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Effects of Cloricromene, a Coumarin Derivative, on Rabbit Dry Eye Model

Claudio Bucolo[1], Angelo Spadaro[2] and Filippo Drago[1]
[1]Department of Experimental and Clinical Pharmacology, School of Medicine, University of Catania, Catania, Italy; [2]Department of Pharmaceutical Sciences, School of Pharmacy, University of Catania, Catania, Italy

Abstract
The aim of the this study was to evaluate the effects of cloricrome, a new coumarin derivative, on dry eye model. Lacrimal gland inflammation-induced dry eye was performed by Concanavalin A (ConA) injection in rabbit, and matrix metalloproteinase-9 (MMP-9), TNFα, IL-1β and IL-8 were evaluated. Tear film integrity was investigated by measuring tear volume and tear breakup time. Elevated levels of MMP-9 and cytokines were detected in the lacrimal gland 72 hours after ConA injection. Cloricromene was effective in this model, restoring tear function and inhibiting MMP-9 and cytokines formation in a dose-dependent manner. This study provides the first evidence that the new coumarin derivative cloricromene attenuates the degree of inflammation and tear film dysfunction in rabbits with experimental dry eye syndrome suggesting that this drug may be useful in clinical practice.
Keywords: coumarin derivative; ocular inflammation; cytokines; matrix metalloproteinase; dry eye

Introduction

Dry eye is a disorder of the tear film due to tear deficiency or excessive evaporation, which causes damage to the interpalpebral ocular surface and is associated with symptoms of ocular discomfort [1]. Dry eye is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface. Epidemiological studies have estimated that it affects between 11% and 17% of the general population, with an increasing prevalence in elderly people [2,3]. This incidence significantly decreases the quality of life and it was found that, among people asking for ophthalmological referral, 29% had a dry eye [4,5]. Dry eye occurs when tears production is insufficient to moisturize and lubricate the ocular surface [6,7]. Dry eye is due to straining of the eyes, reduced blinking and is often accompanied by altered meibomian gland secretion [8,9]. Dry eyes are also a frequent side effect of thyroid diseases, Parkinson disease, Sjögren syndrome (SS), and vitamin A deficiency. Most women experience dry eyes as they enter menopause, due to the changes of the hormonal status[10] Ocular discomfort and visual impairment are the possible consequences of the dry eye condition [5]. The mainstay for dry eye treatment is the use of artificial tears, which is often accompanied by the use of anti-inflammatory eye drops, such as corticosteroids. Anyway, these drugs have a variety of side effects that limit their use in clinical practice. Failure of tear secretion and/or excessive evaporation cause tear hyperosmolarity. This stimulates a cascade of inflammatory events involving mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-kB) signaling pathways and the generation of inflammatory cytokines such as interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNF)-α as well as matrix metalloproteinases (MMP-9) [11,12]. It has recently been shown that experimental dry eye in mouse stimulates expression and production of IL-1a, IL-6, TNFα, MMP-9, MAPK, and ICAM-1, for the predisposition of ocular surface inflammation facilitating the presentation of potential antigens by epithelial cells [13,14].

Cloricromene (ethyl({8-chloro-3-[2-(diethylamino) ethyl]-4-methyl-2-oxo-2H-1-benzopyran-7-yl}oxy) acetate) is a semi-synthetic coumarin derivative (Fig. 1) used as an antiplatelet drug with vasodilating and endothelium-protective activity [15]. It has been demonstrated [16] that cloricromene attenuates chronic inflammation and tissue damage associated with collagen-induced arthritis in rats. Results from our lab [17] showed that cloricromene attenuates the degree of inflammation and tissue damage associated with endotoxin-induced uveitis in the rabbit eye and protects against experimental rat uveitis, reducing the

expression of TNF-α, and adhesion molecules such as P-selectin and ICAM-1. Recently, we demonstrated [18] that cloricromene attenuates the degree of inflammation and tissue damage in rats with experimental diabetic retinopathy.

The aim of the present study was to evaluate the effects of topical cloricrome, a new coumarin derivative, on dry eye model.

Materials and Methods

Materials

Cloricromene hydrochloride were obtained from Fidia (Abano Terme, Italy). Concanavalin A (ConA) was purchased from Sigma-Aldrich (Milano, Italy). In order to obtain an ophthalmic solution at pH comprised in the ocular tolerability range, we formulated cloricromene at pH 6.3 with an isotonic phosphate buffer containing 2% of glycerin and 5% of Tween 80. The surfactant were added to increase the solubility of the cloricromene free base formed at pH 6.3.

Animals

Male albino rabbits of the New Zealand strain (Charles River; Calco, Italy), weighing 1.8–2.2 kg, and with no signs of ocular inflammation or gross abnormality, were used. The animals were housed in a single cage upon arrival in the facilities under standard conditions of temperature (21°C) and 12:12 hours light/dark cycle with tap water and commercial food provided ad libitum. Animal management procedures conformed to the ARVO (Association for Research in Vision and Ophthalmology) resolution on the use of animals in research and the European Community Council Directive 86/609/EEC. Efforts were made to minimize animal suffering and to reduce the number of animals used. After 1 week of adaptation in the facilities, the animals were admitted to the experimental session. In order to exclude any disease that could interfere with the experimental treatment, the eyes of all animals were previously examined with a slit lamp.

Lacrimal gland inflammation-induced dry eye

Rabbits (n = 4-6 per group) were randomized into treatment groups and treated topically 3 times per day with 25 μL of either cloricromene (0.01%, 0.1%, 1% w/v) or vehicle immediately after the ConA injection. Rabbits were anesthetized by an intramuscular injection of 35 mg/kg of ketamine HCl and 5 mg/kg xylazine HCl (KN-93; Sigma-Aldrich, Milan, Italy). Each rabbit received bilateral injections into the lacrimal glands. Injections were made by slightly retracting the lower eyelid and inserting the needle approximately 1 cm from the nasal canthus into the suborbital space to a depth of approximately 6 mm. A single 15μL volume of saline or 300μg Con A was injected into the lacrimal gland using a 30 G needle and an Hamilton syringe. Euthanasia was quickly and painlessly performed by a lethal injection of 0.2 ml/kg of Tanax (Intervet, Milan, Italy).

Tear breakup time (TBUT) assessment

TBUT was determined by instillation of 5μL sodium fluorescein (2% in sterile saline) onto the eye and the lids blinked manually to distribute the fluorescein within the tear film. Under slit-lamp (Sbisà 4179T, Firenze, Italy) observation, the eye was held open and the time until one or more black spots or streaks appeared in the precorneal tear film was recorded.

Tear volume (Schirmer test)

Schirmer strips (Alfa Intes, Casoria, Italy) were placed in the lower eyelid of the rabbit for 15 seconds, and the wetted portion of the strip was measured in millimeters as an index of tear volume.

Measurements of cytokines

Lacrimal glands were collected 72 hours after ConA injection and immediately placed in 1 mL of ice-cold buffer (PBS), bovine serum albumin (BSA) (10 mg / mL), (EDTA) (10 mM), and protease

inhibitors (Complete™ Mini/Roche, 1 tablet/10 mL). The samples were homogenized on ice and centrifuged (2000*g* for 30 min at 4°C) and supernatants were decantedand stored at -80°C and subsequently assayed for IL-1β IL-8 and TNFα by ELISA kits (R&D Systems Minneapolis, MN, USA). Proteins were determined by the Coomassie blue assay (Sigma-Aldrich, Milan, Italy).

MMP-9 assay

Lacrimal gland levels of MMP-9 were detected by a commercial ELISA kit (Invitrogen, Carlsbad, CA; USA). Lacrimal glands were collected and homogenized (1.5 mL PBS, centrifuged 12,000*g*, 4°C) , and supernatants were assayed for MMP-9 levels following the manufacturer's protocol.

Statistical analysis

All values are expressed as mean ± SD. The results were analyzed by one-way ANOVA followed by a Bonferroni *post-hoc* test for multiple comparisons. A value of $p \leq 0.05$ was pre-determined as the criterion of significance.

Results and discussion

Ex vivo assessment of lacrimal gland cytokine levels was conducted to provide biochemical evidence of lacrimal gland inflammation following the Con A injection. Compared to control, the Con A injection elicited statistically significant increases in TNFα, IL-1β and IL-8 (Table 1). Cytokines levels were statistically reduced by cloricromene treatment in a dose-dependent manner. ConA significantly elevated levels of immunoreactive MMP-9 in lacrimal gland homogenates (Table 1). MMP-9 levels elicited by ConA were significantly reduced by cloricromene. Tear film integrity was assessed by determining the interval the tear film remained intact without blinking. Normal TBUT was around 50 seconds in rabbits, In contrast, ConA injection produced a dramatic reduction in TBUT within 72 hours. TBUT decreased to approximately 5 seconds in animals injected with ConA, and it is statistically reversed by cloricromene treatment (Table 2). The volume of tears quantified using the Schirmer test in rabbits injected in the lacrimal gland with either Con A or saline is reported in Table 2. The tear volume (3.0 ± 0.5 mm) at day 3 following a Con A injection was significantly decreased, compared to tear volume in sham group (8.5 ± 1.0 mm). Cloricromene treatment significantly attenuated the tear volume decrease elicited by ConA injection (Table 2). The efficacy of the anti-inflammatory cloricromene on lacrimal gland inflammation-induced dry eye was assessed using clinically relevant parameters such as tear breakup time and tear volume.

Features of this rabbit lacrimal gland inflammation model of dry eye are consistent with the current understanding of dry eye as a local ocular surface inflammatory response to abnormal tear volume and composition. It was recently shown that inflammation is involved as one of the main mechanisms common to all the different forms of dry eye [19]. Therefore, the therapeutic approach to dry eye should take into consideration this aspect. This study demonstrated for the first time that cloricromene treatment was able to reduce, in a dose-dependent manner, the inflammatory process elicited by ConA injection and prevent the tear film integrity. Cloricromene acts as a potent anti-inflammatory drug avoiding the side effects of steroids, therefore in chronic diseases like dry eye syndrome this compound represents an useful pharmacological tool. Cloricromene protects rats from lipopolysaccharide (LPS)-induced endotoxemia by blocking NF-kB activation, leading to inhibition of NO and TNFα overproduction [20], reversing the LPS-induced vascular hyporeactivity. Since cloricromene influences TNFα production, the drug has been recently evaluated in an animal

model of inflammatory bowel disease[21], where TNFα has a key role; cloricromene significantly reduced tissue concentrations of TNFα and myeloperoxidase activity, whereas no effect was seen on blood

Table 1. Effects of cloricromene on inflammatory cytokines and MMP-9. Data are expressed as mean±S.D. (pg/mg protein).

Groups	TNFα	IL-1β	IL-8	MMP-9
Control	1.9±0.2	2.6±0.4	6.7±1.0	99±11
ConA	9.7±0.5	8.9±0.5	88±23	246±29
Cloricromene 0.01%	4.8±0.2**	6.3±0.3*	47±12**	191±26**
Cloricromene 0.1%	3.4±0.1**	4.9±0.2**	29±9.0**	160±19**
Cloricromene 1%	3.0±0.1**	3.1±0.2**	10±1.1**	138±13**

*p<0.05, **p<0.01 *vs.* ConA

Table 2. Effects of cloricromene on tear breakup time (TBUT) and tear volume (Schirmer). Data are expressed as mean±S.D.

Groups (mm)	TBUT (seconds)	Schirmer
Control	53±6	8.5±1.0
ConA	4±1	3.0±0.5
Cloricromene 0.01%	30±4*	6.0±0.5*
Cloricromene 0.1%	36±6*	6.5±0.5*
Cloricromene 1%	44±3*	7.0±1.0*

*p<0.01 *vs.* ConA

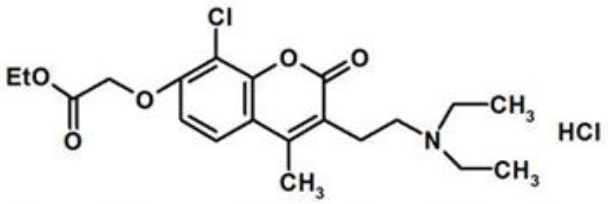

Figure 1. Chemical structure of cloricromene

coagulation parameters [21]. Corsini and colleagues[22] showed that cloricromene inhibits LPS-induced transcription of TNFα and activation of NF-kB by interfering with LPS-induced cellular oxidative activity. These results [22] demonstrated that cloricromene interferes with the early signal transduction pathway triggered by LPS. The mechanism by which cloricromene inhibits activation of NF-kB and subsequent neosynthesis of TNFα could be related to the scavenger effect against ROS. We recently proposed [17] cloricromene for ocular applications, showing that the drug attenuates the degree of inflammation and tissue damage associated with endotoxin-induced uveitis in the rabbit eye and protects against experimental rat uveitis, reducing the expression of adhesion molecules such as P-selectin and ICAM-1. Furthermore, we demonstrated [17] that cloricromene strongly inhibited TNFα production, cell infiltration, protein exudation, and nitrite/nitrate formation. More recently we demonstrated [18] that cloricromene

attenuates the degree of inflammation preserving the blood-retinal barrier in diabetic rats reducing retinal TNFα, ICAM-1, VEGF and eNOS levels.

The anti-inflammatory activity of cloricromene led us to undertake the present study about the effect of this drug in patients with dry eye syndrome where a chronic inflammation of the ocular surface was demonstrated. From the results of our study it appears that cloricromene could be of help in controlling the evolution of signs and symptoms of dry eye. Further, cloricromene could contribute to obtain a good efficacy reducing the risk of both ocular and systemic side effects, which often accompany the use of steroids.

Conclusions

This study showed for the first time that cloricromene, a new coumarin derivative, may be useful in the treatment of dry eye syndrome, and that clinical studies to evaluate this possibility may be warranted.

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