Ocular inflammation and corneal alteration by benzalkonium chlorids in rabbits: an unexpected protective effect of a myosin light chain kinase inhibitor on tarsal glands

Olivier Roche(1), MD, PhD; Eric Belot(2), MD

(1) Service d’ophtalmologie, Hôpital Necker Enfants Malades, APHP, Faculté Paris Descartes, Paris, France
(2) Université Paris Descartes - Sorbonne Paris Cité, Paris, France

Correspondence: Olivier Roche,
Université Paris Descartes - Sorbonne
Paris Cité, Paris, France;
oph.roche@free.fr.
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Abstract

The objectives of this study were to create a dry eye model in New Zealand White rabbits using a daily topical application of benzalkonium chloride (BAC) and then to evaluate the efficacy of 0.1% ML7 in the dry eye model.

The administration of BAC to rabbits two or four times daily for nine weeks did not produce a dry eye model based on tear production measurements and ophthalmic examination, although there were local changes related to irritation and inflammation. Subsequent ocular instillation of 0.1% ML7 three times per day for ten days resulted in an unexpected absence of inflammation or dilatation of the tarsal gland of the eyelid in the ML7 group compared to the controls. For the other tissues, subsequent ocular instillation of 0.1% ML7 three times per day for ten days resulted in no obvious clinical or microscopic differences from the placebo or PBS controls.

Introduction
Dry eye is an ocular surface disorder with a complex interplay of aggressive agents.\(^1\) The anterior segment of the eye, the corneal and conjunctival epithelia, protects the eye against external aggressors. The ocular surface is a transitional mucosa between the deep ocular medium and the external environment. This epithelium acts as a barrier between fluid loss and penetration of pathogens. It also protects the eye from abrasions\(^2\).

To be effective the epithelium’s cells must adhere tightly to each other and to subjacent cellular components. Due to the position of the epithelium on the external surface of the eye, the response of the epithelium to any aggressor is immediate and effective.\(^2\)

This ocular epithelium is the only site of exchange between the external medium of the eye and its internal medium. Water and electrolyte transport of small molecules use a transcellular route. Large molecules are absorbed and antigens and toxins pass through the paracellular route at the level of tight junctions (TJs) located between epithelial cells\(^3,4,5,6,7\). These TJs form a paracellular seal between the lateral membranes of adjacent cells. They are composed of at least three families of transmembrane proteins (occludins, claudins and adhesion proteins) and a cytoplasmic plaque consisting of multiple proteins that form large complexes. The transmembrane protein mediates cell adhesion and constitutes the intermembrane and paracellular diffusion barrier.\(^8\) The cytoplasmic plaque of TJs is formed of multiple protein types including adaptors, such as zonula occludens (ZO) proteins and proteins that contain PDZ domains, as well as regulatory and signaling components.\(^8\) A high density of cytoskeletal actin and myosin filaments surrounds the corneal epithelial cells near the apical region of the cellular borders at the level of the TJs.\(^7\) The disruption of the perijunctional actin-myosin filaments allows for increased penetrability in the epithelium. Myosin light chain (MLC) contraction is regulated by the opposite actions of MLC phosphatase and MLC kinase (MLCK). MLC phosphorylation by MLCK triggers a contraction of the cytoskeleton (actin-myosin filaments) and subsequently an opening of intercellular TJs. This increases paracellular permeability and allows the entry of allergens and toxins.\(^8,9\)

Corneal and conjunctival epithelia are constantly exposed to aggressors known to alter this protective barrier. Different factors such as temperature, humidity, ultraviolet irradiation, bacteria, virus, fungi, allergens, use of contact lens, photorefractive surgery or preservatives can be responsible for corneal epithelial cell disruption. This is linked to some alterations of corneal paracellular permeability. Some factors can also be determined genetically, such as the Gougerot-Sjögren syndrome.\(^10,11\)
The permeability of the ocular surface epithelium can also be altered by preservatives that are present in eye drops or antiseptic substances, such as quaternary ammonium salts. Benzalkonium chloride (BAK), a component of all multi-dose eye drop formulas, is known to induce lysis of cell membranes at the ocular surface, even at very low doses. \textsuperscript{12,13,14,15,16,17}

Alterations of conjunctival and corneal permeability can occur during the healing phases following a trauma to the ocular surface. Consequently, alterations of the anterior eye segment paracellular permeability result in acute or chronic dehydration of the ocular surface.\textsuperscript{18}

This alteration of the epithelial TJs, due to the entry of microorganisms, allergens or a chemical molecule responsible for allergic and inflammatory symptoms, can lead to sensitization. This is often accompanied by pain leading to a chronic pathology.\textsuperscript{19}

In a previous study conducted by the Neuroptis team (O Roche et al, IOVS) we have demonstrated that the ML7 is able to counteract the corneal cytoskeleton contraction. ML7 will be an important candidate to prevent the resulting ocular inflammation.

To this end, we have decided to conduct a new study on a rabbit model of ocular inflammation with chloride benzalkonium.

**Materials and methods**

This study was not conducted in compliance with GLP regulations; however, it was conducted using good scientific practices and following the applicable SOPs of the test facility and applicable test sites.

**Chemicals**

ML7 (1-(5-iodonaphtalene-1-sulphonyl)–1H-hexahydro-1, 4-diazepine), an MLCK inhibitor, was obtained from Provence Technology (Marseille, France). The eyewash with ML7 and the placebo for the present study were manufactured by Octalia Technologies (Nice, France). The Phosphate Buffer Saline was sourced from PCS-MTL (Montréal, Canada) (Table 1).

<table>
<thead>
<tr>
<th>Identification</th>
<th>Test Item</th>
<th>Reference Items</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification</td>
<td>ML7</td>
<td>ML7 Placebo</td>
</tr>
<tr>
<td>Batch (Lot) No.</td>
<td>E821</td>
<td>E822</td>
</tr>
</tbody>
</table>

*Phosphate Buffered Saline (PBS)*
Table 1

Animals and procedures of benzalkonium chloride and ML-7 administration

The test system consisted of 20 male naive New Zealand White rabbits, ranging from 2 to 3 Kg and from 4 to 6 months of age at the start of model generation. Source: Covance Research Products Inc. Denver, USA.

The study design was as follows:

Experimental Design

<table>
<thead>
<tr>
<th>Day</th>
<th>Test Item</th>
<th>Concentration</th>
<th>No. of Applications/Day</th>
<th>Animal No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 36</td>
<td>BAC</td>
<td>0.1%</td>
<td>2</td>
<td>All</td>
</tr>
<tr>
<td>37 to 48</td>
<td>BAC</td>
<td>0.1%</td>
<td>4</td>
<td>All</td>
</tr>
<tr>
<td>49 to 60</td>
<td>BAC</td>
<td>0.15%</td>
<td>4</td>
<td>All</td>
</tr>
</tbody>
</table>

Sourced by PCS-MTL. Details were documented in the data.
Model Generation Phase

All study animals were treated with benzalconium chloride (BAC) by topical ocular instillation during the model generation phase. Weekly measurements of tear production were taken during the model generation phase in order to determine when the model was sufficiently induced.

For the first five weeks, 0.1% BAC was administered twice daily (generally separated by at least six hours) by ocular instillation (50 µL drop size) to both eyes. As measurements showed an increase in tear production the model generation phase was prolonged. BAC administration was increased to four-times daily from days 37 to 48. The concentration of BAC was subsequently increased to 0.15% and administered four times daily from days 49 to 60. Animals were administered buprenorphine SR (0.1 mg/kg) twice weekly, from two weeks after the first BAC administration and throughout the remainder of the study to minimize pain and discomfort due to irritation of the eyelids.

During the model generation phase, gross ocular examinations were performed weekly. Ophthalmic examinations were performed prior to the start of BAC administration and again on days 34 and 48.

Dose Administration Phase

<table>
<thead>
<tr>
<th>Dose Administration Phase</th>
<th>PBS</th>
<th>ML7 Placebo</th>
<th>ML7 0.1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>61 to 70</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3001-3006</td>
</tr>
</tbody>
</table>

Table 2

BAC = benzalconium chloride
The test item (ML7) and the placebo were used as recommended. Vials were transferred to the dosing area on wet ice. PBS was dispensed as daily aliquots. These were handled in the same manner as the test item and placebo.

The test and reference items (placebo and PBS) were administered to the appropriate animals three times daily by ocular instillation (equally spaced during a normal working day) from days 61 to 70. The dose volume was 50 µL/eye/dose. Dose formulations were removed from storage 20-30 minutes prior to dosing to bring them to room temperature. They were kept refrigerated or on wet ice between doses. One spare animal remained on study but was not dosed.

During the dose administration phase, gross ocular examinations were performed on days 61, 63, 65, 67 and 70, prior to the second daily dose. Tear production measurements were performed before the dosing started (on day 61) and again on days 65 and 70. Ophthalmic examinations were performed on the third day of dose administration (day 63) and again prior to necropsy. Images of the eyes and eyelids of all animals were captured when possible.

**Terminal Procedures and Histopathology**

Due to clinical signs observed during the model generation period (skin lesions in the dorsal region), which were unrelated to BAC administration, one animal was euthanized and replaced by a spare. No necropsy or tissues were collected for this animal.

Animals surviving to study termination, including the spare animal, were euthanized on day 71 by intravenous injection of sodium pentobarbital, followed by exsanguination by incision of the axillary or femoral arteries. All study animals were subjected to a limited necropsy examination, which consisted of an evaluation of the retained tissues. Representative samples of the conjunctiva, eyelids, hard erian and lacrimal glands, extraocular muscles and nictitating membrane were collected from all animals and preserved in 10% neutral buffered formalin. The eyes and optic nerves were fixed in Davidson’s fixative for 24 to 48 hours then transferred to and stored in 70% ethanol for at least 18 hours before processing.

Five sagittal sections of each eye were prepared as follows: one section was taken at a target of 150 µm after the first exposure of tissue; one section at a target of 500 µm medially to the first one; two sections in the optic nerve separated by 100 to 300 µm;
one section at least 500 μm medially of the optic nerve. All tissues were then embedded in paraffin, sectioned, mounted on glass slides and stained with hematoxylin and eosin.

A board-certified veterinary pathologist performed the histopathological evaluation.

**Retention of Records, Samples and Specimens**

All study-specific raw data, electronic data, documentation, study plan, retained samples and specimens and final reports from this study will be transferred to a Charles River archive no later than the date of the final report issue. One year after issue of the draft report, the sponsor will be contacted to determine how to dispose of materials associated with the study.

**Results**

**Model generation phase**

Administration of 0.1% BAC resulted in clinical signs of periorbital irritation (ocular redness, clear or white discharge, swelling of the eyelids and partly or completely closed eyes); starting just a few days after the commencement of treatment and persisting for the remainder of the model generation phase. However, tear production was not reduced as anticipated; the overall average was increased by 30% at the end of five weeks (day 34 and 35) of BAC administration. Increasing the frequency of administration to four times a day for two weeks and increasing the concentration to 0.15% did not result in reduced tear production when compared to the baseline values.
## Schirmer Tear Test Results (Table 3)

<table>
<thead>
<tr>
<th>Mod e l Generation Phase</th>
<th>Test Item</th>
<th>Concentration</th>
<th>No. of Applications / Day</th>
<th>Mean Tear Production Measurement* (mm)</th>
<th>Standard Deviation</th>
<th>Change from Baseline (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (Pre-study)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.7</td>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>Week 5 (Day 34)</td>
<td>BAC 0.1%</td>
<td>2</td>
<td>11.2</td>
<td>1.7</td>
<td>+2.5</td>
<td></td>
</tr>
<tr>
<td>Week 5 (Day 35)</td>
<td>BAC 0.1%</td>
<td>4</td>
<td>11.1</td>
<td>2.0</td>
<td>+2.4</td>
<td></td>
</tr>
<tr>
<td>Week 6 (Day 42)</td>
<td>BAC 0.1%</td>
<td>4</td>
<td>10.9</td>
<td>2.0</td>
<td>+2.2</td>
<td></td>
</tr>
<tr>
<td>Week 7 (Day 48)</td>
<td>BAC 0.15%</td>
<td>4</td>
<td>10.3</td>
<td>3.5</td>
<td>+1.6</td>
<td></td>
</tr>
<tr>
<td>Week 8 (Day 55)</td>
<td>BAC 0.15%</td>
<td>4</td>
<td>11.9</td>
<td>3.5</td>
<td>+3.2</td>
<td></td>
</tr>
<tr>
<td>Day 61 (prior to dose)</td>
<td>PBS 0</td>
<td>-</td>
<td>11.9</td>
<td>3.1</td>
<td>+3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ML7 0%</td>
<td>-</td>
<td>10.8</td>
<td>2.3</td>
<td>+2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ML7 0.1%</td>
<td>-</td>
<td>8.8</td>
<td>1.8</td>
<td>+0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBS 0</td>
<td>3</td>
<td>7.4</td>
<td>2.0</td>
<td>-3.8</td>
<td></td>
</tr>
</tbody>
</table>

* Change from baseline is calculated as: (Mean Tear Production Measurement - Baseline Tear Production Measurement) / Baseline Tear Production Measurement.
Similarly, ophthalmic examination indicated extra-ocular irritation related to BAC administration. Findings consisted of ocular discharge, conjunctival and eyelid hyperemia, eyelid swelling, corneal opacities and corneal vascularization. The fundus view was limited in many animals due to the discomfort. On days 34 and 48, multiple eyes had faint positive fluorescein staining, most likely associated with thinning of the corneal epithelium, but none of the eyes had a well-defined corneal ulcer. Overall, ophthalmic findings were similar to those usually found with dry eye, but were secondary to the chronic irritation rather than low tear production (corneal vascularization, conjunctival hyperemia and faint positive fluorescein staining).

Total Incidence of Gross Ocular Examination Findings

<table>
<thead>
<tr>
<th>Group</th>
<th>Grade</th>
<th>Model Generation</th>
<th>Dose Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Study Day</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Redness</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td></td>
<td>Moderate</td>
</tr>
<tr>
<td>ML7</td>
<td></td>
<td></td>
<td>Very Slight/ Slight</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td>Moderate</td>
</tr>
</tbody>
</table>

a6 animals/Group/occasion.
Following the discontinuation of BAC and the start of dosing, the ocular findings related to irritation quickly resolved in all groups. Although the eyes generally remained partially closed, the redness and swelling of the eyelids, as well as ocular discharge, diminished or resolved during the ten-day dosing period. The fluorescein staining, observed in most eyes during model generation, was present in only one or two animals three days after the start of dosing and in none prior to necropsy. However, overall there was no clinical difference between the control or ML7 groups.

Macroscopic and microscopic findings were generally similar across treatment groups and were considered related to the administration of benzalkonium chloride; these included corneal neovascularisation, mixed cell infiltrates at the limbus of the eye and variable in the cornea and inflammation of the conjunctiva of the eyelids (upper, lower and/or nictitating membrane). Inflammation or dilatation of the tarsal gland of the

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Table 4

| BAC only administered between days 1 and 60; ML7, placebo or PBS administered from days 61 to 70. | 12 eyes/group/occasion. |
eyelid was noted in a few animals in group 1 and 2 (PBS and ML7 placebo), but not in group 3 (ML7 0.1%), indicating a possible anti-inflammatory effect of ML7.

Discussion

Dry eye syndrome is a chronic lack of sufficient lubrication and moisture to the ocular surface linked to inflammations affecting the cornea and conjunctiva.

Among the aggressors responsible for dry eyes, such as environmental factors; exposure of the ocular surface to preservatives (antiseptic substances) provokes significant disorders. The most well known preservative salts on the market are the quaternary ammonium salts, such as BAK, which is an ingredient of multi-dose eye drops approved for the treatment of glaucoma. By inducing free radical release and apoptosis of ocular cells, these preservatives reach the corneal epithelium and stimulate the infiltration of inflammatory cells into the conjunctiva. Severe damage to the ocular surface, such as ulcerations, large epithelial defects and neovascularisations can occur. The administration of BAK induces changes similar to dry eye syndrome in humans, accompanied by a decrease in the amount of tears, an increase in corneal fluorescein and the rose Bengal score.

BAK can also affect cell membrane permeability, causing lysis of cell contents and allowing vital substances to escape.

It is now well demonstrated that BAK accelerates the desquamation of corneal epithelium cells with a concomitant depletion of intracellular ATP. Among the varied effects of ATP depletion, phosphorylation of the regulatory light chain of myosin II (MLC) has been reported; it has also been clearly demonstrated that the exposure of corneal epithelial cells to BAK leads to MLC phosphorylation. This contracts the cytoskeleton of epithelial cells, breaking down the corneal barrier integrity. Similar effects are noted in the presence of histamine. This barrier loss contributes to the propagation and exacerbation of the inflammation.

Aggressors, such as BAK, can cause a decrease in the expression of the zonula occludens protein (ZO-1), a key compound of TJs, or alter the organization of the actin cytoskeleton in the apical region of the cell.
In our previous study (Roche et al), we found that the administration of BAK in a rat model will reproduce the symptoms of dry eye observed in humans. This study performed on a rat model of BAK demonstrated that 0.1% BAK administration causes side effects on the corneal membrane through MLC phosphorylation. We concluded that the administration of ML-7 is able to prevent the deleterious effects of BAK.

In this new study, on a rabbit model, we have observed that BAK administration induces a very serious inflammation without decreasing tear production. The deleterious effects observed in our previous study (Roche et al) were not found in the rabbit model.

Following the necropsy analysis, we found subsequent administration of ML7 resulted in an unexpected absence of inflammation or dilatation of the tarsal gland of the eyelid in the ML7 group compared to the controls. This is the first time this effect was observed and no data on these kinds of results were reported in the literature yet.

The tarsal glands (or meibomian glands) are a special kind of sebaceous gland at the rim of the eyelids inside the tarsal plate, responsible for the supply of meibum, an oily substance that prevents evaporation of the eye's tear film.

A recent study conducted by Mudgil P et al (Invest Ophthalmol Vis Sci. 2014 Oct 14;55(11):7272-7), concluded that these lipids, produced by the tarsal glands, possess antimicrobial properties against both Gram-positive and Gram-negative bacteria and are involved in the innate host defense of tears in protecting the ocular surface against microbial pathogens. The alteration of the tarsal glands integrity and functionality is one of the most important factors in ocular inflammation. The protective effect of ML-7 on tarsal glands opens up a large number of possible applications in dry eye disease and beyond.

Our study confirmed that the ML-7 has some positive effects on ocular inflammation and appears to be a candidate for the treatment of other ocular disorders linked to inflammation.
Conclusion

Administration of benzalkonium chloride to New Zealand White rabbits two or four times daily for nine weeks did not successfully result in the generation of a dry eye model, based on tear production measurements and ophthalmic examination, although changes related to irritation and inflammation were present. Subsequent administration of ML7 resulted in an unexpected absence of inflammation or dilatation of the tarsal gland of the eyelid in the ML7 group compared to the controls. For the other tissues, subsequent ocular instillation of 0.1% ML7 three times per day for ten days resulted in no obvious clinical or microscopic differences from the placebo or PBS controls. The alteration of the tarsal glands integrity and functionality is one of the most important factors in ocular inflammation. The protective effect of ML-7 on tarsal glands opens up a large number of possible applications in dry eye disease and beyond.
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