

Topical cis-urocanic acid prevents ocular surface irritation in both IgE -independent and -mediated rat model

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Abstract

Purpose Our purpose was to investigate the effect of locally administered cis-urocanic (cis-UCA) in two experimental models of allergic conjunctivitis.

Methods The compound 48/80 (C48/80)-induced ocular irritation model (IgE-independent) and the ovalbumin (OA)-induced ocular allergy model (IgE-mediated) were used to test and compare the effect of cis-UCA on dexamethasone, ketotifen and olopatadine. In the C48/80 model, clinical severity scoring from photographs, immunohistochemical analysis of nuclear Ki-67 antigen to quantify actively proliferating epithelial cells and of caspase-3 enzyme to identify apoptotic activity in the conjunctival tissue were used. In the OA model, an Evans Blue stain concentration of conjunctival tissue was used to evaluate vascular leakage due to allergic reaction.

Results The cis-UCA was well tolerated and effective in both the IgE-independent and -mediated rat models. Treatment

with C48/80 caused conjunctival hyperaemia, which was significantly inhibited by ketotifen at the 6 h time point ($p = 0.014$) and by dexamethasone and cis-UCA 0.5% at 12 ($p = 0.004$) and 24 ($p = 0.004$) hour time points. In a comparison between the active drug treatments, only ketotifen showed a significant difference ($p = 0.023$) to cis-UCA treatment at the 1 h time point, otherwise there were no statistically significant differences between the active drugs. Ketotifen, dexamethasone and cis-UCA 0.5% significantly inhibited the C48/80-induced nuclear accumulation of Ki-67, without differences between the active treatment groups. In the OA model, cis-UCA 0.5% did not inhibit the vascular leakage of conjunctiva, whereas cis-UCA 2.5% of was at least equally effective compared to olopatadine, abolishing the allergic vascular leakage response almost completely.

Conclusions The present findings in the two AC models suggest that cis-UCA might have anti-allergic potency both in immediate and delayed-type allergic reactions in the eye.

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Keywords Cis-urocanic acid · Ocular allergy · Allergic conjunctivitis · Experimental animal model

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Introduction

Allergic conjunctivitis (AC) is a common and complex ocular inflammatory response that usually coincides with rhinoconjunctivitis, rhinosinuitis, asthma, urticarial and eczema tissue [1]. Typical symptoms and signs in AC are ocular itching, tearing, redness, photophobia, conjunctival oedema and mucus discharge that gradually resolve when exposure to the allergen is terminated. AC can damage the ocular surface permanently in its severe and chronic forms, such as vernal (VKC) and atopic keratoconjunctivitis (AKC) and giant papillary conjunctivitis (GPC).

The pathophysiology of AC is closely linked to the conjunctival mast cells and their secreted products (e.g., histamine, proteases, cytokines, receptors and growth factors) released in immediate type 1 hypersensitivity IgE-related response upon allergen challenge [2]. Infiltrating neutrophils, eosinophils and lymphocytes may also be involved in the responses of acute reactions. In type IV hypersensitivity, chronic conjunctival inflammation, and T cell subsets are known to play a major role.

Today, the first line pharmacological treatment of AC is based on topical antihistamines and mast cell stabilizers or dual-action therapeutic agents and NSAID drops [3–5]. In severe cases, topical corticosteroids may be prescribed with caution, since their long-term use can cause sight-threatening side effects [4]. Immunomodulatory treatments like cyclosporine A and tacrolimus are used in a long-term treatment of severe cases, but they can evoke patient-dependent side effects as well. Thus, there is still a need for safe and effective treatment modalities.

Urocanic acid (UCA) is an important endogenous molecule formed in the epidermis of the human skin. Apart from its function as a natural moisturizing factor component [6] and a photoprotective agent [7], the cis isomer (cis-UCA) is recognized as a modulator of cell-mediated [8–10] and innate immunity [11, 12]. More recently, cis-UCA has been found to improve skin barrier function and to suppress local cutaneous inflammation [13].

The anti-inflammatory properties of cis-UCA have been established both in vitro and in vivo. In the human corneal and conjunctival cell cultures, cis-UCA suppressed IL-6 and IL-8 secretion and cytotoxicity, and restored the metabolic activity of the UV-B-irradiated cells [14]. These effects were observed to be mediated by the inhibition of stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) in the human corneal and conjunctival cell cultures after UV-B exposure [14, 15]. The activation of SAPK/JNK has been linked to ocular inflammatory conditions such as the dry eye disease [16]. These findings encouraged us to investigate the effect of locally administered cis-UCA in two experimental models of allergic conjunctivitis: the compound 48/80 (C48/80)-induced ocular irritation model (IgE-independent) and the ovalbumin (OA)-induced ocular allergy model (IgE-mediated).

Materials and methods

Test animals

The experiments were conducted in compliance with the Council of Europe (Directive 86/609) and the Association of Research in Vision and Ophthalmology guidelines for use of animals in experimental procedures and Finnish guidelines.

The study protocol was approved by the State Provincial Office of Eastern Finland.

In the C48/80 study, male Wistar rats aged 10 weeks were used (six animals in each group). In the OA-induced allergic ocular inflammation model, male Sprague Dawley rats aged 10 weeks were used: seven animals in the immunized placebo group, six animals in the cis-UCA 2.5% group, six animals in the cis-UCA 0.5% group, eight animals in the olopatadine group and five animals in the non-immunized placebo group. The animals were housed in cages in a controlled environment (constant temperature 22 ± 1 °C; humidity 50–60%; lights on 0700–1900 h) with free access to food and water.

Reagents and test formulations

Cis-UCA was manufactured by BioCis Pharma (Turku, Finland). The purity of cis-UCA was above 99% by HPLC analysis, and its chemical identity was characterized by IR spectrometry, melting point analysis, ^1H NMR and ^{13}C NMR spectrometry. The water content of the dry product was 0.1% by the Karl Fischer method, and its endotoxin concentration was below 25 IU/g (EU/g) as assessed with the *Limulus* amoebocyte lysate gel clot method.

In the C48/80 study, cis-UCA was dissolved in a commercial eye drop solution containing hypromellose (Artelac®, Santen, Finland), adjusted to pH 6.5, and sterile filtered. The concentration of 0.5% cis-UCA (w/w) was verified by HPLC. In a pilot study, conjunctival hyperaemia evoked by C48/80 was not inhibited by the vehicle, i.e., the Artelac drop alone, while in combination with cis-UCA, an inhibitory effect was observed (data not shown). As comparator drug formulations, dexamethasone 1 mg/ml (Oftan®Dexa, Santen, Finland) and ketotifen 0.25 mg/ml (Zaditen, Novartis, Switzerland) eye drops were used.

For the OA-induced allergy study, cis-UCA 0.5% and 2.5% (w/v) eye drops were prepared in a vehicle containing water for injection, sodium chloride, and polyvinyl alcohol, pH 6.5–6.7. The eye drops were sterile filtered and filled aseptically in white plastic non-transparent eye drop bottles. The cis-UCA concentration was verified by HPLC. Olopatadine 1 mg/ml (Opatanol, Alcon, UK) was used as a positive comparator and immunized, vehicle-treated (saline) and non-immunized, vehicle-treated as negative controls.

Compound 48/80 induced ocular irritation model

C48/80 is a mast cell degranulator and condensation product mixture of p-methoxy-*N*-methyl phenethylamine and formaldehyde. C48/80 (Sigma-Aldrich, USA) was dissolved in phosphate-buffered saline (PBS). Based on a previous study [17] one drop (30 µl) containing 1000 µg C48/80 was applied topically onto the surface of both eyes to release the histamine from mast cells. At time points 0.5 h, 4 h, and 12 h, the eyes

were treated with a single drop of 0.5% cis-UCA, dexamethasone eye drop or ketotifen eye drop. The positive control animal group was challenged with 1000 µg of C48/80 without further treatment. The negative control animal groups were treated with a single drop of PBS, 0.5% cis-UCA, dexamethasone eye drop or ketotifen eye drop at time points 0.5 h, 4 h, and 12 h. All eyes were photographed at 1 h and 6 h and the eyes of animals completing the 24 h follow-up at 12 h and 24 h and the severity of conjunctival hyperaemia were evaluated in the photographs. Severity scoring (none = 0, mild = 1, moderate = 2; severe = 3) was performed by two independent and treatment-blinded ophthalmologists (HMJ, KK).

Three animals from each group were killed at 6 h (i.e., after two eye drop treatments). The eyes were immediately removed, fixed in 10% formalin, and subjected to immunohistochemical analysis of nuclear Ki-67 antigen to quantify actively proliferating epithelial cells (1:50; clone MIB-5; DakoCytomation, Glostrup, Denmark). Immunohistochemical analysis of caspase-3 enzyme was used to identify apoptotic activity in the conjunctival tissue (1:100; Cleaved Caspase-3, #9661, Cell Signaling Technology). The intensity and the quality of Ki-67 and caspase-3 staining were evaluated in the conjunctival histological sections by counting at least 300 adjacent squamous epithelial cells under a light microscope. The rest of the animals were killed after the final evaluation at 24 h.

Ovalbumin (OA) -induced allergic ocular inflammation model

The rats were first immunized with intraperitoneal injection of 100 µg OA (Sigma-Aldrich, USA) and 20 mg aluminum hydroxide (Sigma-Aldrich, USA) dissolved in PBS. After two weeks, 10 µl of DL-dithiotreitol (DTT) freshly prepared in PBS was applied topically to both eyes of the rats. Fifteen minutes after DTT application, 10 µl of 100 mg/ml OA in PBS was applied topically to both eyes to induce an allergic reaction. Test formulations (10 µl, cis-UCA 0.5% and 2.5%) positive control product (10 µl olopatadine 1 mg/ml) and in immunized placebo group saline (negative control) were applied to both eyes 30 min prior to and 30 min after the OA challenge. After OA challenge, the animals were anesthetized with an intramuscular combination of ketamine, medetomidine and buprenorphine. During anesthesia, immediately prior to the second instillation of the test formulations, 15 mg/kg of Evans Blue (1.5%, Sigma-Aldrich, USA) was injected into the cervical vein of the animals. One hour after OA challenge, the animals were sacrificed and the palpebral and bulbar conjunctiva was collected into a solution of 0.5% sodium sulfate and acetone (1:2.3). In the non-immunized placebo group, only saline was applied to both eyes. The solution was kept at room temperature for 24 h and then centrifuged. Evans Blue was detected in the supernatant by measuring the absorbance at 620 nm.

Statistical analysis The statistical analyses were performed with SPSS Statistics (version 22, IBM, NY, USA). Due to small group size, the non-parametric Kruskal-Wallis test was used to test differences between the active treatment groups in conjunctival hyperemia. T-tests were performed to compare Ki-67 and caspase-3 cell counts in active treatment groups to control group as well as in ovalbumin test to compare Evans Blue concentrations between all groups, assuming different variances. In addition, the Bonferroni correction was performed in Evans Blue concentration comparisons due to multiple comparisons.

Results

Treatment with C48/80 caused moderate conjunctivitis in all animals treated. Conjunctival hyperaemia evoked by C48/80 was significantly inhibited by ketotifen at 6 h time point ($p = 0.014$) and by dexamethasone and cis-UCA 0.5% at 12 ($p = 0.004$) and 24 ($p = 0.004$) hour time points. When the comparison was made between the active drug treatments, only ketotifen treatment showed a significant difference ($p = 0.023$) to cis-UCA treatment at the 1 h time point, otherwise there were no statistically significant differences between the active drugs (Fig. 1 and Table 1).

Immunohistochemical analysis of the conjunctiva from three animals in the control group killed at 6 h showed up-regulation of nuclear Ki-67 expression in response to C48/80. Ketotifen, dexamethasone and cis-UCA 0.5% significantly inhibited the C48/80-induced nuclear accumulation of Ki-67 (Fig. 2). There were no statistically significant differences between the active treatment groups (Kruskal-Wallis test). Caspase-3 activity did not show a statistically significant

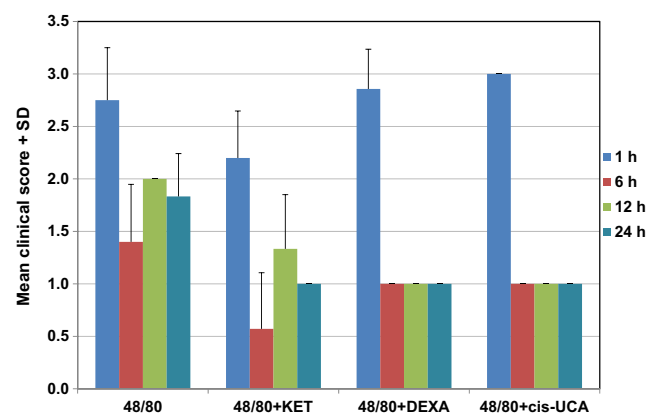


Fig. 1 Conjunctival hyperemia in response to topical C 48/80 1000 µg drops or combined with anti-allergic drugs applications. The eyes were treated with eye drops containing ketotifen (KET), dexamethasone (DEXA) or 0.5% cis-UCA at the time points of 0.5, 4 and 12 h after the application of C 48/80. Severity scoring was performed from photographs taken at the time points of 1 h, 6 h, 12 h and 24 h. Severity scoring: none = 0, mild = 1, moderate = 2; severe = 3; mean + SD; $n = 6$

Table 1 Comparison of conjunctival hyperemia between the groups (Kruskal-Wallis Test), *p*-values

	48/80 1000µg	48/80 1000µg + 0,5% cis-Uca	48/80 1000µg + Zaditen
1 h			
48/80 1000µg + 0,5% cis-Uca	1.0		
48/80 1000µg + Zaditen	0.432	0.023	
48/80 1000µg + Oftan Dexa	1.0	1.0	0.083
6 h			
48/80 1000µg + 0,5% cis-Uca	0.998		
48/80 1000µg + Zaditen	0.014	0.545	
48/80 1000µg + Oftan Dexa	0.998	1.000	0.545
12 h			
48/80 1000µg + 0,5% cis-Uca	0.004		
48/80 1000µg + Zaditen	0.141	1.000	
48/80 1000µg + Oftan Dexa	0.004	1.000	1.000
24 h			
48/80 1000µg + 0,5% cis-Uca	0.004		
48/80 1000µg + Zaditen	0.141	1.000	
48/80 1000µg + Oftan Dexa	0.004	1.000	1.000

difference between any of the groups either (Kruskal-Wallis test, data not shown).

Immunization with OA and subsequent topical ocular challenge with OA induced conjunctival vascular leakage in Sprague Dawley rats as an indication of an allergic reaction. In this model, cis-UCA 0.5% did not inhibit the vascular leakage of conjunctiva, whereas the higher 2.5% concentration of cis-UCA was at least equally effective compared with the antihistamine olopatadine, abolishing the allergic vascular leakage response almost completely (Fig. 3). The non-effective cis-UCA 0.5% differed significantly from the non-

immunized baseline ($p = 0.0001$) as well as from cis-UCA 2.5% ($p = 0.0003$) (Table 2). Due to relatively large variation in vascular leakage response in the immunized and OA-challenged control group, no statistical significance was found compared to other treatment groups.

In all experiments, topical cis-UCA eye drops were well tolerated and caused no local irritation or systemic toxic effects (data not shown).

Discussion

The C48/80 model of AO is an IgE-independent ocular irritation model that acts by a non-immunologic mechanism [18].

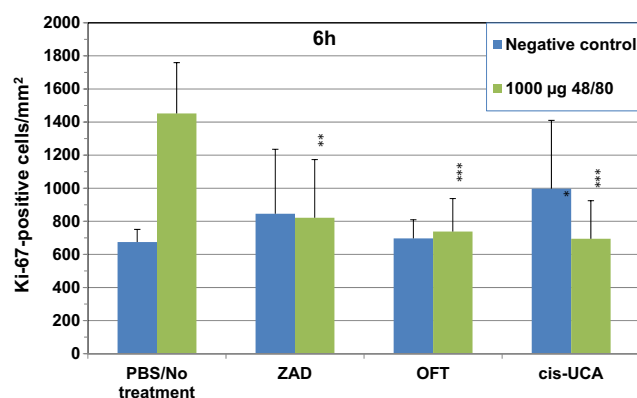


Fig. 2 Ki-67 staining on the conjunctival epithelium in response to vehicle only (PBS), topical C48/80 drop (no treatment) or combined with anti-allergic drug applications. The eyes were treated with eye drops containing ketotifen (KET), dexamethasone (DEXA) or 0.5% cis-UCA at the time points of 0.5 and 4 h after the application of C 48/80 1000 µg in the rat eye. The animals were killed at the 6 h time point. (pairwise t-test, comparison to PBS/no treatment, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

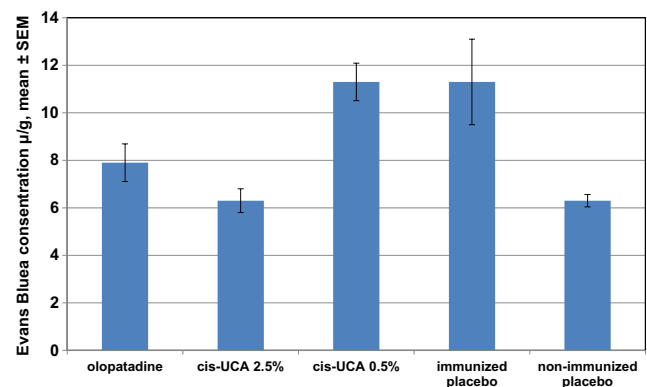


Fig. 3 Effect of cis-UCA eye drops on conjunctival vascular leakage in OA sensitized rats. The eyes were treated with saline (immunized placebo and non-immunized placebo), 0.5% or 2.5% cis-UCA eye drops or antihistamine olopatadine. Vascular leakage was measured by Evans Blue accumulation in the conjunctiva

Table 2 Ovalbumin test, comparison of Evans Blue concentrations between the groups (t-test, different variances assumed), *p*-values

	cis-UCA 2.5%		cis-UCA 0.5%		immunized placebo		non-immunized placebo	
	<i>p</i> -value	Bonferoni corrected	<i>p</i> -value	Bonferoni corrected	<i>p</i> -value	Bonferoni corrected	<i>p</i> -value	Bonferoni corrected
Optanol	0.0981	0.9812	0.0064	0.0637	0.1485	1.0000	0.0653	0.6528
cis-UCA 2.5%			0.0000	0.0003	0.0513	0.5130	1.0000	1.0000
cis-UCA 0.5%					1.0000	1.0000	0.0000	0.0001
immunized placebo							0.0499	0.4993

C48/80 strongly induces histamine release from mast cells and modulates cell proliferation [19, 20], resembling hypersensitivity type I reaction taking place in AC [19, 21, 22]. Khosravi and co-workers [23] evaluated the clinical signs of conjunctivitis in the C48/80-induced immediate hypersensitivity model in the rat. They found that the antihistamines (levocabastine and chlorpheniramine) were more effective than the mast cell stabilizers (lodoksamide and sodium cromoglycate) in inhibiting lid swelling. We used the ocular irritation model to study the effect of topically administered cis-UCA on ocular signs and local tissue stress reactions. As expected, the comparator anti-histamine and mast cell stabilizer ketotifen was effective in alleviating the clinical signs of inflammation at an early stage of 1 h, but at the later time points, cis-UCA showed similar efficacy to ketotifen and dexamethasone. As Khosravi et al. performed the clinical scoring at 10 and 20 min after induction, we scored the reaction in photographs taken at 1 to 24 h. Even though the visual evaluation is always somewhat subjective, the results of the two studies are similar in that they indicate a fast mode of action by antihistamines.

Ki-67 is a nuclear protein which is strongly associated with cell proliferation and is, therefore, widely used as a proliferation marker [24]. The Ki-67 labeling index has been used for evaluation of histopathology and malignancy in ocular surface neoplasia [25]. Elevated nuclear Ki-67 immunoreactivity in the basal and supra-basal cells in VKC specimens may indicate a role of Ki-67 in conjunctival remodeling in VKC [26]. In the present study, nuclear Ki-67 expression was used for quantifying proliferating conjunctival epithelial cells. The data indicate that cis-UCA decreases the number of nuclear Ki-67 positive cells during the C48/80 challenge, suggesting anti-proliferative and cytoprotective effects of cis-UCA in this model. The interpretation is consistent with previous findings in vitro [14]. Cell proliferation is strongly regulated by JNK/SAPK and AP-1 signaling cascades and found to be suppressed in ocular cell cultures by cis-UCA [15].

Ovalbumin is a model protein antigen, which induces an IgE-mediated delayed-type hypersensitivity reaction. It causes oedema at the site of challenge, a frequent sign of allergic inflammation. The amount of extravasated Evans Blue dye can be extracted from the ocular conjunctiva and its concentration in the tissue measured [27, 28]. In an ovalbumin model

in the guinea pig [29], spaglumic acid and emedastine were found more effective than the olopatadine in reducing Evans Blue extravasation, but olopatadine was equally effective in reducing the clinical signs of allergic conjunctivitis. Olopatadine shows selective histamine H1 receptor antagonistic action and conjunctival mast cell stabilization action [30]. In a later study, olopatadine was superior to levocabastine and tranilast in preventing vascular leakage in the rat AC model, exerting its anti-leakage effect already 5 min after antigen challenge [30]. In our study, the potency of cis-UCA 2.5% was comparable to that of olopatadine, bringing the measured vascular leakage to the base-line level of the non-immunized control animals. This may indicate a cytoprotective effect and, similarly to olopatadine, a mast cell stabilization or histamine antagonistic action by cis-UCA in the ocular conjunctiva.

The cis-UCA was well tolerated in both the IgE-independent and -mediated rat models, as there was no increase in clinical signs or tissue stress markers. This observation is in line with results from our clinical trial with cis-UCA recently conducted in healthy human subjects [31]. The previous and present data gathered indicate that cis-UCA has a beneficial profile in cell cycle control, regulation of inflammatory responses and cytoprotection in ocular surface cells [14, 15, 31]. The present findings in the two AC models suggest that cis-UCA might have anti-allergic potency both in immediate and delayed-type allergic reactions in the eye.

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Compliance with ethical standards

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

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