added to the mixtures.

Plaque assay to determine the viral titers after treatment

Immediately following the addition of the tissue culture medium to the tubes, the samples were serially diluted in tissue culture medium containing 10% FBS for five 10-fold dilutions to dilute the BAK and stop the reaction. One hundred microliters of the samples were inoculated onto duplicate A549 cell monolayers in 24-well multiplates. After 3 h of adsorption, 1 mL of tissue culture medium containing 0.5% methylcellulose was added to each well that did not contain the Ad8 samples. Wells with Ad8 samples were filled with normal tissue culture medium. The plates were incubated for 7–10 days at 37°C in 5% CO_2. Following incubation, the media was removed, and the cells were fixed with formalin and stained with 0.5% gentian violet. The plates were dried, and the number of plaques was counted under a dissecting microscope (25×). The viral titers were calculated and presented as PFU/mL.

Data analysis

The viral titers +1 (PFU/mL +1) were converted to Log_{10}, and the Log_{10} decreases in titers compared with the negative control were determined for each trial. The mean ± standard deviation (SD) Log_{10} decrease in titer for each BAK concentration and virus was determined from the data of the 2 trials. The results are displayed as Log_{10} differences in titer compared to the negative control for each virus and BAK concentration. Mean decreases in titer of 3 Log_{10} (99.9%) or more were considered virucidal decreases, mean decreases in titer less than 3 Log_{10} but greater than 1 Log_{10} were considered effective decreases, while decreases in titers of less than 1 Log_{10} were considered ineffective decreases.

Results

Table 1 depicts the decreases in titers of the different Ad types to the different concentrations of BAK. The decreases are presented as the mean ± SD Log_{10} reductions in titer for each BAK concentration and Ad isolate. Concentration-dependent antiviral activity was produced in this study. BAK 0.1% produced mean virucidal decreases in titers for Ad3, Ad5, Ad7a, Ad19/64, and Ad37, while it reduced titers more than 1 Log_{10} but less than 3 Log_{10} for Ad4 and Ad8. However, the decreases in titers produced by BAK 0.1% for the Ad8 isolates were 1–4 Log_{10} less than the other Ad types tested.

Greater than 1 Log_{10} mean decreases in titers were produced for Ad5 by BAK 0.003%, 0.005%, and 0.01%. Mean decreases in titers for Ad types other than Ad5 were ≤0.53 Log_{10} for those concentrations, which by our definition were considered ineffective. BAK 0.001% produced minimal to no decreases in titers for all Ad types.

Discussion

Based on the results of the current study, it appears unlikely that BAK, at concentrations used to preserve common ophthalmic medications, would be effective as an antiviral

TABLE 1. THE DECREASES IN ADENOVIRUS TITERS COMPARED TO THE NEGATIVE CONTROL OF THE DIFFERENT ADENOVIRUS TYPES TO THE DIFFERENT CONCENTRATIONS OF BENZALKONIUM CHLORIDE

BAK concentration, %	*Ad3*	*Ad4*	*Ad5*	*Ad5*	*Ad7a*	*Ad8*	*Ad8*	*Ad19/64*	*Ad37*
			Isolate MC	*Isolate MA*		*Isolate ED*	*Isolate CR*		
0.1	**−5.02 ± 1.57 (V)**	**−2.94 ± 0.80 (E)**	**−5.27 ± 0.73 (V)**	**−3.65 ± 1.04 (V)**	**−3.71 ± 1.87 (V)**	**−1.80 ± 1.12 (E)**	**−1.01 ± 0.50 (E)**	**−3.66 ± 1.46 (V)**	**−4.23 ± 0.21 (V)**
0.01[a]	−0.20 ± 0.11 (I)	−0.53 ± 0.00 (I)	**−2.66 ± 0.49 (E)**	**−1.59 ± 0.02 (E)**	−0.36 ± 0.13 (I)	−0.06 ± 0.13 (I)	−0.45 ± 0.41 (I)	−0.06 ± 0.13 (I)	−0.24 ± 0.04 (I)
0.005	−0.09 ± 0.06 (I)	−0.17 ± 0.18 (I)	**−2.31 ± 1.42 (E)**	**−1.20 ± 0.31 (E)**	−0.17 ± 0.30 (I)	−0.02 ± 0.05 (I)	−0.51 ± 0.36 (I)	+0.12 ± 0.26 (I)	−0.23 ± 0.02 (I)
0.003	+0.06 ± 0.09 (I)	−0.15 ± 0.23 (I)	**−1.41 ± 0.46 (E)**	**−1.09 ± 0.01 (E)**	−0.08 ± 0.04 (I)	−0.10 ± 0.10 (I)	−0.45 ± 0.45 (I)	+0.16 ± 0.06 (I)	+0.04 ± 0.09 (I)
0.001	+0.10 ± 0.36 (I)	+0.12 ± 0.04 (I)	−0.17 ± 0.33 (I)	−0.08 ± 0.08 (I)	+0.03 ± 0.04 (I)	+0.08 ± 0.24 (I)	−0.35 ± 0.01 (I)	+0.06 ± 0.05 (I)	+0.07 ± 0.11 (I)

Mean decreases in Ad titers of 3 Log_{10} (99.9%) or more were considered virucidal decreases (V), decreases in Ad titers of less than 3 Log_{10}, but greater than 1 Log_{10} were considered effective decreases (E), while mean decreases in Ad titers of less than 1 Log_{10} were considered ineffective decreases (I). Values that are in bold are considered virucidal or effective decreases in Ad titers.

[a]0.01% is the highest concentration of BAK found in ophthalmic medications.

Ad, adenovirus; BAK, benzalkonium chloride.

in patients with adenoviral conjunctivitis. The highest concentration of BAK used in ophthalmic medications, 0.01%, demonstrated minimal antiviral activity against Ad types other than Ad5 at 1 h of incubation, which is much longer than the residence time of most ophthalmic medications on the ocular surface. Furthermore, it appears that the BAK could be ineffective for reducing the amount of adenovirus in multidose bottles of medications if the solutions became contaminated. We previously demonstrated that adenovirus could survive for several weeks in multidose bottles of fluorescein.[19] These results do not suggest that BAK is an ineffective preservative. The United States Pharmacopeia (USP) Antimicrobial Effectiveness Testing evaluates the preservative's efficacy against 3 bacterial species (*Staphylococcus aureus, Pseudomonas aeruginosa*, and *Escherichia coli*) and 2 fungal species (*Candida albicans and Aspergillus niger*).[20] Efficacy testing of the preservatives against adenovirus is not included in these tests.

In contrast, BAK at 0.1% demonstrated virucidal antiviral activity against a number of Ad types. However, the *in vitro* antiviral activity demonstrated against Ad8, the most commonly occurring adenovirus type seen in human conjunctivitis cases, was not virucidal. The decreases in viral titers with the Ad8 isolates were 1–4 Log_{10} less than the other Ad types tested but were still considered effective by our stated criteria. Therefore, hand sanitizers containing at least 0.1% BAK may be effective in reducing adenovirus from the hands of adenovirus patients. It is very important to sanitize the hands of these patients to prevent transmission of the virus to uninfected patients. Our laboratory has shown that 46% of patients with adenoviral conjunctivitis had infectious adenovirus on their hands.[21] It may be prudent to have all patients sanitize their hands when entering an ophthalmology practice.

The current study demonstrated differences in the efficacy of BAK against the Ad types tested. BAK was deemed effective at 0.01%, 0.005%, and 0.003% against Ad5 and ineffective against the other Ad types tested. As described above, BAK was not as active against Ad8 at 0.1% as the other types tested. The original study design included only 1 Ad5 isolate (Ad5 isolate MC) and 1 Ad8 isolate (Ad8 isolate ED). After these results were produced, a second isolate each of Ad5 (Ad5 isolate MA) and Ad8 (Ad8 isolate CR) were added to the study to determine whether these effects were isolate dependent. The second isolates confirmed the activity of BAK against Ad5 and Ad8 that was demonstrated with the first isolates. We concluded that BAK produced type-dependent activity against adenovirus. This study supports a previous finding, in which our group demonstrated that 50 ppm (50 μg/mL) of polyhexamethylene biguanide was effective against multiple adenovirus types, but was ineffective against Ad8.[18]

Further *in vitro* studies with BAK 0.1% evaluating shorter times of incubation and concentrations between 0.01% and 0.1% would build upon this preliminary study to determine whether BAK has the potential as an antiviral against adenovirus. This is contingent upon ocular toxicity evaluation. Ocular toxicity is commonly attributed to BAK contained in ocular medications.[22–26]

In conclusion, BAK, at concentrations used in common ophthalmic medications, was not consistently effective as an agent against adenovirus, but higher concentrations could be further investigated as a topical treatment for adenoviral ocular infections, as long as ocular toxicity is not an issue.

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Author Disclosure Statement

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