Ocular Inflammation and Corneal Permeability Alteration by Benzalkonium Chloride in Rats: A Protective Effect of a Myosin light chain kinase Inhibitor.

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Abstract

Purpose

The aim of this study was to evaluate the interest of an ophthalmic eye-drop preparation containing a myosin light chain kinase (MLCK) inhibitor, ML-7, in the treatment of ocular surface. The local protective effect on the inflammation and the increase of corneal permeability induced by benzalkonium (BAK) was evaluated.

Methods

An ocular instillation of 10 μl of BAK at a concentration of 0.1% in phosphate buffered saline (PBS) was performed on rats. The eyes were rinsed with sterilized water, 10 mins after BAK preceded by instillation at T -24, - 12 and - 0.5 hours of 10 μl of ML-7: 100 μg (10 μl) into a gel form vehicle. All animals were sacrificed 6 hours after BAK instillation. The eyes were isolated for study in masked manner. The assessment of ocular surface inflammation was done by measuring the inflammatory cell infiltration by a histological quantitative analysis and for total ocular myeloperoxidase (MPO) activity. The tight junction permeability was tested.

Results

Instillation of 0.1% BAK increased the inflammation of the eye. The quantitative analysis showed an increase in the number of eosinophil and neutrophil polynuclears and MPO activity. Pretreatment with ML-7 reduced inflammation (P<0.05). The vehicle alone produced no notable effects. BAK instillation also thickened the fluorescent corneal front on frozen sections indicating an increase of tight junction permeability. Pretreatment with ML-7 suppressed BAK-induced alterations of paracellular permeability while the vehicle had no visible effects.

Conclusions

This study indicates that the inhibition of corneal cytoskeleton contraction by a MLCK inhibitor prevents BAK-induced ocular inflammatory response and that ML-7 may be a new and original preparation in the treatment of ocular surface pathologies.
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 the conjunctival epithelia, protects the eye against external aggressors; the ocular surface being a transitional mucosa between the deep ocular medium and the external environment. In fact, this epithelium is a competitive barrier between fluid loss and penetration of pathogens. It also protects the eye from abrasions (2).

In order to be effective, the cells constituting this epithelium must adhere tightly to each other and must also adhere to subjacent cellular components. Considering the vulnerable position of the epithelium at 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 the internal medium. Water and electrolyte transport of small molecules use a transcellular route. The absorption of large molecules and the passage of antigens and toxins occur through the paracellular route at the level of tight junctions located between epithelial cells (3-7). These tight junctions (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 many different proteins which form large complexes. The transmembrane protein mediates cell adhesion and constitutes the inter-membrane and paracellular diffusion barrier (8). The cytoplasmic plaque of Tjs is formed by different types of proteins that include adaptors such as the zonula occludens (ZO) proteins and the proteins which contain PDZ domains as well as regulatory and signaling components (8). There is a high density of cytoskeletal actin and myosin filaments, which surround 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 an increase in the epithelium penetrability. Myosin light chain contraction is regulated by the opposite actions of myosin light-chain phosphatase and myosin light-chain kinase. Myosin light chain (MLC) phosphorylation by MLC kinase (MLCK) triggers a contraction of the cytoskeleton (actinomyosin filaments) and subsequently an opening of intercellular tight junctions, giving rise to an increase of paracellular permeability favoring the entry of allergens and toxins (8, 9).

Corneal and conjunctival epithelia are always exposed to many aggressors, known to alter this competitive barrier. Different factors, such as temperature, humidity, ultraviolet irradiation, bacteria, virus, fungi, allergens, contact lens wear, photo-refractive surgery or preservatives can be responsible for corneal epithelial cell disruption linked to some alterations of corneal paracellular
permeability. Some factors can also be genetically determined, such as the Gougerot-Sjögren syndrome (10, 11).

Furthermore, the permeability of the ocular surface epithelium can be altered by preservatives which are present in eye drops or antiseptic substances, such as quaternary ammonium salts. Benzalkonium chloride (BAK), an component of all multi-dose eye drop formulae such as those used in the treatment of glaucoma, is known to induce the lysis of cell membranes at the ocular surface, even at very low doses (12 - 17).

In addition, alterations of both conjunctival and corneal permeability can occur after a trauma to the ocular surface, during the healing phases. Consequently, alterations of the anterior eye segment paracellular permeability result in acute or chronic dehydration of the ocular surface (18).

This alteration of the epithelial tight junctions can also lead to sensitization due to the entry of microorganisms, allergens or a chemical molecule, responsible for allergic and inflammatory phenomena often accompanied by pain leading to a chronic pathology (19).

Therefore the purpose of this study is to determine the protective effect of a selective MLCK inhibitor, ML-7 (20 - 22), on the inflammation and the increase of corneal permeability induced by BAK and its implication on the regulation of the paracellular permeability linked to MLCK activation which provokes epithelial tight junction opening. We specifically looked for the direct effect of this MLCK inhibitor on the corneal barrier function.

**Methods**

**Chemicals**

ML-7, a MLCK inhibitor, was obtained from (Sigma Aldrich Chimie, L’Isle D’Abeau Chesnes, France). ML-7 is a 1-(5-iodonaphtalene-1-sulphonyl) – 1H-hexahydro-1, 4-diazepine.

**Animals and procedures of Benzalkonium chloride and ML-7 administration.**

Four groups of male Wistar rats (Janvier, Le Genest St Isle, France), weighing between 300 and 350g, were used, BAK + Sodium carmellose, BAK + ML-7, PBS + Sodium carmellose, PBS + ML-7; PBS and Sodium carmellose being the solvents for Benzalkonium (BAK) and ML-7, respectively.
The rats were housed in polycarbonate cages with lights (12/12 hour cycle) set at a temperature of 20-22°C. The rats were fed with Standard pellets (Safe 003 Epinay sur Orge, France). All the procedures were performed in accordance with the relevant recommendations for animal care from ARVO.

The animals received a local application of ML-7 (Sigma, France), 24 hours, 12 hours and 30 minutes, before chemical induction of ocular inflammation. Thus each eye was treated with 100 μg of ML-7 in 10 μl of eye drop solution (sodium carmelloose 4 mg / 0.4 ml) or with 10 μl of unmodified eye drop solution.

**Inflammation induction**

Thirty minutes after the third application of ML-7 or of the unmodified eye drop solution, each eye was treated with 10 μl of 0.1 % benzalkonium chloride (Sigma-Aldrich, Steinheim, Germany) in PBS or 10 μl of unmodified PBS. After 10 minutes, the eyes of all the rats were rinsed with 250 μl of sterile water.

**Eye excision**

Six hours after the application of benzalkonium chloride (BAK) or PBS, the animals were anesthetized with pentobarbital (80 mg/kg/ip) (CEVA, Libourne, France), sacrificed by decapitation. The eyes were then enucleated and frozen immediately or after surface biotinylation (tight junction permeability Test). All observations were performed in a masked manner.

**Measurement of polynuclear eosinophil infiltration**

The polynuclear eosinophil leukocytes were stained with Direct Red and counted in the venous plexus region of the sclera. Immediately after excision, the eyes were embedded in a protective tissue freezing medium (Tissue Tek® OCT compound, Sakura Finetek, Inc., CA, USA), frozen in liquid nitrogen and stored at -80°C. 6μm thick slices were prepared with a cryostat and fixed in cold acetone for 10 minutes. After being dried, the slices were rehydrated by successive baths in toluene (5, 3 and 2 minutes), then in a 100 % ethanol solution (3 and 2 minutes), a 95 % ethanol solution (3 and 2 minutes) and a 50 % ethanol solution (2minutes). The sections were then bathed for 20 minutes in a staining solution of 0.03% Sirius red in 50 % ethanol (Direct Red 75 dye content 30% Sigma-Aldrich, Steinheim, Germany), rinsed with running water for 5 minutes and mounted in an aqueous medium (glycerol/PBS, 50/50 V/V).
The eosinophils, bright pink stained on an illuminated background, were counted in the venous plexus region of the sclera under a Nikon Eclipse 90 I microscope equipped with a Nikon DXM1200F digital camera. The area of the zone to be counted was determined with Nikon Lucia image analysis software (release 4.8) and counts were expressed as the number of eosinophils per mm².

The results obtained for the four experimental groups were compared using a one-way analysis of variance, followed by a Bonferroni multiple comparison test with statistical significance set at p<0.05.

**Measurement of polynuclear neutrophil infiltration**

Neutrophil polynuclear cells were specifically labeled by immunochemistry using an anti-myeloperoxidase (MPO) monoclonal antibody as primary antibody, a horseradish (HRP) - conjugated secondary antibody and a HRP-diamino benzidine (DAB) reaction as staining step.

The cold acetone-fixed sagital frozen sections (6 μm thick) were first incubated with hydrogen peroxide (0.6 % in methanol) during 30 minutes, to inhibit endogenous peroxidases. Non specific linking sites were saturated by a solution of normal goat serum (2% in PBS-Tween- 1% BSA) during 10 minutes. Sections were then incubated with primary anti-MPO antibody (IgG1 Mouse Monoclonal [8F4] to MPO, Abcam plc, Cambridge, UK), 2000-fold diluted in Tween-PBS-1% BSA, overnight, 4 °C).

After having rinsed with Tween-PBS, incubation with secondary antibody [Stabilized Goat Anti-Mouse HRP-conjugated, Pierce, Rockford, IL, USA] (2000 fold diluted in Tween-PBS-1% BSA) was performed for one hour at room temperature.

Sections were then incubated with a HRP-chromogen substrate solution [3,3’- Diaminobenzidine Tetrahydrochloride (DAB) kit, MP Biomedicals, Aurora, Ohio, USA ] for 5 minutes at room temperature.

Sections were counterstained with Mayer’s hematoxylin (20 seconds), dehydrated and mounted in Depex medium.

Counting was done using a Nikon DXM 1200F digital camera as with eosinophils.

**Measurement of Tight junction permeability – surface Biotinylation**
The permeability of tight junctions in the cornea was evaluated by biotinylation of surface proteins. The chosen biotinylation reagent was water-soluble and contained an aminocaproyl spacer group, which lowered steric hindrance during avidin coupling. Immediately after excision, the eyes were incubated for 30 minutes at room temperature with gentle stirring in a solution containing sodium biotinamidohexanecarboxylate and 3-sulfo-N-hydroxysuccinimide at 1 mg/ml in PBS (Sigma-Aldrich, Steinheim, Germany). The eyes were then rinsed three times with PBS, embedded in a protective tissue freezing medium (Tissue Tek® OCT compound, Sakura Finetek, Inc., CA, USA), frozen in liquid nitrogen and finally stored at -80°C.

Six μm thick slices were prepared with a cryostat and fixed in cold acetone for 10 minutes. After being dried out, the slices were labeled for 30 minutes in the dark with avidin D-FITC (Vector Laboratories, Inc., Burlingame, CA, USA) 250-fold diluted in PBS-Tween containing 1% BSA, then rinsed three times for 5 minutes with PBS-Tween, in the dark. The slices were then mounted in a fluorescent medium (Cappel fluorostab embedding medium, MP Bomedicals, Inc. Aurora, OHIO, USA) and examined under a Nikon Eclipse 90 I fluorescence microscope equipped with a Nikon DXM1200F digital camera. The images were analyzed with the Nikon Lucia image analysis software (release 4.8). As no significant differences in corneal thickness were observed between the different groups (102 ± 10 μm, 110 ± 9 μm, 115 ± 13 μm and 124 ± 8 μm for BAK+ sodium carmellose, BAK +ML-7, PBS +sodium carmellose and PBS+ML-7 groups respectively), the depth of fluorescence labeling reflected the permeability of external corneal epithelial tight junctions to the biotinylation reagent.

**Measurement of Myeloperoxidase activity**

The activity of myeloperoxidase (MPO), which is found in polymorphonuclear neutrophil granules, was assessed according to Bradley’s method (23). Samples of the eyes were suspended in a potassium phosphate buffer (50mM, pH6.0) and homogenized in ice. Three cycles of freeze-thaw were undertaken. Suspensions were then centrifuged at 10,000g for 15 min at 4°C. Supernatants were discarded and pellets were resuspended in hexadecyl trimethylammonium bromide buffer (HTAB 0.5% w/v, in 50 mM potassium phosphate buffer, pH 6.0). These suspensions were sonicated on ice, and centrifuged again at 10,000g for 15 min at 4°C. The supernatants obtained were diluted in potassium phosphate buffer (pH 6;0) containing 0.167 mg ml⁻¹ of O-dianisidine dihydrochloride and 0.0005 % of hydrogen peroxide. Myeloperoxidase from human neutrophils (0.1 units per 100 μl) was used as standard. The kinetic changes in absorbance at 450 nm, every 10s over 2 mins, were recorded with a spectrophotometer. One unit of MPO activity was defined as the quantity of MPO degrading 1 μmol of hydrogen peroxide min⁻¹ ml⁻¹ at 25 °C. Protein concentration
was determined with a commercial kit using a modified method of Lowry (Detergent Compatible Assay, Bio-Rad, Marnes la Coquette, France). MPO activity was expressed as units per gram of protein.

**Statistical analysis**

Data were presented as means +/- standard error of the mean. To compare the groups, we used the Student’s impaired t test and the Bonferroni multiple comparison test. Statistical significance was accepted at P<0.05.

**Results**

**Effect of local application of ML-7 on Polynuclear infiltration induced by corneal instillation of Benzalkonium chloride (BAK).**

The instillation of 10μl of 0.1% BAK in the eye led to a highly significant increase in the number of inflammatory cells as determined by the significant increase of Direct Red stained polynuclear eosinophils in the venous plexus region of the sclera, showing evidence of a severe ocular inflammation (Figure 1).

This polynuclear eosinophil infiltration was significantly inhibited after local application of ML-7 100μg in a carbomer gel. The vehicle (unmodified carbomer gel) produced no notable effects (Figure 1).

Similarly, instillation of 10 μl 0.1% BAK led, after 6 hours, to a very significant increase of MPO activity in the sclerous veinous plexus. The vehicle (unmodified carbomer gel) had no notable effects (Figure 2).

These BAK increased MPO immunoreactivity cells were significantly inhibited by a pretreatment with 100 μg of ML-7 in a carbomer gel. Histological observations confirmed these results. (Figure 1).

**Effect of local application of ML-7 on tight junction permeability alteration induced by corneal instillation of Benzalkonium chloride (BAK).**

On the frozen sections, the permeability study showed that instillation of 10 μl 0.1% BAK induces an alteration of tight junction opening as demonstrated by a thickening of the fluorescent marker at the external of the cornea (Figure 3).
Pretreatment with 100 μg of ML-7 in a carbomer gel protects against this alteration of paracellular permeability induced by BAK instillation (Figure 3A, B).

These observations are confirmed by a quantitative analysis showing that ML-7 in a carbomer gel significantly protects against this alteration of paracellular permeability after 0.1% BAK instillation. The vehicle (carbomer gel) had no notable effects on paracellular permeability (Figure 3 A, B).

**Discussion**

Dry eye syndrome is a chronic lack of sufficient lubrication and moisture of 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 benzalkonium chloride (BAK), which is an ingredient of multidose eye drops approved for the treatment of glaucoma. The said preservatives, by inducing free radical release (24) and apoptosis of ocular cells (24,25), reach the corneal epithelium and stimulate the infiltration of inflammatory cells into the conjunctiva (26,16,27). Severe damages to ocular surface, such as ulcerations, large epithelial defects and neovascularizations can occur (25). The administration of BAK induces changes similar to the dry eye syndrome in humans accompanied with a decrease in the amount of tears, an increase of the corneal fluorescein and the rose Bengal score (25). BAK can also affect cell membrane permeability, causing lysis of cell contents and allowing vital substances to escape (28).

It is now well demonstrated that Benzalkonium chloride (BAK) accelerates the desquamation of corneal epithelium cells with a concomitant depletion of intracellular ATP. Among the varied effects of ATP depletion, phosphorylation of regulatory light chain of myosin II (MLC) has been reported and it has been clearly demonstrated that the exposure of corneal epithelial cells to BAK leads to MLC phosphorylation (29) which contracts the cytoskeleton of epithelial cells thus breaking down the corneal barrier integrity. Similar effects are noted in the presence of histamine (30). This barrier loss contributes to the propagation and the exacerbation of the inflammation (31).

Furthermore, aggressors such as BAK can cause a decrease in the expression of the Zonula Occludens protein (ZO-1), a key compound of tight junctions (32), or alter the organization of the actin cytoskeleton in the apical region of the cell (32).

Our results confirm that 0.1% BAK administration causes side effects on the corneal membrane through MLC phosphorylation. 0.1% BAK significantly provokes eye inflammation. We observe an
increase of MPO immuno-reactivity in the sclera venous plexus. We also note a significant increase in the number of infiltrated eosinophil polymnuclears. Pretreatment of rat eyes with ML-7, an inhibitor, significantly reduces the number of infiltrated neutrophil and eosinophil polymnuclears (P<0.05).

Concomitantly to the corneal inflammation, following 0.1% BAK administration, we observe a significant increase of the paracellular permeability in the corneal epithelium, as demonstrated on frozen sections of rat cornea after biotinylation and amplification by Avidine Fluorescein. This increase of paracellular permeability is prevented by ML-7, which attenuates MLCK activity, suggesting that it limits the entry of allergens and pathogens (9). Consequently to this, ML-7 limits the ocular inflammation response, as seen previously, at the level of the gut colonic epithelium (9).

Furthermore, similar protective effects of ML-7 are described using ethanol as a barrier aggressor. ML-7 treatment attenuates both the ethanol-mediated increase of paracellular permeability and MLCK activity (33).

In conclusion, ML-7, a MLCK inhibitor, by preventing the deleterious effects of Benzalkonium chloride (BAK) preservatives on corneal cytoskeleton and the consecutive inflammation, may be a new and original preparation in the treatment of ocular surface pathologies such as dry eye.

References


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FIGURE 1

Legend Figure 1: Application of 10 μl BAK (0.1 %) in the eye led, after 6 hours, to a significant increase in the number of direct red stained polynuclear eosinophils in the venous plexus of the sclera.

This polynuclear eosinophil infiltration was significantly inhibited by local application of ML-7.

Effect of ML-7 on polynuclear eosinophilic infiltration induced by benzalkonium chloride in the sclera veinous plexus following benzalkonium chloride treatment in rats (mean +/- SEM; n=8).

* : p<0,05; significantly different from “BAK”

+ : p<0,05; significantly different from “Vehicle”
BAK

BAK + ML7

PBS

**Bar Graph:**

- Total number of eosinophils
- Categories: Vehicle, Veh + ML7, BAK, BAK + ML7

* : p<0.05; significantly different from "BAK"
+ : p<0.05; significantly different from "Vehicle"
FIGURE 2

Legend Figure 2: Effect of ML-7 on the number of MPO-immunoreactive cells (neutrophils) accumulated in the sclera venous plexus following benzalkonium chloride treatment in rats (mean±/SEM; n=8).

*: p<0.05; significantly different from “BAK”

+: p<0.05; significantly different from “Vehicle”
FIGURE 3A

Legend Figure 3A : Effect of ML-7 on the increase of permeability to the fluorescent dye induced by BAK.

The application of 10 μl BAK (0.1%) in the eye led, after 6 hours, to an opening of corneal epithelial tight junctions as manifested by deeper penetration of the fluorescence and by its diffusion.

The influence of BAK was inhibited by ML-7, which suppressed this increase in the diffusion and thickening of the fluorescent zone.
FIGURE 3B

Legend Figure 3B: Quantitative analysis showing that ML-7 significantly protects the corneal barrier in rats against the strong alteration of the paracellular permeability after 0.1% BAK instillation. The corneal barrier is evaluated by deeper penetration of the fluorescence. [\( n=6 \) (PBS and PBS ML-7), \( n=11 \) (BAK : ML-7), \( n=20 \) (BAK)]

\* : \( p<0.05 \); significantly different from “BAK”

\+ : \( p<0.05 \); significantly different from “PBS”