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HANNA-MARI JAUHONEN

EFFECTS OF CIS-UROCANIC ACID ON THE OCULAR SURFACE

Cell Culture, Experimental Animal and Clinical Studies

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ABSTRACT

The ocular surface is constantly exposed to environmental challenges, such as UV-light, dust and pollen that can exacerbate the symptoms of anterior ocular inflammatory diseases (AOID). In the United States, AOID affect over 40% of the population i.e. AOID are a source of both individual suffering and a huge economic burden. It is crucial that the immunopathology of certain AOID e.g. ocular allergy and dry eye disease (DED) should be clarified so that more targeted treatments can be developed.

Urocanic acid (UCA) is a major UV-absorbing chromophore naturally existing in the skin. UCA suppresses UV-induced damage in cells and alleviates hypersensitivity reactions in the epidermis. These inflammatory reactions can also be detected in diseases affecting ocular surface. The aim of this study was to explore how cis-UCA affects the ocular surface.

The human corneal epithelial cells (HCE-2) and human conjunctival epithelial cells (HCECs) were incubated with different concentrations of cis-UCA and irradiated with a single dose of UV-B. An ELISA assay was used to evaluate DNA-binding, protein expression and secretion levels. Cytotoxicity was monitored with colorimetric methods. Treatment with 100 μg/ml cis-UCA completely suppressed UV-B irradiation induced interleukin (IL)-6 and IL-8 secretion. Cis-UCA inhibited the DNA-binding of c-Jun and c-Fos but not that of NF-κB. Whereas UV-B increased the levels of phospho-c-Jun and phospho-JNK, the expressions of both were attenuated by cis-UCA. Cis-UCA also alleviated the UV-B-induced apoptosis and proliferative decline in HCE.

Treatment with an allergy-inducing compound, C48/80, evoked conjunctival hyperaemia in a rat model; this was significantly inhibited by administration of ketotifen at the 6 hour time point and by dexamethasone and cis-UCA 0.5% at the 12 and 24 hour time points. In a comparison between the active drug treatments, only ketotifen achieved a significant improvement ($p=0.023$) over cis-UCA treatment at the 1 hour time point. In the ovalbumin (OA) induced rat model, cis-UCA 2.5% was at least as efficacious as olopatadine, abolishing the allergic vascular leakage response almost completely.

Finally, the tolerability of topical administration of cis-UCA was evaluated in a phase I, double-blinded, placebo-controlled trial. Healthy volunteers were randomized to three treatment arms: 0.5% cis-UCA (12 subjects), 2.5% cis-UCA (12 subjects) and placebo eye drops (13 subjects). In the first part, the subjects were dosed topically with one eye drop three times during one day. In the second part, the subjects self-administered three daily drops into both eyes for 14 days. There were no significant adverse events (AEs). The most common treatment-related ocular AE was eye irritation (62.2% of subjects). Topical ocular dosing led to transient systemic exposure to cis-UCA that did not cause any systemic AEs.

In summary, cis-UCA possesses anti-inflammatory and cytoprotective properties; these are regulated through the JNK signaling pathway in response to UV-B-irradiation *in vitro* conditions. Cis-UCA is well tolerated and efficacious in both IgE-independent and IgE-mediated rat models. Our clinical trial also revealed the good tolerability of cis-UCA.

National Library of Medicine Classification: QU 65, QU 375, WW 166, WW 212, WW 220
Medical Subject Headings: Urocanic Acid; Cornea; Epithelium, Corneal; Conjunctiva; Ultraviolet Rays; MAP Kinase Signaling System; Cells, Cultured; Disease Models, Animal; Clinical Trials, Phase I as Topic

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TIIVISTELMÄ

Silmän pintaosat altistuvat jatkuvasti ultraviolettivalolle (UV), pölylle, siitepölylle ja muille ympäristövaikutuksille. Nämä pahentavat silmän etuosan tulehduksellisia sairauksia, joista tavallisimpia ovat allerginen sidekalvontulehdus ja kuivasilmäoireyhtymä. Näistä sairauksista on USA:ssa arvioitu kärsivän n. 40 % väestöstä ja koska silmänpintaosan sairauksien aiheuttama haitta niin sairastavuutena kuin taloudellisina menetyksinä on merkittävä, tarvitaan näiden sairauksien immunopatologian parempaa tuntemusta ja tarkoin kohdennettuja hoitoja.

Urokaanihappo (UCA) on ihon luontainen kromofori, joka absorboi UV-valoa. Se lievittää UV-valon aiheuttamaa soluvauriota ja vaimentaa yliherkkyysreaktioita ihossa. Samat tulehdusreaktiot ovat havaittavissa myös silmän pintaosan sairauksissa. Tämän tutkimuksen tarkoitus oli selvittää cis-UCAn vaikutusta silmän pintaosassa.

Ihmisen sarveiskalvo- ja sidekalvosoluviljelmiin lisättiin eri pitoisuuksia cis-UCA ennen UV-B valoaltistusta. ELISA-menetelmää käytettiin immunologisten reaktioiden, kuten DNA:han sitoutumisen, proteiinien ilmenemisen ja erittymisen arviointiin. Haitallisia soluvaikutuksia arvioitiin spektrometrisellä menetelmällä. Cis-UCA altistus esti kokonaan UV-B valon aiheuttaman interleukiini-6 ja -8 erittymisen. cis-UCA esti myös c-Jun ja c-Fos proteiinien sitoutumisen DNA:han mutta ei Nf-κB:n. Lisäksi UV-B lisäsi fosforoituneen c-Jun ja c-Fos proteiininen määrää, mutta cis-UCA altiste lievensi tätä reaktiota. Cis-Uca vähensi myös UV-B:n aiheuttamaa solukuolemaa ja -kasvun häiriintymistä.

Rotta-mallisssa allergiainduktori C48/80 aiheutti huomattavan hyperemian koe-eläinten sidekalvolla. Tämä lievittyi ketotifeeninlla kuusi tuntia ja myös deksametasonilla ja cis-UCA 0,5%:lla 12 ja 24 tuntia hoidon alusta. Ketotifeeni oli ensimmäisen tunnin kuluttua hoidon alusta cis-UCAa tilastollisesti tehokkaampi (p=0.023), muutoin hoitojen välillä ei ollut tilastollisesti merkitseviä eroja. Ovalbumiinilla aikaan saadussa rotan sidekalvon allergiamallissa cis-UCA 2,5% oli vähintään yhtä tehokas verrattuna antihistamiini olopatadiiniin ja esti allergiaan liittyvän verisuonten läpäisevyyden lisääntymisen lähes kokonaan.

Cis-UCA valmisteen siedettävyyttä silmään annosteltuna tutkittiin faasi 1, kaksoissokkoutetussa, plasebo-kontrolloidussa kokeessa. Kolme ryhmää terveitä vapaaehtoisia sai seuraavia silmätippoja: 0.5% cis-UCAa (12 henkilöä), 2.5% cis-UCAa (12 henkilöä) ja plaseboa (13 henkilöä). Ensimmäisessä osassa tipat annosteltiin jompaan kumpaan silmään kolme kertaa vuorokauden aikana. Toisessa osassa koehenkilöt annostelivat tippoja kumpaankin silmään kolmesti päivässä 14 vrk ajan. Vakavia haittavaikutuksia kokeen aikana ei havaittu. Yleisin valmisteeseen liittyvä silmähaitta oli ohimenevä ärsytys (62,2%). Paikallinen annostelu aiheutti hetkellisen systeemisen altistumisen cis-UCA:lle, joka ei aiheuttanut systeemisiä haittavaikutuksia.

Yhteenvetona voidaan todeta cis-UCA:lla olevan tulehdusta hillitseviä ja soluja suojaavia vaikutuksia, joita säädellään UV-B altistuksen jälkeen JNK signalointireittiä *in vitro*-olosuhteissa. Cis-UCA on hyvin siedetty ja tehokas sekä IgE:stä riippumattomassa ja IgE-välitteisessä rottamallissa. Myös kliinisessä kokeessa cis-UCA oli hyvin siedetty.

Luokitus: QU 65, QU 375, WW 166, WW 212, WW 220
Yleinen suomalainen asiasanasto: lääkeaineet; silmät; sarveiskalvo; ultraviolettisäteily; tulehdus; soluviljely; koe-eläinmallit; kliiniset kokeet

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Kuopio, October 2017

Hanna-Mari Jauhonen

List of the original publications

This dissertation is based on the following original publications:

I Viiri J, JauhonenH-M, Kauppinen A, Ryhänen T, Paimela t, Hyttinen J, Sorri I, Laihia JK, Leino L, Kaarniranta K. Cis-urocanic acid suppresses UV-B-induced interleukin-6 secretion and cytotoxicity in human corneal and conjunctival epithelial cells in vitro. *Molecular Vision 15:1799-1805, 2009.*

II Jauhonen H-M, Kauppinen A, Paimela T, Laihia JK, Leino L, Salminen A, Kaarniranta K. Cis-Urocanic acid inhibits SAPK/JNK signaling pathway in UV-B exposed human corneal epitheliala cells in vitro. *Molecular Vision 15:2311-2317, 2011.*

III Jauhonen H-M, Laihia JK, Oksala O, Viiri J, Sironen R, Alajuuma P, Kaariranta K, Leino L. Topical cis-urocanic acid prevents ocular surface irritation in both IgE - independent and -mediated rat model. *Graefes Arch Clin Exp Ophthalmol* (*Epub ahead of print 24 Aug 2017).*

IV Jauhonen H-M, Kari E, Pylkkänen L, Poutanen J, Laihia JK, Kaariranta K, Leino L. A randomized phase I clinical study of cis-urocanic acid eye drops in healthy adult subjects. *Acta Ophthalmologica 93:368-376, 2015.*

Contents

Abbreviations

5-HT	5-hydroxytryptamine, serotonin
AC	Allergic conjunctivitis
AD	Atopic dermatitis
AEs	Adverse events
AKC	Atopic keratoconjunctivitis
AP-1	Activator protein-1
APC	Antigen presenting cell
AOID	Anterior ocular inflammatory diseases
BCR	Bulbar conjunctival redness
BMI	Body Mass Index
C48/80	Compound 48/80
CALT	Conjuntiva-associated lymphoid-tissue
CDK	Climatic droplet keratopathy
cis-UCA	Cis-urocanic acid
CSE	Corneal staining extent
CST	Corneal staining type
CTA	Clinical Trial Application
CXCL1	chemokine (C-X-C motif) ligand 1
DED	Dry eye syndrome
DSS	Dextran sodium sulfate
DTT	DL-dithiotreitol
EGFR	Epidermal growth factor
ELISA	Enzyme-linked immune sorbent assay
GDNF	Glial derived neurotrophic factor
GLP	Good laboratory practice
GMP	Good Manufacture Practice
HCE	Human corneal epithelial cells
HCEC	Human conjunctival epithelial cells
HLEC	Human limbal epithelial cells
HPLC	High-performance liquid chromatography
HPV	Human papilloma virus
IBD	Inflammatory bowl disease
IER	Institute of Eye research
IFN-gamma	Interferon gamma
Ig	Immunoglobulin
IKK	IκB kinase
IL	interleukin
JNK	C-Jun N-terminal kinase
LC	Langerhans cell
LC-MS/MS	Liquid chromatography tandem mass-spectrometry
LD	Lactate dehydrogenase
LESC	Limbal epithelial stem cells
logMAR	logarithm of the minimal angle of resolution

MAPK	Mitogen activated protein kinase
MCP	Monocyte chemoattractant protein
miR	mikroRNA
MMP	Matrix metalloproteinase
MnSOD	Manganese superoxide dismutase
MTT	3-(4,5-dimethyldiazol-2yl)-2,5-diphenyltetrazolium bromide
NF-kappa-B	Nuclear factor -kappaB
OA	Ovalbumin
OSSN	Ocular surface squamous neoplasia
PAC	Perennial allergic conjunctivitis
PAF	Platlet activating factor
PGE	Prostaglandin
PKCδ	Protein kinase C delta
RANTES	Regulated and Normal T-cell Expressed and Secreted)
ROS	Reactive oxygen spcies
ROS	Reactive oxygen spcies
SAC	Seasonal allergic conjunctivitis
SAPK/JNK	Stress activated protein kinase/c-Jun N-terminal kinase
SS	Sjögren syndrome
TFs	Transcription factors
TLR	Toll-like receptor
TNF-alpha	Tumor necrosis factor alpha
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UV	Ultraviolet light
VA	Visual acuity
VKC	Vernal keratoconjunctivitis

1 Introduction

The ocular surface is composed of corneal and conjunctival epithelia that protects and shields the underlying structures. Since the ocular surface is constantly exposed to external challenges, such as UV-light, dust and mollen, it is not surprising that anterior ocular inflammatory diseases are common. In the United States, they affect over 40% of the population (Bielory, Syed 2013). Inflammatory allergic conjunctivitis (AC) and dry eye syndrome (DED) are the most common ocular surface diseases. Since one cannot conduct experiments in healthy eyes, cell cultures and experimental animal models are often used to investigate the basic mechanisms of ocular surface inflammatory diseases and to search for novel therapies.

Due to the constant exposure to UV-B radiation, damage to both nucleic acids and proteins may be detected in cornea cells. The clinical manifestations of this damage are photokeratitis and climatic droplet keratopathy. The corneal defects are also often accompanied by conjunctival changes such as pterygium and ocular surface squamous neoplasia (OSSN) (Delic et al. 2016, Willmann 2015).

Urocanic acid (UCA) is a major UV-absorbing chromophore in the epidermis. It has been observed to suppress UV-induced damage in cells (Gibbs, Tye & Norval 2008). The cis-UCA, formed from trans-UCA upon UV-B exposure, has also been implicated in several physiological processes e.g. the down-regulation of hypersensitivity reactions (Prater, Blaylock & Holladay 2003, Lauerma, Aioi & Maibach 1995), in the actions of epidermal antigen-presenting cells (Hart, Grimbaldeston & Finlay-Jones 2000, el-Ghorr, Norval 1997) and the activation of neutrophils (Kivisto et al. 1996, Rinaldi et al. 2006) which are all inflammatory reactions that have been detected in ocular surface diseases. In human keratinocytes, cis-UCA has been shown to increase prostaglandin (PGE)-2 secretions as well as cytokine protein production (TNF-alpha, IL-6, and IL-8) (Kaneko et al. 2008). In acute and subacute mouse skin irritation models, cis-UCA has been shown to suppress mouse ear skin swelling (Laihia et al. 2012). In chronic skin allergy (atopic dermatitis (AD)), cis-UCA has been reported to reduce transepidermal water loss and improve skin barrier functions as well as suppressing inflammation (Peltonen et al. 2014).

In the present work, the effects of cis-UCA were studied on UV-B induced inflammatory ocular surface cell models and in two different rat models of experimental allergic conjunctivitis. The safety, ocular tolerability and pharmacokinetics of cis-UCA during topical ocular administration were evaluated in healthy adult subjects in a phase I clinical trial.

2 Review of the Literature

2.1 ANTERIOR OCULAR SURFACE STRUCTURES AND IMMUNOLOGY

The anterior part of the eye is covered by the eyelids. Eyelids spread tears over the cornea and the bulbar conjunctiva and provide a shield against external exposures. The blinking reflex protects the eye against superficial trauma and it is triggered by visual or sensation stimulus. The ocular surface is composed of the tear film and conjunctival and corneal epithelia that are responsible for defence against external exposures.

2.1.1 Tear film

The tear film over the ocular surface is secreted by lacrimal glands and accessory lacrimal exocrine glands (see figure 1). It is composed of soluble mucus secreted by conjunctival goblet cells and fluid and proteins secreted by the lacrimal glands and conjunctival epithelial cells. It also contains electrolytes, metabolites and organic molecules (Azkargorta et al. 2016). One of the main functions of the tear film is to protect the ocular surface from external environmental factors as well as lubricating and nourishing the underlying cornea and conjunctiva. In addition, it has crucial role in maintaining the optical integrity of the eye (Tiffany 2003).

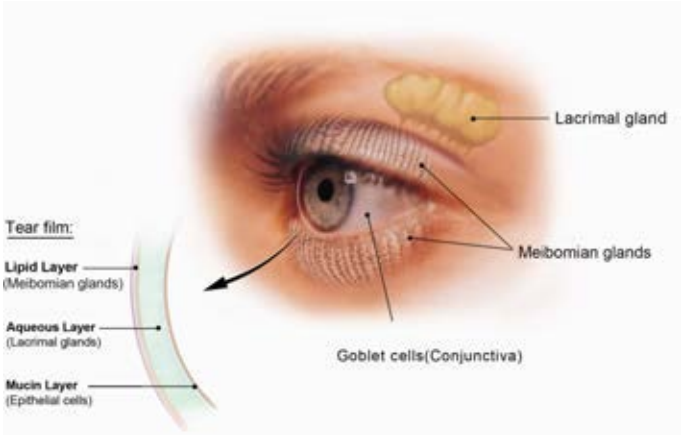

Figure 1. Structures involved in tear production. Source: National Eye Institute, image is unchanged and licensed under Creative Commons 4.0.

The tear film contains nonspecific defence compounds like immunoglobulin A (IgA), lysozyme and lactoferrin to confer protection against microbes. The cytokines and the chemokines of the tear film are important participants and mediators of the immunological reactions caused by antigens, such as microbes, viruses or allergens.

They are derived from conjunctival fibroblasts and epithelial cells as well as from inflammatory cells in the tear fluid and conjunctiva (Leonardi et al. 2006).

2.1.2 The conjunctiva

The conjunctiva is composed of the epithelium and the stroma, which is called also the substantia probria. Goblet cells in the conjunctival epithelium are specialized secretory cells that release protective mucin into the tear film (McCauley, Guasch 2015). There are many mast cells in the conjunctiva that regulate immediate hypersensitivity reactions by secreting histamine (Bonini 2006). Langerhans cells (LC) are the main antigen presenting cells (APC) in the conjunctiva. These cells control the innate and adaptive immunity on the ocular surface (Baudouin et al. 1997, Yoshida et al. 1997). The conjunctiva-associated-lymphoid-tissue (CALT) contains lymphocytes and other leukocytes; CALT is an essential part of the immune defense against microbes, interacting with mucosal epithelial cells via cytokines and chemokines (Siebelmann et al. 2013). The immunoregulatory mechanisms are an essential feature of the ocular surface epithelium to maintain homeostasis with commensal microbes and self-antigens (Stern et al. 2010).

2.1.3 The cornea

The cornea is composed of five layers: epithelium, Bowmans layer, stroma, Descemet's membrane and endothelium (see figure 2). The tight junctions of the corneal epithelium create a mechanical barrier against microbes and environmental factors like allergens (Mantelli, Mauris & Argueso 2013). The blood and lymphatic vessels as well as APC are absent in the central cornea that represent the foundation for the cornea's privileged immunological status (Ellenberg et al. 2010). The central cornea is transparent and immunological reactions are effectively down-regulated in that tissue in order to maintain this feature (Stern et al. 2010, Zhang et al. 2012). The limbal region between the conjunctiva and the cornea is the main area of immunological reactions in the cornea. Corneal stem cells grow in the limbal area and there are APC Langerhans cells, small numbers of lymphocytes and regulatory T cells (Treg) (Hazlett 1993, BCSC 2013-2014 2013), which are all key players in immunological defence.

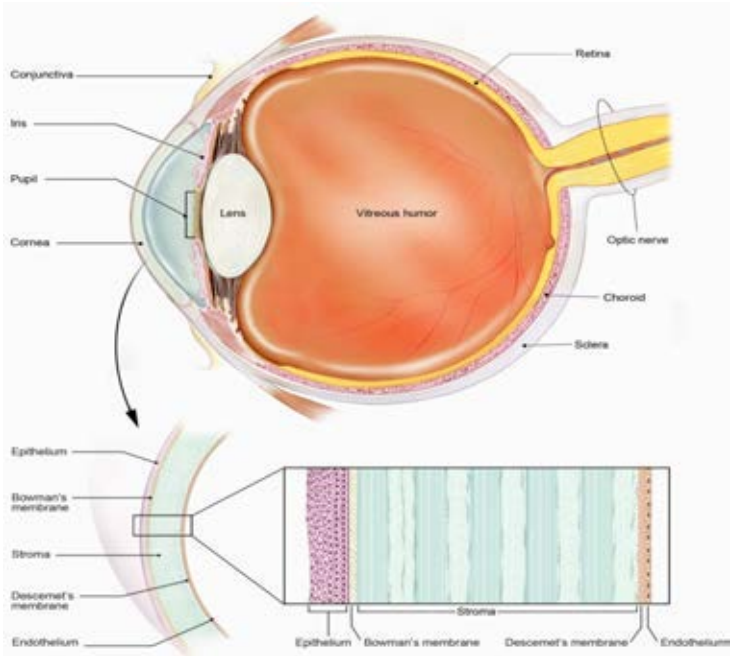

Figure 2. The structure of the eye and the cornea. Source: National Eye Institute, image is unchanged and licensed under Creative Commons 4.0.

2.2 TRANSCRIPTION FACTORS AND OCULAR SURFACE IMMUNOLOGY

Transcription factors (TFs) are nuclear proteins that regulate gene transcription in a cell nucleus. They are part of the complex signal cascades that mediate cellular signaling in response to various extra- and intra-cellular stimuli. Thus, TFs are involved in cell proliferation, differentiation and cell death. Two of the most extensively studied stress related TFs are nuclear factor-kappaB (NF-kappaB) and activator protein-1 (AP-1) (Cooper, Bowden 2007). They are present on ocular surface and tear fluid and one of their many actions is to enhance the resistance of epithelial cells to bacterial invasion and cytotoxicity by regulating innate immunity defence pathways (Mun et al. 2011).

AP-1 transcription factor is a heterodimer protein complex in mammalian cells. Its proteins belong to Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) subfamilies (Murphy, Travers & Walport 2008, 236, Shaulian, Karin 2002). The AP-1 activation is induced by physical or chemical stress, such as UV-radiation or cytokines. In simplified terms, stimulation of cell surface receptors activates the mitogen activated protein kinase (MAPK) cascade (JNK, p38), resulting in phosphorylation of c-Jun and heterodimerization with another AP-1 subunit. This protein complex binds to the AP1-target genes promoting changes in cell function (Shaulian, Karin 2002) (see Figure 3).

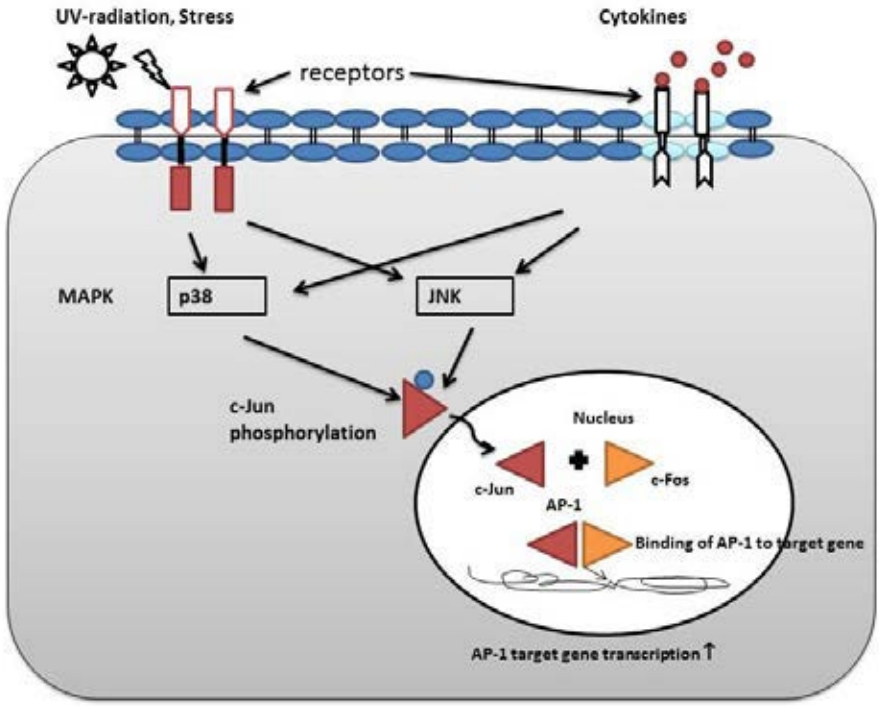

Figure 3. Activation pathway of AP-1.

NF-kappaB is also a protein complex that consists of dimeric proteins (e.g. p50, p52, and p65/RelA)). The NF-kappaB regulation is both tissue and cell specific (Halsey et al. 2007). In the canonical pathway, NF-kappaB is activated by a specific cell surface receptor which evokes the release of inhibitory IκB kinase (IKK) complex, induces phosphorylation and degradation of IκB in cytoplasmic proteasomes (Srivastava, Ramana 2009). Subsequently, the active form of the NF-kappaB protein complex translocates to cell nucleus, binds to target genes and enhances gene transcription (Lan et al. 2012) (see Figure 4).

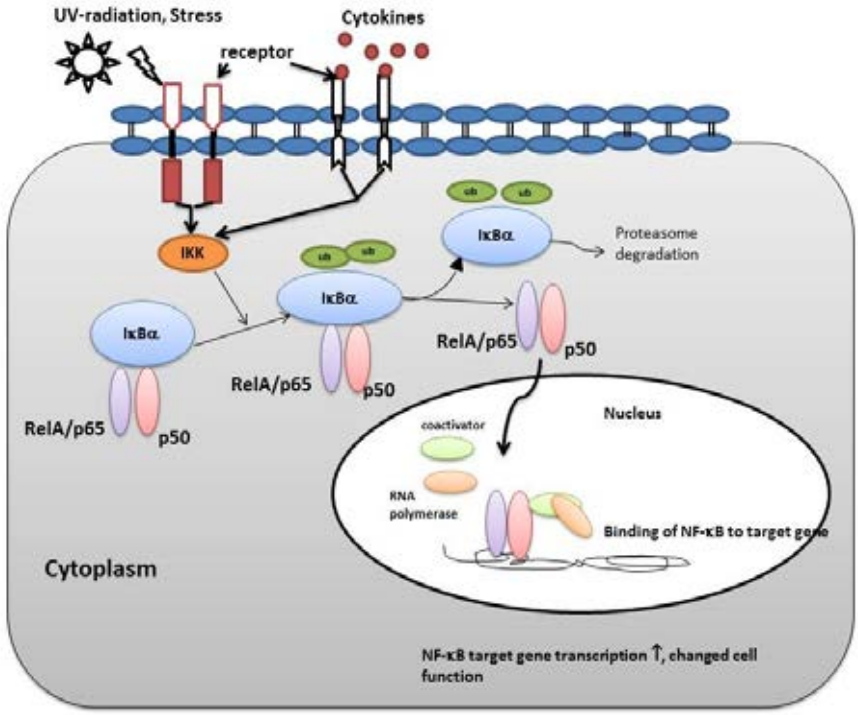

Figure 4. Canonical activation pathway of NF-kappaB.

2.2.1 AP-1 and ocular surface immunology

AP-1 is already present in the embryonic period on the mouse corneal epithelium. AP-1 is involved in the development and maturation of the corneal epithelium as well as in eyelid closure (Okada et al. 2003b, Geh et al. 2011). The AP-1 monomers c-Fos, Fra-2, and c-Jun have been found on the healthy limbal and bulbar conjunctival epithelia, whereas on the normal human corneal epithelium, c-Fos, Fra-2, FosB, c-Jun or JunB are absent (Saika et al. 1999). Furthermore, JunD is absent from normal epithelia of cornea and conjunctiva, but in the dysplastic epithelium, it is present throughout its thickness which reveals its role in tumorigenesis and cell differentiation (Saika et al. 1999). The multilayered corneal epithelium is formed through the differentiation of corneal limbal stem cells. This differentiation is regulated by two AP1 factors - Fra-1 and JunB (Adhikary et al. 2005).

AP-1 has a role in corneal wound healing and in the regulation of inflammation evoked by the damage insults on the corneal epithelium (Lake et al. 2013, Lee, Heur 2013, Shirai et al. 2014). Corneal epithelium damage results in the secretion of fibronectin and induces the expression of its integrin receptor alpha5 beta gene by altering the nuclear ratio of transcription factors such as AP-1(Gingras et al. 2009). After damage, the AP-1 (c-Fos/c-Jun) is required for the corneal epithelial healing in the early

phase, because it is implicated in the initiation of cellular movement (Okada et al. 2003a). In the corneal cells, the activation of AP-1 has been shown to be involved in inflammatory cytokine interleukin-1beta (IL-1beta)-induced matrix metalloproteinase-9 (MMP-9) expression (Tseng et al. 2013). AP-1 has a role in the differentiation of corneal epithelial cells. The absence of the AP-1 subunit, c-Fos, has been shown to disturb the formation of desmosomes which leads to weakness of the adhesions between the superficial layers of the corneal epithelium (Okada et al. 2008). This may evoke the clinical entity of recurrent corneal erosion.

In addition, AP-1 is involved in the activation of innate immunity in the conjunctival epithelial cells in a response to bacterial invasion. Bacterial infection with S. aureus activates JNK (C-Jun N-terminal kinase) by phosphorylating c-Jun which triggers the activation of innate immunity, such as IL-8 production in conjunctival epithelial cells (Venza et al. 2007). Environmental or genotoxic stress, such as UV-radiation, hypoxia and hyperosmotic pressure can also induce the AP-1 activation on the ocular surface via polo-like kinase 3 (Plk3) activation (Wang, Dai & Lu 2007, Wang et al. 2008, Wang, Dai & Lu 2011, Wang et al. 2016).

2.2.2 NF-κB and ocular surface immunology

Nuclear factor-kappaB (NF-kappaB) pathway is involved in many biological processes on the ocular surface, such as inflammation, apoptosis, stress response, corneal wound healing, angiogenesis and lymphangiogenesis. The NF-kappaB is inducible in all cell types including conjunctival, limbal and corneal epithelial cells. It mediates innate immunity response via the toll-like receptors (TLR) 2, 3, 4, 5 and 7 (Lan et al. 2012). The NF-kappaB activity is enhanced by proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), i.e. it participates adaptive immunity pathway as well (Bonizzi, Karin 2004).

In the conjunctival epithelial cells, the innate immune response to Gram-negative bacteria is initiated by toll-like receptor 4 (TLR4), which elicits the activation of the NF-kappaB pathway (Chung et al. 2009). The NF-kappaB pathway is also triggered when there are viral infections of the cornea; in this case it promotes the secretion of cytokines and chemokines from the corneal fibroblasts (Orita et al. 2013). The NF-kappaB is a master regulator of IL-6 and IL-8 expression.

NF-kappaB has also been associated in anterior ocular surface allergic reactions. In the mouse model of the allergic conjunctivitis (AC), a pollen challenge with short ragweed was shown to attenuate microRNA (miR-146a) expression and that NF-kappaB is associated in this down-regulation (Sun et al. 2015). Moreover, ragweed pollen has been reported to up-regulate thymic stromal lymphopoietin (TSLP) that induces allergic conjunctivitis in mice (Sun et al. 2015). TSLP has also been associated in activation of mucosal dendritic cells (like Langerhans cells in cornea and conjunctiva), which is a phenomenon of innate immunity.

Dry eye disease (DED) is a common anterior ocular surface inflammatory disorder. A desiccating stress has been shown to cause the NF-kappaB dependent disruption of the ocular surface immune tolerance in mouse model of dry eye (Guzman et al. 2016). The high osmotic pressure of the tear fluid is one exacerbating factor in the DED that evokes the secretion of reactive oxygen species (ROS) and leads to activation of JNK (c-Jun N-

terminal kinase) inflammatory signaling pathway. An in vitro study of rabbit corneal epithelial cells showed the above mentioned activation of the JNK pathway leading further to NF-kappaB expression and the generation of inflammatory cytokines like IL-1beta and TNF-alpha (Chen et al. 2013).

2.2.3 TNF-alpha, IL-6 and IL-8 on ocular surface

Cytokines and chemokines are multifunctional molecules secreted by different cell types including inflammatory cells, epithelial and endothelial cells and fibroblasts. These soluble chemicals are crucial participants in a large variety of biological phenomena, when regulating immune responses (D'Ambrosio, Panina-Bordignon & Sinigaglia 2003, Dinarello 2007)

Corneal epithelial cells differentiate from the limbal epithelial stem cells (LESC). LESC are under investigation as a potential treatment option of ocular surface disease. A large variety of molecules have been found in LESC cultures; these take part in cellular movement, proliferation, development, cell death, survival, adhesion and cell-to-cell signaling. In these cell cultures, IL-6 and IL-8 have been shown to be important participants in stemness, differentiation and angiogenesis (Vereb et al. 2013). In addition, the limbal stem cells are capable of producing immunoprotective glial cell-derived neurotropic factor (GDNF), which has been shown to suppress interleukin (IL) -17 -mediated inflammation via the NF-kappaB signaling pathway (Bian et al. 2010). IL-17 stimulation on the cornea surface has been implicated in the expression and secretion of inflammatory cytokines TNF-alpha, IL-6, and IL-1beta and chemokine IL-8.

Human corneal epithelial cells contain toll-like receptors ST2 that regulate Th2 type (T-cell) innate immune response. ST2 receptors are activated by interleukin 33 (IL-33), which in turn promotes expression and secretion of inflammatory cytokines, such as TNF-alpha, IL-1beta and IL-6 and chemokine IL-8 by corneal epithelial cells (Lin et al. 2013).

Conjunctival epithelial cells play an important role in immunity and inflammation in the ocular surface. These cells are known to secrete IL-6 and IL-8 and they are regulated by several factors e.g. IFN-gamma, TNF-α and IL-4 (Enriquez-de-Salamanca et al. 2008). Conjunctival epithelial cells form the first line barrier and defence against the pathogens presented to the innate immune system. For instance, gram-negative bacteria promote an inflammatory response via a toll-like receptor 4 (TLR4) on the conjunctival epithelial cells. These inflammatory reactions are mediated by NF-kappaB and subsequent IL-6 and IL-8 expressions in the conjunctival epithelial cells (Chung et al. 2009). In the response to TNF-alpha exposure, upregulation of IL-6 and IL-8 has been detected in primary conjunctival fibroblasts, (Leonardi et al. 2006) primary human conjunctival epithelial cells and immortalized conjunctival cell line (HC0597) (Smit et al. 2003). In both conjunctival fibroblasts and epithelial cell cultures, TNF-alpha stimulation has been found to induce the activation of NF-kappaB as well as the expression and secretion of IL-8 and RANTES (Regulated and Normal T cell Expressed and Secreted) (Sakai et al. 2013).

2.3 UV-RADIATION AND OCULAR SURFACE IMMUNOLOGY

In addition to the skin and its epithelial cells i.e. keratinocytes, the ocular surface cells are constantly exposed to terrestrial sunlight that contains ultraviolet (UV) visible and infrared (IR) radiation. The shortest UV wave lengths of UVC (100-280 nm) and UVB (under 295 nm) are totally filtered by stratospheric ozone layer, while UVB (295-315 nm), UVA (315-400 nm), visible and IR (800nm-1 mm) radiations influence human body. In the ey visible radiation is used for sight, while IR is detected only byt skin (Young 2006). The corneal epithelial cells are highly sensitive to UVB light-induced oxidative stress, a process that results in the production of inflammatory mediators, which have been implicated in the tissue injury (Cejkova et al. 2004). UVB modulates the expression of antioxidants and proinflammatory mediators in corneal epithelial cells. Alterations in the expression of these mediators are thought to be important in the regulation of inflammation and protection of the cornea from UVB-induced oxidative stress (Black et al. 2011). Moreover, UV is known to evoke NF-kappaB-mediated apoptosis of the human corneal epithelial cells (Lu, Wang & Shell 2003, Lee, Kim & Joo 2005, Alexander, Carlsen & Blomhoff 2006, Strozyk et al. 2006, Tong et al. 2006). In addition, UVB irradiation induces the expression of mRNA of the c-jun, c-fos and JUNB that enhance the expression of IL-6 (Isoherranen et al. 1998).

The acute clinical symptom evoked by UV radiation on the cornea is photokeratitis. It is a painful condition attributable to inflammatory damage to corneal epithelium caused by UVC and UVB (Cullen 2002, Dolin, Johnson 1994). UV radiation promotes an acceleration of the physiological loss of surface cells (Ren, Wilson 1994). Suprathreshold radiation exposure results in the full thickness loss of the stratified epithelium on the basement membrane and exposed nerve fiber endings, resulting in the severe pain of photokeratitis (Cullen 2002).

A climatic droplet keratopathy (CDK) is strongly associated with chronic UV radiation. One characteristic feature of CDK is scarring and elevated matrix metalloproteinase (MMP)-2 and MMP-9 levels in tears that have been implicated in the failure of corneal re-epithelialization (Holopainen et al. 2009). Decreased levels of tissue inhibitors of MMP (TIMP-1) have been measured in the tears of CDK patients (Holopainen et al. 2009).

An excess exposure to sunlight (UV-light) may predispose to the development of pterygium, which is a proliferative, inflammatory fibrovascular tissue on the conjunctiva and superficial cornea. Both NF-kappaB pathways, canonical and non-canonical have been shown to be activated in the pterygia tissues (Siak et al. 2011, Torres et al. 2011). UV-induced inflammatory cascade mediated by ERK1/2, JNK, and p38 mitogen-activated protein kinases (MAPK) lead to increased production of IL-6, IL-8, and VEGF in the pterygium epithelial cell cultures (Di Girolamo, Wakefield & Coroneo 2006).

The ocular surface squamous neoplasia (OSSN) typically arises at the limbus within the interpalpebral fissure and it is strongly associated with UV-radiation induced mutations in tumor-suppressor gene p53. The UV-radiation causes photo-immunosuppression both locally and systemically that may lead to reactivation of latent OSSN-associated viruses, such as human papilloma virus (HPV) (Gichuhi et al. 2014).

2.4 OCULAR SURFACE INFLAMMATORY DISEASE

2.4.1 Ocular surface allergy

If the eye becomes exposed to various environmental allergens, it can trigger inflammatory processes, such as the mast cell degranulation, histamine release, and the production of inflammatory regulating chemokines. Ocular allergy can be divided into IgE- and cell mediated allergies (Friedlaender 2011) or acute and chronic forms. Allergic conjunctivitis (AC) can be subdivided into the acute Ig-E mediated seasonal allergic (SAC) and perennial allergic conjunctivitis (PAC) and to the chronic Ig-E and cell mediated vernal (VKC) and atopic keratoconjunctivitis (AKC) (Friedlaender 2011, Bielory et al. 2016).

The symptoms of allergic conjunctivitis in its acute forms are itching, redness (hyperemia), swelling (chemosis) of the conjunctiva and eyelids as well as tearing and discharge. In chronic AKC and VKC, there may be Horner- Trantas dots in the limbus, giant papillae on the tarsal conjunctiva and corneal plaque (BCSC 2013-2014 2013, Friedlaender 2011, Bielory 2011). AC can cause permanent damage to the ocular surface in the severe and chronic forms of AKC and VKC. The pathophysiology of the AC is closely linked to mast cell penetration and their secreted products (e.g. histamine, proteases, cytokines and growth factors) released in immediate type 1 hypersensitivity IgE response upon allergen challenge (Irkec, Bozkurt 2012). The interaction between conjunctival epithelial cells and intraepithelial mast cells leads to degranulation of mast cells and the release of histamine as well as the production of chemokines (Iwamoto et al. 2013). Therefore, the mast cells are an important target of drug treatment in allergic conjunctivitis. In addition to anti-histamine actions, certain anti-allergic drugs have an ability to inhibit the release of pro-inflammatory cytokines (Yanni et al. 1999, Galatowicz et al. 2007).

Infiltrating immune-cells (neutrophils, eosinophils and lymphocytes) may also be involved in the responses of the acute reactions. They are capable of producing soluble mediators of inflammation and thus, the tear fluid cytokine and chemokine levels are increased in all allergic conjunctivitis patient groups: SAC, AKC and VKC. In particular, the levels of IL-1beta, IL-2, IL-5, IL-6, IL-12, IL-13, monocyte chemoattractant protein (MCP)-1 are increased in all subtypes of allergic conjunctivitis. Some of these soluble molecules, such as IL-6, IL-8, MCP-1, RANTES and eosinophil chemotactic proteins (eotaxin), can be detected from conjunctival fibroblasts (Leonardi et al. 2006).

2.4.2 Dry eye disease

Dry eye disease (DED) is the most common reason for ocular surface inflammation (Gilbard 2005). DED is a multifactorial disease of the ocular surface characterized by loss of homeostasis of the tear film such as tear film instability and hyperosmolarity. DED is associated with inflammation of the ocular surface and an increased risk of secondary infections. In DED, there is increased production of proinflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and proteolytic enzymes released not only from the ocular surface and glandular epithelial cells but also from the infiltrating inflammatory cells.

The levels of immunological mediators are increased in the tears of DED patients (Lam et al. 2009, Yoon et al. 2007). IL-6 and IL-8 display an association with clinical symptoms and signs of DED such as pain (Enriquez-de-Salamanca et al. 2010), an altered Schirmer test result and conjunctival staining (Enriquez-de-Salamanca et al. 2010, Huang et al. 2012), and irritation symptoms (Lam et al. 2009). Instead, no association between the TNF-alpha concentration and clinical signs and symptoms were detected in two clinical studies (Lam et al. 2009, Yoon et al. 2007). In severe forms of DED, Sjögren syndrome (SS), there is a loss of secretory function and a decrease in the numbers of mucin secreting goblet cells. A dysfunction of goblet cells is suggested to be related to over-expression of pro-apoptotic and inflammatory markers (Brignole et al. 2000). The IL-6 and the TNF-α levels are higher in non-SS dry eye patients compared to healthy subjects. Furthermore, the IL-6 level in the tears of SS dry eye patients is significantly higher compared to the corresponding value in non-SS dry eye patients (Yoon et al. 2007). In a mouse model mimicking SS, the apoptosis of goblet cells was enhanced by TNF-α and IFN-gamma, which also inhibited mucin production (Contreras-Ruiz et al. 2013). In this model, IL-6 was found to increase the mucin production and the proliferation of goblet cells (Contreras-Ruiz et al. 2013). The lowered mucin level (Mucin 5AC) is correlated to the more severe clinical symptoms as well as to the higher IL-6 level in DED (Zhang, Yan & Li 2013, Na et al. 2012). Thus, the higher IL-6 level might be a protective reaction to the lowered mucin secretion.

Hyperosmolarity is a common finding in DED. Even a short term hyperosmolar stress causes the increased levels of IL-6 secretion in human corneal epithelial (HCE) cell cultures (Igarashi et al. 2014). Similarly, hyperosmolarity has been linked with increased levels of IL-8 and TNF-α in human limbal epithelial cell (HLEC) cultures. This signaling is mediated through the JNK and ERK/MAPK signaling pathways (Chen et al. 2013, Li et al. 2006). The hyperosmolarity may cause ocular surface cell-death in inflammatory disorders, which occurs via mitochondrial dysfunction related to apoptosis mediated by caspase-3/7 and -9 (Wang, Dai & Lu 2011, Png et al. 2011).

2.4.3 Cell culture models of ocular surface diseases

Ocular surface cell cultures, primarily conjunctival and corneal cell cultures, have been widely used to explore the basic cell biology and the molecular responses of these tissues under different exposures as well as pharmacological and toxicological mechanisms of drugs (Barar et al. 2009, Castro-Munozledo 2008, Civiale et al. 2003). The cell proliferation and viability tests are the basic biology assessments conducted in these experiments (Rougier et al. 1992, Seaman et al. 2010) and these methods have been used to test the effects of external exposures like UV-radiation (Buron et al. 2006). Human conjunctival epithelial cells (HCEC) have been shown to produce proinflammatory cytokines in a dose- and time-dependent fashion after stimulation; for this reason they have been widely exploited as a platform of ocular surface disease pathology (Igarashi et al. 2014, Gamache et al. 1997) and drug target studies. The human conjunctival epithelial cells have also commonly been used to test the antihistaminic activity of ocular agents (Yanni et al. 1999). The protection capability of artificial tears used in DED against desiccation stress has been studied in a human conjunctival cell culture as well (Paulsen et al. 2008). Human corneal epithelial cells (HCE) exposed to hyperosmotic stress have been used to examine the effects of osmoprotectant substances on MMPs

functions, which have been implicated to aggrevate DED (Deng et al. 2014). It is mandatory to undertake these in vitro experiments before embarking on in vivo tests in an experimental animal model and further on to clinical trials.

2.4.4 Experimental animal models of ocular allergy

Experimental animal models have been developed in attempts to elucidate the etiopathogenesis of the ocular allergy and in the search for new therapies. They can be divided into non-IgE- and IgE-mediated models of allergic conjunctivitis. IgE- mediated allergic models are based on active immunization with an antigen challenge (see Figure 5). The most common route of active immunization in allergic conjunctivitis models is systemic injection i.e. intravenous, intraperitoneal or intramuscular administration (Calonge, Siemasko & Stern 2003). In this model, the first systemic dose of antigen initiates the production of antibodies and after the time required to produce antibodies (7-15 days), the second challenge dose of same antigen produces the clinical signs and symptoms of allergic reaction. The challenge dose in allergic conjunctivitis models is typically dosed topically to the conjunctiva (Lee, Kwon & Joo 2016, Cordova et al. 2014). Ovalbumin has been the most frequently used antigen in systemic immunization but also ragweed pollen extract has been utilized (Magone et al. 2000). Topical immunization, such as is conducted in the ragweed pollen exposure model of conjunctiva, mimics better human allergic conjunctivitis, but it has been less extensively used (Merayo-Lloves, Calonge & Foster 1995).

Non-IgE mediated model of allergic conjunctivitis can be produced with a mast-cell degranulating agent like compound 48/80 (Udell, Abelson 1981, Udell et al. 1989). It is dosed topically to the conjunctiva of the experimental animal and it evokes the signs and symptoms of acute allergic conjunctivitis (Tiligada et al. 2000, Li et al. 1996). This model has been exploited to examine the mast-cell degranulation inhibitory effects of drugs (Whitcup et al. 1996, Khosravi, Elena & Hariton 1995, Janssens 1992).

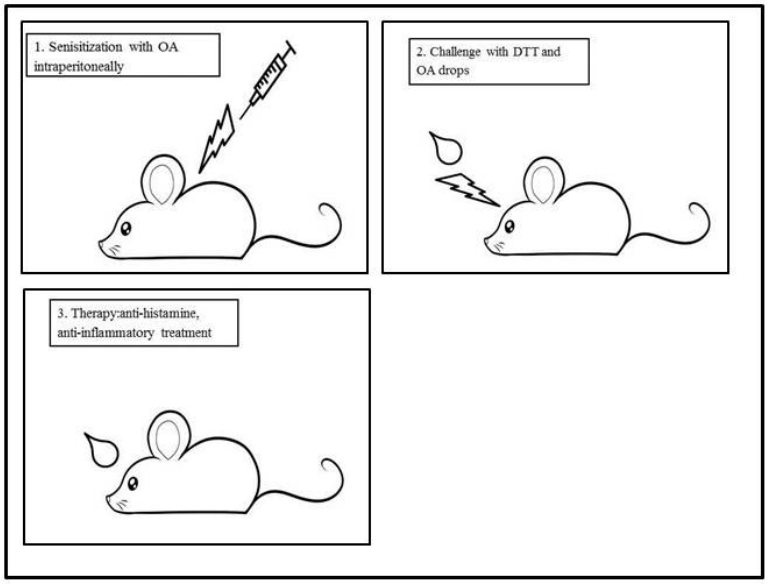

Figure 5. Ig-E mediated experimental animal model of ocular allergy.

2.5 CIS-UROCANIC ACID

2.5.1 Basic mechanisms of action

Urocanic acid (UCA) is a chromophore, which is present in mammalian epidermis. It is formed from histidine in the outermost layers of epidermis, when keratohyalin granules degrade and the enzyme, histidase (histidine ammonia-lyase), is activated (Norval, El-Ghorr 2002). It undergoes photoisomerizion from epidermal trans-urocanic acid (trans-UCA) into cis-urocanic acid (cis-UCA; 3-(1H-imidazol-4-yl)prop-2-enoic acid) upon terrestrial UV radiation exposure consisting of UV-A (320-40 nm) and UV-B (290-320 nm) wavelengths (see Figure 6). The action spectrum is 280-310 nm for maximal cis-UCA production (McLoone et al. 2005). UV exposure may elevate the dermal cis-UCA concentration for 1-3 weeks; its elimination occurs by desquamation and excretion into sweat and urine (Norval, El-Ghorr 2002). Thus, cis-UCA can be detected also in serum (Moodycliffe et al. 1993) and urine (Kammeyer et al. 1995) due to its diffuse penetration into subepidermal tissue.

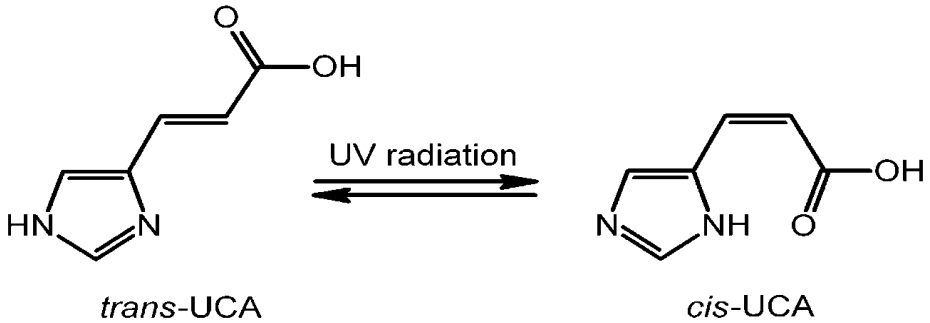

Figure 6. Transformation of urocanic acid from trans- to cis-UCA

The immunosuppressive effect of cis-UCA was initially thought to be mediated through histamine H1 and H2-like receptors in the skin (Norval, Gilmour & Simpson 1990, Gilmour et al. 1992). Subsequently, it has been postulated that cis-UCA might be a serotonin (5-HT) receptor ligand. It has been suggested that its immunosuppressive effects could be mediated through activation of the 5-HT2A receptor (Walterscheid et al. 2006). However, Kaneko with his co-workers have observed that cis-UCA stimulates prostaglandin E(2) (PGE(2)), tumor necrosis factor-alpha (TNF-alpha), and IL-6 secretion independent of 5-HT and platelet activating factor (PAF) receptors in human keratinocytes (Kaneko et al. 2008, Kaneko et al. 2009). cis-UCA induces intracellular reactive oxygen species (ROS) production in human keratinocytes, which in turn has been shown to modulate epidermal growth factor (EGFR) signaling, leading to prostaglandin E2 (PGE(2)) synthesis and apoptotic cell death (Kaneko et al. 2011). This intracellular ROS production is proposed to be mediated by cis-UCA's direct action on NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidase in cell membranes of keratinocytes (Kaneko et al. 2011). On the other hand, one experiment conducted using bovine neutrophils showed that cis-UCA inhibited the generation of extracellular superoxide upon neutrophil activation, but had no effect on the generation of intracellular superoxide or other ROS, i.e. it preserved the bactericidal activity of neutrophils (Rinaldi et al. 2006).

Cis-urocanic acid suppresses the development of contact type hypersensitivity and leucocyte adherence inhibition. Cis-UCA has been shown to stimulate T suppressor cells and cell-mediated immunosuppression, at least after short-term irradiation (Harriott-Smith, Halliday 1988). Moreover, the contact hypersensitivity suppression is caused by local release of TNF-alpha and alteration of morphology of epidermal Langerhans cells, which prevents the immunogenic signal reaching draining lymph nodes and inhibits the activation of adaptive (Ig-mediated) immunity (Kurimoto, Streilein 1992a, Kurimoto, Streilein 1992b). This APC presenting cell impairment has been observed in vivo in mice at one week after cis-UCA exposure, indicating that it represents a delayed type reaction (Noonan, De Fabo & Morrison 1988). In addition, dermal mast cells are essential in mediating both UVB-induced systemic contact hypersensitivity and delayed-type immunosuppressive reactions. Cis-UCA has been found to exert immunomodulatory effects on both the dermal mast cell number and their susceptibility to cis-UCA (Hart et al. 1999). The wide spectrum of cellular effects of

cis-UCA includes also inhibition of IL-10 secretion in T lymphocytes (Bi, Xia & Ni 1999). Moreover, cis-UCA has been postulated to be involved in the downregulation IL-1 production in monocytes and IL-2 production in T-cells (Rasanen et al. 1989) and thus it also influences humoral immune responses.

The effects of cis-UCA seem to be cell type dependent and the molecular mechanisms of action of cis-UCA are not fully understood. It has been hypothesized that induction of immune suppression by cis-UCA may involve the initiation of gene transcription of immunomodulatory mediators. The molecular mechanisms of cis-UCA have been investigated in primary human keratinocytes to examine changes in gene expression and synthesis of cytokines. Cis-UCA has been found to upregulate many of the genes that are associated with apoptosis, cell growth arrest, cytokine production and oxidative stress reactions (Kaneko et al. 2008). Moreover, the effects of cis–UCA on gene transcription have been investigated in Jurkat cells (human T-lymphocyte cell line) and in normal human epidermal keratinocytes. The main finding was that cis-UCA upregulated the expression of a gene encoding a beta-galactoside-binding lectin, galectin-7 (LGALS7B), which has been implicated in increasing apoptosis and inhibiting the expression of mRNA coding for interleukin-2 (IL-2) and interferon-gamma (IFNG) (Yamaguchi et al. 2013).

2.5.2 Clinical studies

Cis-UCA has been of interest from a clinical perspective and it has been evaluated in many experimental models and clinical trials. The effects of topical cis-UCA cream (2.5% and 5% concentrations) have been compared to tacrolimus 0.1% and hydrocortisone 1% in the acute and subacute mouse skin irritation models. Cis-UCA suppressed in both models mouse ear skin swelling more potently than either tacrolmius or hydrocortisone when tested 1 hour after the start of the treatment (Laihia et al. 2012). Subsequently, cis-UCA's efficacy, safety and tolerability in inflammatory skin disease was tested in a randomized vehicle-controlled double-blinded clinical trial; this revealed that cis-UCA was well tolerated both among healthy subjects and in patients mild to moderate atopic deramtitis (AD) (Peltonen et al. 2014). It also reduced transepidermal water loss both in healthy subjects and AD patients. Furthermore, the AD group was evaluated clinically by physicians with the eczema area severity index and a global assessment score. Both indicators of AD severity improved from baseline and cis-UCA also improved skin barrier function and suppressed inflammation (Peltonen et al. 2014).

Inflammatory bowel disease (IBD) has been modelled in mice by evoking colitis by administering DSS (dextran sodium sulfate) and using genetically modified IL10(-/-) mice. These two models have been used to investigate cis-UCA's effects on multiple inflammatory reactions, which are apparent in these models as well as in clinical IBD. The administration of cis-UCA subcutaneously was able to reduce weight loss and to attenuate changes in colon length and weight. It also reduced colonic expression of CXCL1 (chemokine (C-X-C motif) ligand 1) and increased the expression of IL-17A. Cis-UCA also preserved the numbers of splenic T-regulatory cells (CD4+CD25+FoxP3+), which were reduced with DSS treatment (Albert et al. 2010). These cell reactions may be

beneficial in suppressing inappropriate innate immunity activity, which is mimicked in the DSS mouse model of IBD.

Cis-UCA is capable of acidifying the cytosol by transporting protons into the cell in a mildly acidic extracellular environment. This protodynamic property influences many cellular activities such as causing an inhibition of cell proliferation and promoting the induction of apoptosis (Laihia et al. 2009); this phenomenon has been utilized in cancer cell culture experiments. The effects of cis-UCA treatment have been investigated in 5367 bladder carcinoma cell culture. A significant antiproliferative effect was exhibited: cis-UCA caused dose-dependent irreversible termination of the proliferation of cancer cells already two hours after treatment (Laihia et al. 2009). Cis-UCA's antiproliferative characteristics were further investigated in the same cell culture and cis-UCA was demonstrated to cause both apoptotic and necrotic cell death (Peuhu et al. 2010). A blockade of the cell cycle and reduced metabolic activity with increased ERK1/2 and JNK1 activities were also detected after cis-UCA treatment causing cytostatic and cytotoxic effects in bladder carcinoma cells (Peuhu et al. 2010). In addition, the protodynamic action of cis-UCA has been shown to dose-dependently diminish the number of viable human melanoma, cervical carcinoma, and fibrosarcoma cells at a weakly acidic extracellular pH. Further experiments with A2058 melanoma cells showed that cis-UCA caused apoptosis also in this carcinoma cell population (Laihia et al. 2010).

3 Aims of the Study

The general aim of this thesis was to investigate mechanisms and effects of cis-UCA treatment on ocular surface cells, experimental animal models and healthy volunteer subjects.

The specific aims were as follows:

I To examine the effects of cis-UCA on immunological reactions after UV-B exposure in ocular surface cell cultures (Publication I and II)

II To investigate the effects of cis-UCA on clinical, histopathological and biological parameters in IgE- and non-IgE- mediated experimental animal models of ocular allergy (Publication III)

III To evaluate the safety, tolerability and pharmacokinetics of cis-UCA 0.5% and 2.5% eye drops in healthy adult subjects (Publication IV)

4 Cis-Urocanic Acid Suppresses UV-B-induced Interleukin-6 Secretion and Cytotoxicity in Human Corneal and Conjunctival Epithelial Cells in Vitro

ABSTRACT

Purpose: Urocanic acid (UCA) is a major ultraviolet (UV)-absorbing endogenous chromophore in the epidermis and is also an efficacious immunosuppressant. The anti-inflammatory and cytoprotective effects of cis-UCA were studied in ocular surface cell cultures exposed to UV-B irradiation.

Methods: Human corneal epithelial cells (HCE-2) and human conjunctival epithelial cells (HCECs) were incubated with 10, 100, 1,000, and 5,000 μg/ml cis-UCA with and without a single UV-B irradiation dose. The concentrations of IL-1β, IL-6, IL-8, and TNF-α in the culture medium and caspase-3 activity in the cell extract sampled were measured by enzyme-linked immunosorbent assay (ELISA). Cell viability was measured by the colorimetric MTT (3-(4,5-dimethyldiazol- 2-yl)-2,5-diphenyltetrazolium bromide) assay.

Results: UV-B irradiation multiplied interleukin IL-6 and IL-8 secretion levels in HCE-2 cells and HCECs as analyzed with ELISA. Cell viability as measured by the MTT assay declined by 30%–50% in HCE-2 cells and by 20%–40% in HCECs after UV-B irradiation. Moreover, UV-B increased caspase-3 activity in both cell types as analyzed with ELISA. Treatment with 100 μg/ml cis-UCA completely suppressed IL-6 and IL-8 secretion, decreased caspase-3 activity, and improved cell viability against UV-B irradiation. No significant effects on IL-6 or IL-8 secretion, caspase-3 activity, or viability of the non-irradiated cells were observed with 100 μg/ml cis-UCA in both cell types. The 5,000 μg/ml concentration was toxic.

Conclusions: These findings indicate that cis-UCA may represent a promising anti-inflammatory and cytoprotective treatment option to suppress UV-B-induced inflammation and cellular damage in human corneal and conjunctival epithelial cells.

4.1 INTRODUCTION

Urocanic acid (UCA) is a major ultraviolet (UV)-absorbing chromophore in the epidermis, and it has been proposed to function as a regulator of UV-induced damage in photoimmunology (Gibbs, Tye & Norval 2008). The cis-UCA, formed from trans-UCA upon UV-B exposure, has been implicated in the downregulation of hypersensitivity reactions (Prater, Blaylock & Holladay 2003, Lauerma, Aioi & Maibach 1995) in the actions of epidermal antigen-presenting cells (Hart, Grimbaldeston & Finlay-Jones 2000, el-Ghorr, Norval 1997), the activation of neutrophils (Kivisto et al. 1996, Rinaldi et al. 2006), and the prolonged survival of organ transplants (Guymer, Mandel 1993), but the mechanisms of action still remain to be resolved.

Ocular surface cells including corneal and conjunctival cells are frequently exposed to UV radiation, which may evoke epithelial damage, cell death, and inflammation (Lu, Wang & Shell 2003, Gaton et al. 2007). Photokeratoconjunctivitis, pinguecula, pterygium, nodular band keratopathies, and epidermoid carcinoma are believed to be the result of exposure to intense UV radiation of the ocular surface (Wittenberg 1986). The dry eye syndrome is the most common reason for ocular surface inflammation (Gilbard 2005, Bielory 2004). Dry eye is associated with irritation of the ocular surface and increased risk of secondary infections (Versura et al. 1999). In the multifactorial dry eye syndrome, there appears to be increased production of proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α), and proteolytic enzymes. These cytokines are released not only from the ocular surface and glandular epithelial cells but also from the infiltrating inflammatory cells (Yoon et al. 2007, Tishler et al. 1998, Pflugfelder et al. 1999, Solomon et al. 2001, Narayanan, Miller & McDermott 2003). In this study, we have investigated the role of cis-UCA in IL-1β, IL-6, IL-8, and TNF-α immune response and cytotoxicity that are important factors of inflammatory reactions in ocular cell types. We show that cis-UCA suppresses UV-B-induced inflammation and cellular damage in human corneal and conjunctival epithelial cells.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture

Human corneal epithelial cells (HCE-2) and human conjunctival epithelial cells (HCECs) were obtained from American Type Culture Collection (Manassas, VA). The HCE-2 cells were cultured in Keratinocyte-SFM medium with supplements (25 mg bovine pituitary extract and 2.5 μg human recombinant epidermal growth factor; Gibco Invitrogen, Paisley, UK) including 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco Invitrogen), and 0.005 mg/ml insulin (Sigma-Aldrich, St. Louis, MO). HCECs were cultured in Medium 199 (Gibco Invitrogen) supplemented with serum and antibiotics as described above. Confluent cultures of both cell lines were treated with concentrations of 10, 100, 1,000, and 5,000 μg/ ml cis-UCA (BioCis Pharma, Turku, Finland) and exposed to a UV-B irradiation dose of 153 mJ/cm2 (four TL 20W/12 tubes, Philips, Eindhoven, The Netherlands) in 12

well plates. The irradiation was performed at room temperature for 1 min using a source-to-target distance of 30 cm.

4.2.2 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-1β, IL-6, IL-8, and TNF-α in the culture medium and caspase-3 activity in the cell extract sampled at 24 h, 48 h, or 72 h were measured by enzyme-linked immunosorbent assay (ELISA) using OptEIA™ sets from BD PharMingen (San Diego, CA) according to the manufacturer's instructions.

4.2.3 MTT assay

Cell viability was assessed at 24 h, 48 h, or 72 h after the start of the treatments by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test (Sigma-Aldrich) in 12 well plates. Briefly, 25 μl of MTT solution (10 mg/ml in PBS) was added to 500 μl of culture medium and incubated for 1.5 h at 37 °C. Then, 525 μl of MTT lysis buffer (20% SDS, 50% dimethylformamide, pH 4.7) was added to the wells to induce cellular lysis. The colorimetric assay is based on the ability of viable cells to metabolize MTT. The absorbance was measured at 550 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA).

4.2.4 HPLC analysis

Culture medium samples taken at the end of the experiment were assayed for cis- and trans-UCA. Proteins in the 200 μl medium samples were precipitated with 400 μl of trichloroacetic acid on ice for 10 min, centrifuged (12,800× g, 10 min, 4 °C), and analyzed with HPLC using the Agilent 1100 UV detection system (Agilent Technologies, Santa Clara, CA) at 268 nm.

4.2.4 Statistical analysis

The statistical significance was analyzed with SPSS for Windows software (v. 11.5; SPSS, Chicago, IL, USA) using Mann–Whitney U-test. p-values below 0.05 were considered significant.

4.3 RESULTS

In non-irradiated cells, the 10 and 100 μg/ml concentrations of cis-UCA had only a negligible effect on IL-6 production while treatment with 1000 μg/ml of cis-UCA evoked a mild but significant elevation on IL-6 levels in both cell types. A major decrease in IL-6 secretion was observed with 5,000 μg/ ml cis-UCA in all time points (Figure 7A,B). Exposure of the HCE-2 cells and HCECs to UV-B irradiation induced a maximum sevenfold to ninefold increase and twofold increase in the measured IL-6 concentrations, respectively. Treatment of both cell lines with cis-UCA at all studied concentrations significantly decreased the UV-B-induced IL-6 secretion (Figure 8A,B). The 100 μg/ml concentration of cis-UCA completely restored the UV-B-induced increase in IL-6 secretion back to the level detected in the non-irradiated cells after the 24-, 48-, and 72-h follow-up. IL-1β could not be detected in the control, cis-UCA-treated, or UV-B-irradiated cells (data not shown).

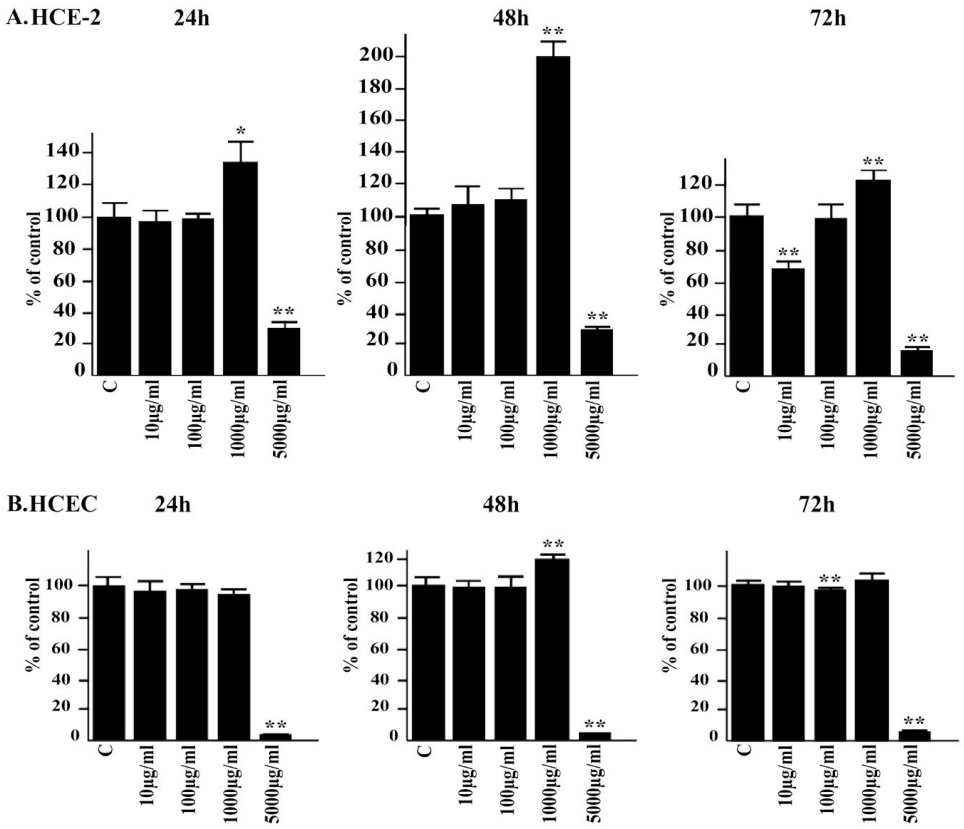

Figure 7. Effect of cis-UCA on IL-6 secretion. The HCE-2 cells (A) and HCEC cells (B) were either untreated (C) or exposed to different concentrations of cis-UCA for 24, 48 or 72 hours. For statistical analysis, cis-UCA treted samples were compared with C samples. An asterisk indicates $p < 0.05$, and double asterisk denotes $p < 0.001$ ($n = 6$ dishes).

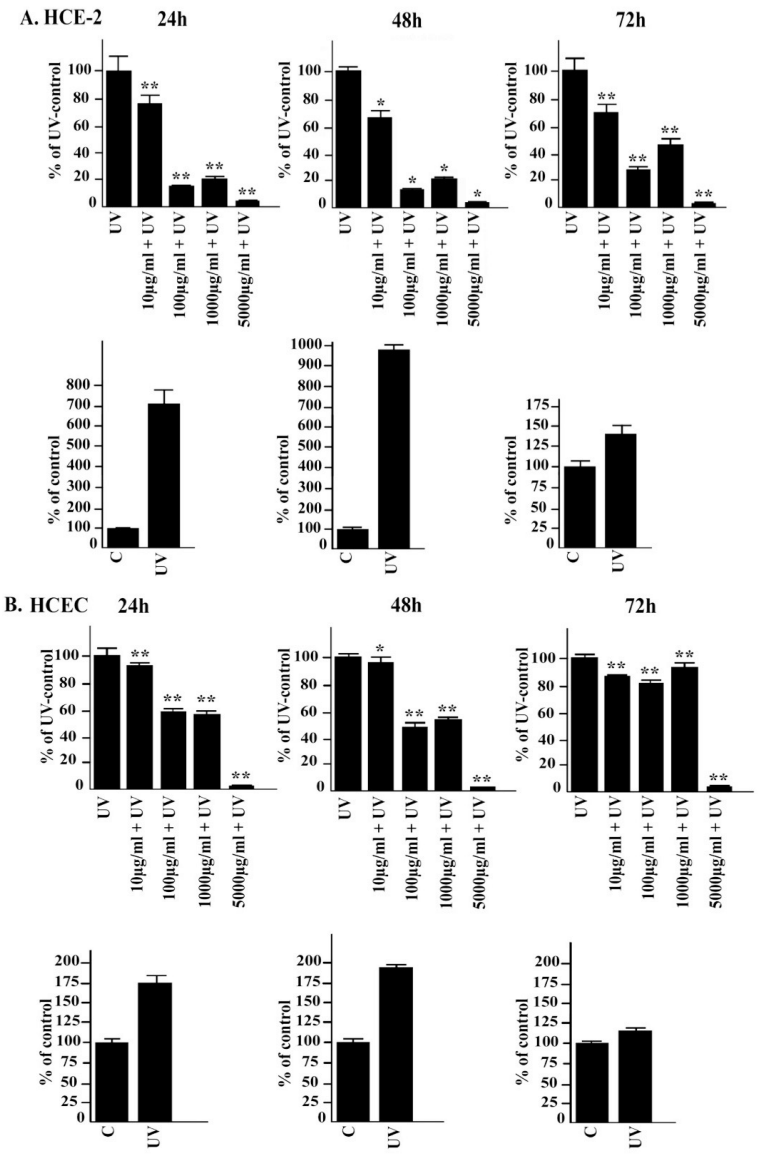

Figure 8. Effect of cis-UCA on UV-irradiation-induced IL-6 secretion. The HCE-2 cells (**A**) and HCEC cells (**B**) were non-irradiated (C) or UV-irradiated (UV;lower panels), or exposed to different concentrations of cis-UCA or UV irradiated and treated with cis-UCA for 24 , 48, or 72 hours (upper panels). For statistical analysis, cis-UCA+UV samples were compared with UV samples. An asterisk indicates $p < 0.05$, and double asterisk denotes $p < 0.001$ (n = 6 dishes).

Analysis of cell viability by the MTT assay revealed no significant effect in response to 10 and 100 μg/ml cis-UCA in the non-irradiated cells during the 24-, 48-, and 72-h follow-up (Figure 9A,B). The 1,000 μg/ml concentration evoked a slight decrease in viability in the HCE-2 cells but not in HCECs while exposure to 5,000 μg/ml cis-UCA caused a clear reduction of MTT metabolism, evidence of increased cytotoxicity in both cell types (Figure 9A,B). Exposure of the cells to UV-B decreased the viability by 20%–50%. Interestingly, the 100 μg/ml concentrations of cis-UCA restored the metabolic activity of the UV-irradiated cells to the level of the non-irradiated cells in all time points (Figure 9A,B), pointing to the presence of a cytoprotective effect of cis-UCA.

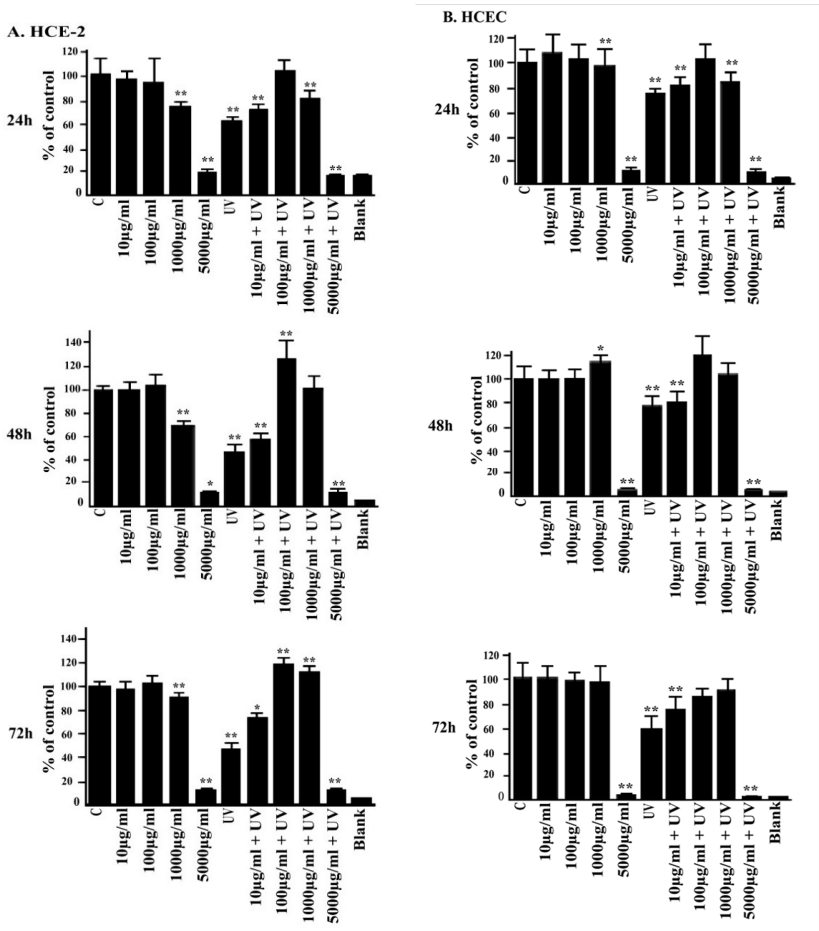

Figure 9. Effects of cis-UCA and UV irradiation on viability. The non-irradiated (C) and UV-irradiated (UV) HCE-2 cells (**A**) and HCEC cells (**B**) were exposed to different concentrations of cis-UCA (c-UCA) for up to 72 hours. For statistical analysis, cis-UCA samples and cis-UCA+UV samples were compared with C samples. An asterisk indicates $p < 0.05$, and double asterisk denotes $p < 0.001$ ($n = 6$ dishes).

In the functional assays with HCE-2 cells and HCECs, cis-UCA was present in the culture medium at the time of irradiation and during the 24 h recovery period. Since the UCA isomers absorb in the UV-B wavelength region and can photoisomerize to each other (Figure 10A), it was investigated whether photoisomerization had taken place in the experiments and could have affected the biological response. A total of 16 medium samples from the assays were subjected to HPLC analysis. The mean concentration of cis-UCA in the cis-UCA-treated (100 μg/ml) medium samples was 87.8 μg/ml (HCE-2) and 90.5 μg/ml (HCEC). The rest of the cis-UCA had apparently been taken up by the cells. Trans-UCA was detected in the non-irradiated samples at levels of 6.4% in HCE-2 cells and 4.0% in HCECs from the total UCA (Figure 10B,C). After exposure to UV-B irradiation as shown above, the net photoisomerization to trans-UCA was 13.1% in HCE-2 cells and 11.1% in HCECs. The cis-UCA concentrations were 72.3 μg/ml in HCE-2 cells and 74.9 μg/ml in HCECs in response to UV-B (Figure 10). This level of photoisomerization was estimated to have a negligible effect on cytokine secretion and viability.

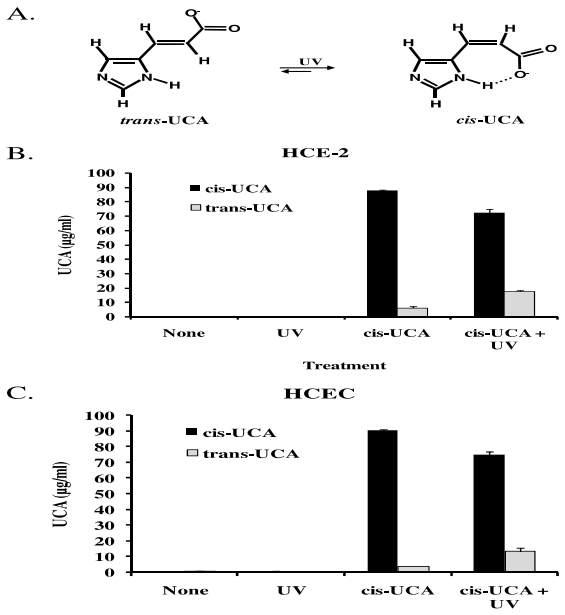

Figure 10. Photoisomerisation of UCA. UV excitation of one isomer leads to formation of the other isomer (A). The concentrations of UCA isomers are measured in the cell culture medium of HCE-2 (**B**) and HCEC cells (**C**) treated with 100 μg/ml cis-UCA for 24 hours with or without of UV irradiation.

Next, the effect of phenol red on IL-6 secretion in response to UV-B irradiation of the cells was examined. The cells were irradiated in a colorless buffer solution or in a normal culture medium prior to change of medium without or with 100 μg/ml cis-UCA and were then followed for 24 h. As shown in Figure 11, no significant differences between the effects of the phenol red-containing medium and the colorless solution on IL-6 secretion can be seen in ocular surface cells.

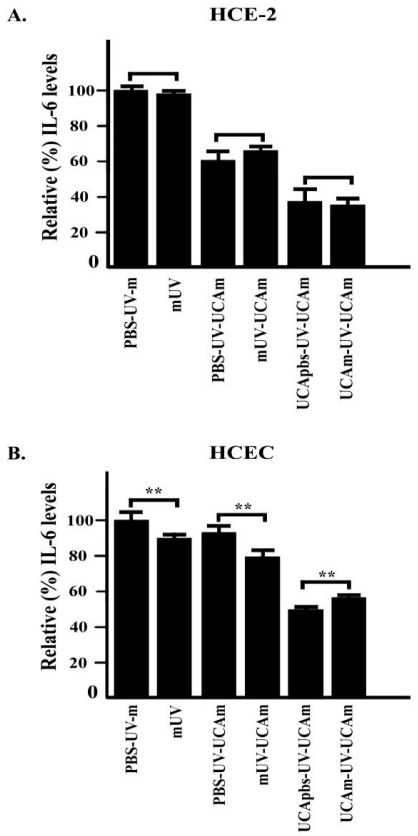

Figure 11. UV absorption effect of culture medium on IL-6 secretion. The HCE-2 cells (**A**) and HCEC cells (**B**) were irradiated in a colorless buffer solution or in normal culture medium prior to change of medium without or with 100 μg/ml cis-UCA and were then followed for 24 hours. An asterisk indicates $p < 0.05$, and a double asterisk denotes $p < 0.001$ ($n = 6$ dishes). Abbreviations: C, control; m, medium; PBS, phosphate buffered saline; UCA, urocanic acid; UV, ultraviolet.

The 100 μg/ml concentration of cis-UCA completely restored the UV-B-induced increase in IL-6 secretion (Figure 8). Therefore, we wanted to analyze IL-8 and TNF-α secretion in similar conditions. Exposure of the HCE-2 cells and HCECs to UV-B irradiation induced a four- to fivefold and threefold increase in the measured IL-8 concentrations, respectively. Treatment of both cell lines with 100 μg/ml cis-UCA significantly decreased the UV-B-induced IL-8 secretion (Figure 12A,B). TNF-α could not be detected in the control, cis-UCA-treated, or UV-B-irradiated cells (data not shown).

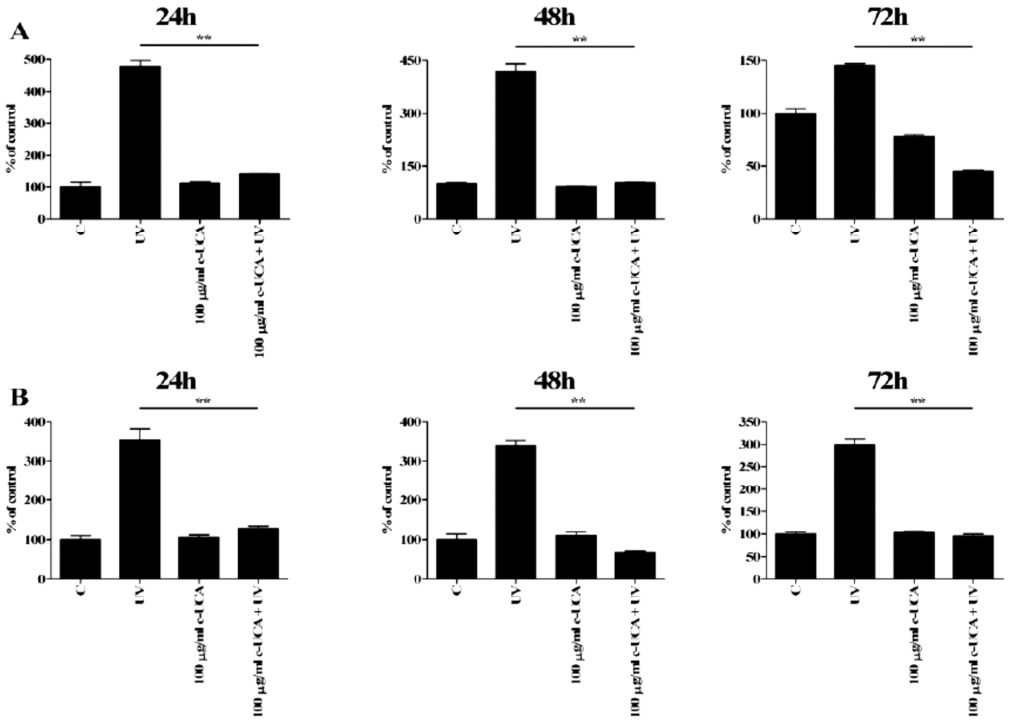

Figure 12. Effect of cis-UCA on UV-irradiation-induced IL-8 secretion. The HCE-2 cells (**A**) and HCEC (**B**) were non-irradiated (C), UV-irradiated (UV), treated with 100 mg/ml cis-UCA (C-UCA), or UV-irradiated and treated with with cis-UCA (100mg/ml c-UCA+UV) for 24, 48, or 72h. Statistical significance is shown by an asterisk ($p<0.05$) or a double asterisk ($p<0.001$; n=6 dishes). Compared samples are shown by the horizontal lines.

Since the 100 μg/ml concentration of cis-UCA restored the metabolic activity of the UV-irradiated cells (Figure 9), we finally analyzed caspase-3 activity. Treatment of both cell lines with 100 μg/ml cis-UCA significantly decreased the UVB-induced caspase-3 activity after 24 h follow-up (Figure 13A,B). This is in line with the MTT assay results (Figure 9) and reveals anti-apoptotic effects of cis-UCA in the HCE-2 cells and HCECs.

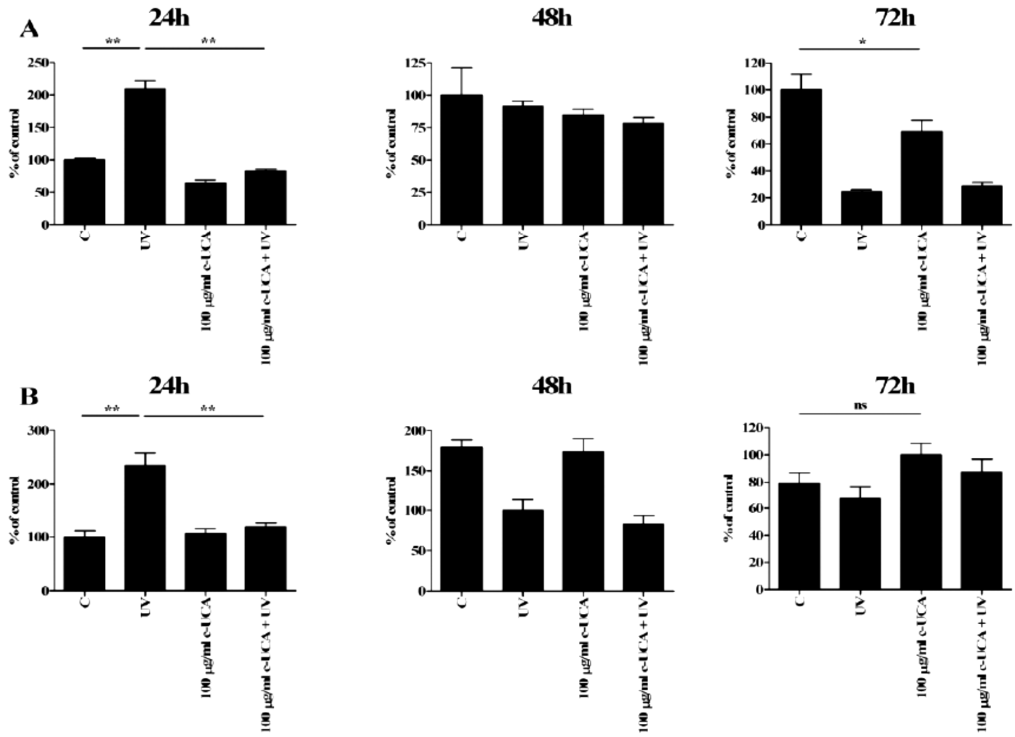

Figure 13. Effect of cis-UCA on UV-irradiation-induced caspase-3 activity. The HCE-2 cells (**A**) and HCEC (**B**) were non-irradiated (C), UV-irradiated (UV), treated with 100 mg/ml cis-UCA (C-UCA), or UV-irradiated and treated with with cis-UCA (100mg/ml c-UCA+UV) for 24, 48, or 72h. Statistical significance is shown by an asterisk ($p<0.05$) or a double asterisk ($p<.001$;n=6 dishes). Note that here is no significant difference (ns) between the control sample and the c-UCA-treated sample after 72h follow-up.

4.4 DISCUSSION

Our results demonstrate that cis-UCA possesses both anti-inflammatory and cytoprotective effects against an external stress, in this case UV-B irradiation, in human corneal (HCE-2) and conjunctival epithelial (HCEC) cells. Many previous reports have described cell type dependent responses of cytokine expression after treatment with cis-UCA. Cis-UCA at concentrations of 10–100 µg/ml has been documented to increase IL-6, IL-8, and TNF-α secretion in human keratinocytes (Kaneko et al. 2008). One study revealed an increased IL-10 production in activated CD4+ T cells (Holan et al. 1998) while another indicated that cis-UCA could inhibit secretion of IL-10 in T lymphocytes (Bi, Xia & Ni 1999). It has been suggested that cis-UCA may stimulate the cytokine cascade by induction of prostaglandin-E2 (PGE2) in keratinocytes (Kaneko et al. 2008, Shreedhar et al. 1998) while in peripheral blood monocytes, it attenuated the PGE2-

induced release of TNF-α (Hart et al. 1993). However, there are findings that low doses of cis-UCA have not had an effect on IL-1 or IL-6 production in monocytes (Hart et al. 1993, Redondo et al. 1996). Similarly, IL-1 could not be induced by 10–100 μg/ml cis-UCA in human keratinocytes (Kaneko et al. 2008). In agreement with these reports, our results indicate that low doses of cis-UCA did not affect IL-1β, IL-6, IL-8, or TNF-α secretion in human corneal and conjunctival cells. The data also show that 100 μg/ml cis-UCA significantly decreased the UV-B-induced secretion of IL-6 and IL-8 in these cells. It is of interest to note that the 1,000 μg/ml concentration of cis-UCA increased the IL-6 levels in the non-irradiated cells. However, our findings are evidence of clear anti-inflammatory activity of cis-UCA on UV-irradiated ocular surface cell types.

It is well known that UV radiation can cause cell damage and even the death of ocular surface cells (Lu, Wang & Shell 2003, Pauloin et al. 2009). In human and animal skin, UCA is the major UV-absorbing chromophore, but how it causes this effect is still far from clear. According to a recent view, cis-UCA may induce immune suppression via cells that express the 5-hydroxytryptamine (serotonin) receptor 2A (5-HT2A) receptor (Walterscheid et al. 2006). In addition to the limited signaling data, only a few studies have determined the toxicity of cis-UCA in cell cultures (Kim et al. 2003). Our findings demonstrated good tolerability of HCE-2 cells and HCECs to low concentrations (10–1,000 μg/ml) of cis-UCA whereas the highest concentration studied (5,000 μg/ml) was clearly toxic. For comparison, a cis-UCA concentration of 3,000 μg/ml in a mildly acidic medium decreased the mitochondrial metabolic capacity by about 90% in a bladder carcinoma cell line (Laihia et al. 2009). With respect to both toxicity and biological effects, the optimal concentration in the present investigation seemed to be 100 μg/ml cis-UCA since this was able to prevent as well as to fully restore both of the measured UV-B-induced changes, i.e., the decrease in metabolic activity and the increase in IL-6 secretion, back to the level of the non-irradiated control cells. Thus, 100 μg/ml cis-UCA was optimally anti-inflammatory and cytoprotective against cellular damage caused by UV-B radiation in the present experimental setting. It should be noted that the decrease in mitochondrial metabolic activity measured by the MTT assay after UV-B irradiation may not be caused by the irradiation only because the UV-B dose induced IL-6 secretion at the nanomolar level, which can directly decrease MTT metabolism (Wang et al. 2005). The UV-B irradiation induced caspase-3 activity in both cell types, which revealed apoptotic cell death and supported the MTT assay data. Treatment with 100 μg/ml cis-UCA also effectively prevented caspase-3 activity in the UV-B treated cells. Since elevated IL-1β secretion levels were not observed in any of the treatments, inflammasomes and caspase-1 are obviously not involved in the UV-B or cis-UCA responses. Caspase-1 is the principal caspase found in human inflammasomes that cleave the precursors of pro-inflammatory cytokines such as IL-1β to mature and active cytokines, which are subsequently secreted from cells (Salminen et al. 2008).

The UV-B irradiation dose used in the study evoked a low level of photoisomerization from cis- UCA to trans-UCA. This may have caused some effect on IL-6 secretion, although we consider it unlikely. The presence of very low concentrations (<4 μg/ml) of trans-UCA in the non-irradiated samples treated with 100 μg/ml cis-UCA may reflect unintentional exposure to laboratory lighting. In previous reported studies with cis-UCA, analyses of the actual concentrations of the isomers have rarely been conducted, and often various mixtures of the cis and trans isomers

have been used. It is possible that the very highest UCA concentrations (1000 and 5000 μg/ml) may have partially blocked the transmission of the UV-B photons in the culture medium, thereby preventing the full cell-irritating action of the irradiation. A smaller absorbing effect may have been caused by the medium ingredients, although no differential effect on IL-6 levels could be observed when using a bright buffer solution instead of the phenol red-containing colored medium. The prolonged exposure of the ocular surface to UV light can cause cellular damage. There are different stages of inflammation in the ocular surface due to alterations in the volume and composition of tear fluid (Yoon et al. 2007, Tishler et al. 1998, Pflugfelder et al. 1999, Perry 2008, Versura et al. 2009). Current clinical therapy for ocular surface inflammation consists of anti-inflammatory agents that do not offer any protection against UV radiation-induced damage (Pflugfelder 2004). Our in vitro findings suggest that cis-UCA may provide a safe and effective anti-inflammatory and cytoprotective treatment option against UV radiation-induced inflammation on the ocular surface.

Acknowledgments

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5 Cis-Urocanic Acid Inhibits SAPK/JNK Signaling Pathway in UV-B Exposed Human Corneal Epithelial Cells in Vitro

ABSTRACT

Purpose: The cornea is sensitive to ultraviolet B (UV-B) radiation-induced oxidative stress and inflammation. Its clinical manifestations are photokeratitis and climatic droplet keratopathy. Urocanic acid (UCA) is a major endogenous UVabsorbing chromophore in the epidermis and it is also an efficacious immunosuppressant. We have previously shown that cis-UCA can suppress UV-B-induced interleukin-6 and −8 secretion and cytotoxicity in human corneal epithelium (HCE) cells. In the current study, we further wanted to investigate the effects of cis-UCA on UV-B-induced inflammatory and apoptotic responses in HCE-2 cells, focusing on the nuclear factor kappa B (NF-κB) and AP-1 (subunits c-Fos and c-Jun) signaling pathways.

Methods: After exposing HCE-2 cells to UV-B and cis-UCA, DNA binding of c-Fos, c-Jun and NF-κB was measured with ELISA. In addition, the endogenous levels of phosphorylated stress-activated protein kinase/c-Jun N-terminal kinase (phospho-SAPK/JNK) and phospho-c-Jun were determined. The proliferative capacity of HCE-2 cells was also quantified, and the cytotoxicity of the cis-UCA and UV-B treatments was monitored by measuring the release of lactate dehydrogenase enzyme in the culture medium.

Results: UV-B irradiation induced the binding of transcription factors c-Jun, c-Fos, and NF-κB to DNA. Cis-UCA inhibited the binding of c-Jun and c-Fos but not that of NF-κB. Moreover, UV-B increased the levels of phospho-c-Jun and phospho-JNK, and the expression of both was attenuated by cis-UCA. Cis-UCA also alleviated the UV-B-induced apoptosis and proliferative decline in human corneal cells.

Conclusions: The results from this study suggest that cis-UCA suppresses JNK signaling pathway, which provides potential for treating UV-B-induced inflammatory defects in human corneal cells.

5.1 INTRODUCTION

In addition to skin and its epithelial cells, keratinocytes, the eye and its corneal epithelial cells are constantly exposed to ultraviolet (UV) radiation. The acute clinical effect of UV radiation on the cornea is photokeratitis, also known as "snow blindness" or "welder's flash." It is a painful inflammatory damage of corneal epithelium caused by UV-B (Cullen 2002, Dolin, Johnson 1994). UV radiation accelerates the physiologic loss of surface cells (Ren, Wilson 1996, Kitaichi et al. 2008). Exfoliation takes place by two mechanisms; shedding where whole cells detach into the tear film and apoptosis in which cells disintegrate into the tear film (Cullen 2002). Suprathreshold radiant exposure results in full-thickness loss of the stratified epithelium to the basement membrane and, consequently, exposed nerve fiber endings result in severe pain (Cullen 2002).

Climatic droplet keratopathy (CDK) is a degenerative condition characterized by the accumulation of translucent material in the superficial corneal stroma within the interpalpebral strip (Gray, Johnson & Freedman 1992). The corneal deposits are thought to be derived from plasma proteins which diffuse into cornea, and may become photochemically damaged by excessive exposure to UV (Gray, Johnson & Freedman 1992). Corneal deposits have been shown to contain various oxidative stress and inflammation–related agents (Holopainen et al. 2009, Menegay et al. 2008, Kaji et al. 2007, Kaji et al. 2009).

The transcription factors activator protein-1 (AP-1) and nuclear factor-kappaB (NF-κB) are known to be induced by UV-B (Cooper, Bowden 2007, Black et al. 2011, Lu, Wang & Shell 2003). These two transcription factor families have been identified to be involved in the processes of cell proliferation, cell differentiation and cell survival as well as having important roles in tumorigenesis (Cooper, Bowden 2007).

The transcription factor NF-κB comprises a family of proteins that are activated in response to inflammatory signals or cellular stress. In NF-κB-dependent gene expression analyses with human keratinocytes, tumor necrosis factor-alpha (TNF-α) and UV-B treatments resulted in the activation and inhibition of different genes, evidence of the stimuli and cell-type specific nature of NF-κB function (Lewis, Spandau 2007). NF-κB is activated by direct UV-B exposure and in different pathological conditions of the cornea (Alexander, Carlsen & Blomhoff 2006). During aging, the cellular capacity to respond to environmental stress via NF-κB-mediated signaling can be attenuated (Helenius, Makelainen & Salminen 1999).

The heterodimeric AP-1 is a transcription factor that is composed of proteins belonging to several families, the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and Fra2) subfamilies being the major AP-1 proteins (Shaulian 2010). The AP-1 regulation has been shown to be affected by all forms of mitogen-activated protein kinase (MAPK) cascades, e.g., p38 and JNK (c-Jun N-terminal kinase) (Shaulian 2010, Chang, Karin 2001), which activate in response to cellular stress. Study results with

human keratinocytes suggest that the activation of p38 MAPK is required for UV-B-induced AP-1 activation. A potential mechanism of UV-B-induced AP-1 activation through p38 is to enhance the binding of the AP-1 complex to its target DNA (Chen, Bowden 2000). Besides p38 activation, a potential UV-B signaling cascade for AP-1 activation in human keratinocytes involves c-Fos gene expression (Chen et al. 1998, Chen, Bowden 1999). The role of JNK in UV induced apoptosis is still controversial, studies suggesting either an anti-apoptotic or a pro-apoptotic effect. The biphasic function of JNK can be dependent on cell type, type of stimuli, crosstalk with other signaling pathways, and the intensity and duration of activation (Chen et al. 1996, Karin, Gallagher 2005, Wisdom, Johnson & Moore 1999).

UV-B has been shown to induce dose-dependent oxidative stress as well as MAP kinase activation, including JNK, in human corneal epithelium (HCE) cells (Black et al. 2011). In addtion, reactive oxygen species can induce phosphorylation of cell surface receptors, which results in the activation of the MAPK signaling pathway (Assefa et al. 1997). JNK phosphorylates c-Jun (Ser63/73 and Thr91/93) and potentiates the transcriptional capacity of c-Jun (Derijard et al. 1994, Hibi et al. 1993, Morton et al. 2003, Hayakawa et al. 2003). The JNK-initiated phosphorylation of c-Jun has been suggested to increase the half-life of c-Jun by protein stabilization, thus enabling potent and prolonged expression under stressful conditions such as UV irradiation (Derijard et al. 1994, Hibi et al. 1993, Musti, Treier & Bohmann 1997, Fuchs et al. 1996, Shaulian et al. 2000, Anzi, Finkin & Shaulian 2008). However, this mechanism seems to depend on the cell type (Anzi, Finkin & Shaulian 2008, Gao et al. 2004).

Urocanic acid (UCA) is the major UV-absorbing chromophore in the skin and it has been proposed to function as a regulator of UV-induced damage (Gibbs, Tye & Norval 2008). Cis-UCA, formed from trans-UCA upon UV-B exposure, has been implicated in the down-regulation of hypersensitivity reactions (Prater, Blaylock & Holladay 2003, Lauerma, Aioi & Maibach 1995), in the actions of epidermal antigen-presenting cells(Hart, Grimbaldeston & Finlay-Jones 2000, el-Ghorr, Norval 1997), in the activation of neutrophils (Kivisto et al. 1996, Rinaldi et al. 2006), and in the prolonged survival of organ transplants (Guymer, Mandel 1993, Filipec et al. 1998). Moreover, cis-UCA neither photobinds to DNA (Yarosh et al. 1992, IJland et al. 1998) nor is able to enhance UV photocarcinogenesis (Macve, Norval 2002), whereas it may suppress immunological recognition of tumor antigens in specific experimental conditions (Beissert et al. 2001). In our previous study, we have showed that cis-UCA suppresses UV-B-induced interleukin (IL)-6 and IL-8 secretion and cytotoxicity in human corneal and conjunctival cells in vitro (Viiri et al. 2009). However, the molecular targets of cis-UCA action remain to be resolved.

In this study we explored the hypothesis that UV-B radiation causes cell damage through an increase in transcription factor activity and that cis-UCA may protect the exposed corneal epithelial cells through alterations in this activity.

5.2 METHODS

5.2.1 Cell culture

Human corneal epithelial (HCE-2) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultivated on 6-well cell culture plates (Cellstar®, Greiner Bio-One, Frickenhausen, Germany) in Keratinocyte-SFM medium (Gibco, Invitrogen, Paisley, UK) supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml human recombinant epidermal growth factor, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco), 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), and 10% fetal bovine serum (HyClone, Logan, UT). Confluent cell cultures were treated with 100 μg/ml of cis-UCA (BioCis Pharma, Turku, Finland) when indicated in Results, and/or exposed to a UV-B irradiation dose of 153 mJ/cm2 (four TL 20W/12 tubes; Philips, Eindhoven, The Netherlands) at room temperature for 1 min using a source-to-target distance of 30 cm. Thereafter, the cell cultures were incubated in a humidified 10% CO2 incubator at 37 °C for 3, 6, or 24 h.

5.2.2 ELISA assays

For determining the DNA binding of transcription factors and for analyzing the activation of AP-1, cell lysates were prepared by scraping the cells into Buffer C (25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM Hepes in double-distilled water). To detect the binding of AP-1 and NF-κB to DNA, c-Fos and c-Jun TransAM™ kits (Active Motif, Rixensart, Belgium), and NF-κB p65 ELISA Kit (Enzo Life Sciences, Farmingdale, NY) were used. Phosphorylated c-Jun and phosphorylated stress-activated protein kinase/Jun-N-terminal kinase were measured using PathScan® Phospho-c-Jun (Ser63), and PathScan® Phospho- SAPK/JNK (Thr183/Tyr185) Sandwich ELISA Kits (Cell Signaling Technology, MA), respectively. All assays were performed according to the manufacturers' protocols. The absorbance values were measured at 450 nm with a reference wavelength of 655 nm using a BIO-RAD Model 550 microplate reader (BIO-RAD, Hercules, CA).

5.2.3 Proliferation assay

For the proliferation test, 100,000 cells/ ml were plated in 200 μl on 96-well flat-bottomed cell culture plates (Cellstar®, Greiner Bio-One). After 3 h of incubation in a humidified 10% CO2 incubator at 37 °C, cells in eight replicate wells were treated with cis-UCA and UV-B irradiation as described above. The cell cultures were incubated in a humidified 10% CO2 incubator at 37 °C for 24 or 48 h, and the proliferation of HCE-2 cells was quantified using the CyQUANT® Cell Proliferation Assay Kit (Invitrogen) according to the manufacturer's instructions. After 2 min incubation at room temperature, fluorescence intensity of the samples was measured at the ex/em wavelength of 485/530 nm using VICTOR™ 1420 multilabel counter (PerkinElmer/Wallac, Turku, Finland).

5.2.4 Cytotoxicity assay

Cytotoxicity of the cis-UCA and UV-B treatments was monitored by measuring the amount of lactate dehydrogenase (LDH) enzyme in duplicate from the culture medium

samples. Cyto-Tox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) was used for detection according to the instructions of the manufacturer. Absorbance values after the colorimetric reaction were measured at the wavelength of 490 nm with a reference wavelength of 655 nm using a BIO-RAD Model 550 microplate reader (BIO-RAD).

5.2.5 Statistical analysis

Statistical differences between groups were assessed using the Kruskall-Wallis test, and post hoc comparisons were made using the Mann–Whitney U-test. P values below 0.05 were considered significant.

5.3 RESULTS

To examine the activity of central transcription factors following UV-B and cis-UCA treatments, the DNA binding of AP-1 and NF-κB were determined. The DNA binding of c-Fos and c-Jun subunits of the transcription factor AP-1 heterodimer increased following UV-B irradiation (Figure 14). After 6 h of incubation, cis-UCA significantly decreased the UV-B-induced binding of both subunits (Figure 14). The decrease was not yet observed at 3 of incubation, and it was negligible after 24 h. UV-B irradiation also approximately doubled the binding activity of the p65 subunit of NF-κB when compared to non-irradiated control cells after 6 h of incubation. However, cis-UCA did not affect this activation at any of the 3, 6 or 24 h time points studied (Figure 15 and data not shown). No significant change in the activity of AP-1 or NF-κB was observed in non-irradiated cells treated with cis- UCA (Figure 14 and Figure 15).

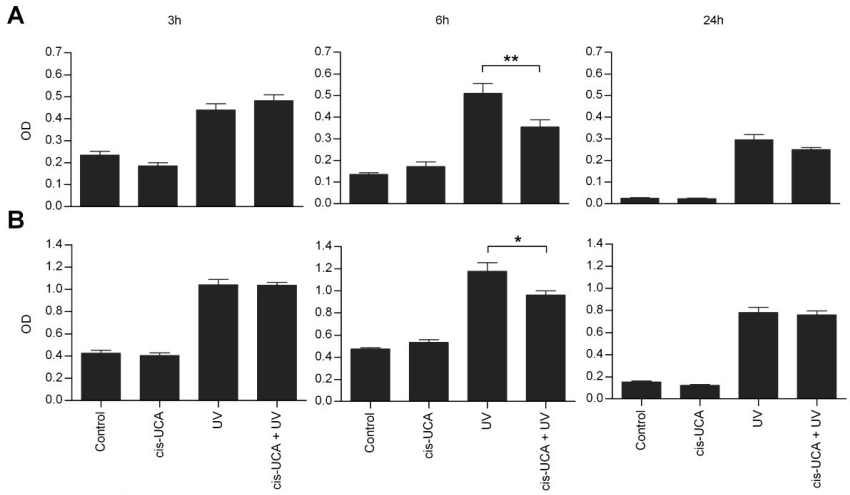

Figure 14. DNA binding of c-Fos and c-Jun subunits of the transcription factor AP-1 heterodimer. Binding of c-Fos (A) and c-Jun (B) to DNA. Results are presented as mean optical density (OD) ± SEM. cis-UCA concentration was 100 µg/ml. Seven parallel samples were measured in control and cis-UCA, and nine parallel samples in UV and cis-UCA + UV treatments. *, $p<0.05$; **, $p<0.01$ (Mann-Whitney).

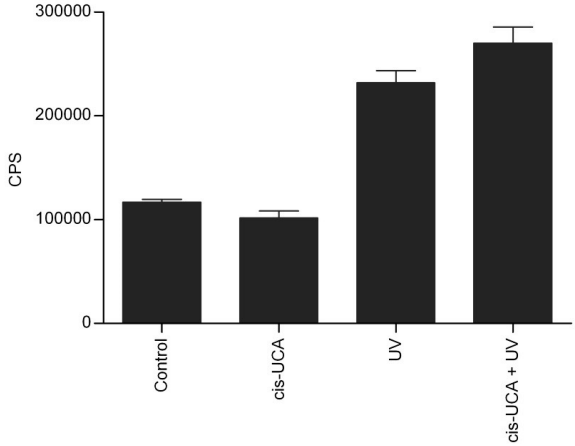

Figure 15. Binding of NF-κB (p65) to DNA 6h after stimulation. Results are presented as mean counts per second (CPS)± SEM. cis-UCA concentration 100 µg/ml. Five parallel samples were measured in control and cis-UCA, and seven parallel samples in UV and cis-UCA + UV treatments.

To verify our observation that cis-UCA inhibits the activity of AP-1 in UV-B-irradiated HCE-2 cells, we measured phosphorylated c-Jun from the cell extracts. As

shown in Figure 16, cis-UCA significantly decreased the level of phospho-c-Jun in UV-B-irradiated HCE-2 cells after 6 h of incubation. Moreover, cis-UCA also significantly decreased the amount of phosphorylated JNK in those cells (Figure 17). In non-irradiated cells cis-UCA had no effect on the expression of these phosphoproteins.

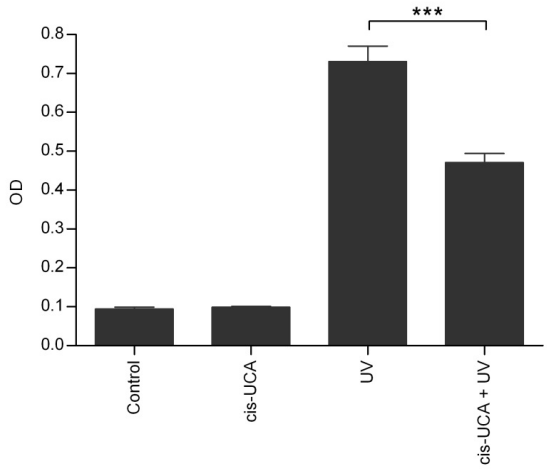

Figure 16. Phosphorylation of c-Jun (Ser63) 6h after stimulation. Results are presented as mean optical density (OD) ± SEM. cis-UCA concentration was 100 µg/ml. Seven parallel samples were measured in control and cis-UCA, and nine parallel samples in UV and cis-UCA + UV treatments. ***p<0.001 (Mann-Whitney).

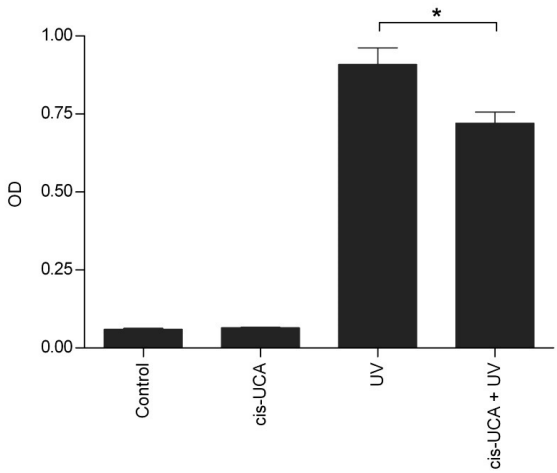

Figure 17. Phosphorylation of SAPK/JNK (Thr183/Tyr185) 6h after stimulation. Results are presented as mean optical density (OD) ± SEM. cis-UCA concentration was 100 µg/ml. Seven parallel samples were measured in control and cis-UCA, and nine parallel samples in UV and cis-UCA + UV treatments. *$p<0.05$ (Mann-Whitney).

Since JNK signaling can result in either cellular proliferation or apoptosis (Pimienta et al. 2007), we examined the influence of cis-UCA on cell survival. As shown in Figure 18, cis-UCA significantly prevented the loss of viability of HCE-2 cells in normal cell culture conditions. Also after UV-B irradiation, cell survival was increased by cis-UCA after 24 h of incubation and reached statistical significance after 48 h (Figure 18). Concomitantly, cis-UCA decreased cell damage. The decreased release of LDH from cis-UCA-treated cells was observed at 3 and 6 h of incubation and was statistically significant after 24 h both in non-irradiated and in UV-B exposed HCE-2 cells (Figure 19).

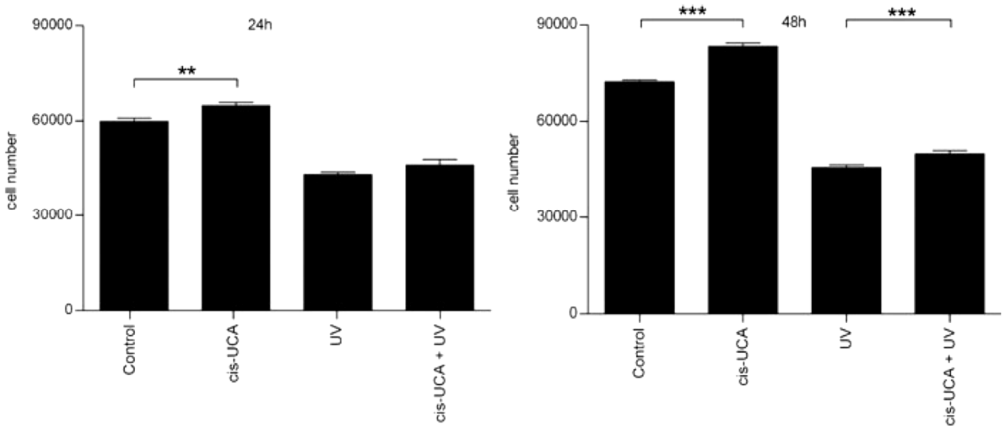

Figure 18. Proliferation of HCE-2 cells. Results are presented as mean cell numbers ± SEM. cis-UCA concentration was 100 µg/ml. Eight parallel samples were measured in all groups. **p<0.01; ***p<0.001 (Mann-Whitney).

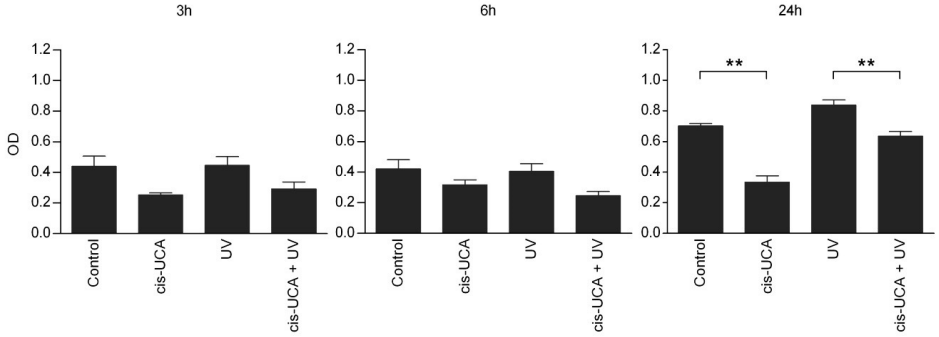

Figure 19. Release of lactate dehydrogenase (LDH). Results are presented as mean optical density (OD) ± SEM. cis-UCA concentration was 100 µg/ml. Six parallel samples in all groups. **p<0.01 (Mann-Whitney).

5.4 DISCUSSION

We have previously shown that cis-UCA suppresses UV-Binduced cytokine expression and improves cell viability against UV-B irradiation in human ocular cells (Viiri et al. 2009). In the present study, we further investigated the mechanisms of these actions. As the cornea is frequently exposed to solar UV radiation, we wanted to elucidate the role of JNK in apoptotic regulation in HCE-2 cells. Our data demonstrates that cis- UCA inhibits the phosphorylation of c-Jun (Ser63) and JNK (Thr183/Tyr185) as well as the binding of c-Fos and c-Jun to DNA in response to UV-B stimulation. The findings that cis-UCA reduced the phosphorylation of both JNK and c-Jun, and had no effect on basal

level of these phosphoproteins in nonirradiated cells, suggests that the molecular target of cis-UCA action is up-stream of JNK in UVB-stressed cells.

UV-induced generation of reactive oxygen species and subsequent TNF-α formation activates, besides JNK signaling, also the NF-κB pathway (Nakano et al. 2006, Papa et al. 2006). JNK activation by TNF- α activates pro-apoptotic effects; however, TNF-α-induced NF-κB activation prevents apoptosis through the suppression of the JNK pathway and the activation of antioxidant genes, such as manganese-superoxide dismutase (MnSOD) (Nakano et al. 2006, Papa et al. 2006). Interestingly, in epidermal cells, JNK activates cell proliferation, and the inhibition of JNK by NF-κB has a tumor-suppressing function (Zhang et al. 2005).

Conversely, following UV stimulus, p65/RelA directly results in the expression of protein kinase C delta (PKCδ), which leads to activation of JNK (Liu et al. 2006). In addition, after UV stimulation, MnSOD expression has been shown to be UV dose-dependent, exerting diminishing expression in high UVB doses (Black et al. 2011). However, at the same time, UV-B exposure induces the NF-κB-related proinflammatory cytokines IL-6 and IL-8 in HCE-2 cells (Viiri et al. 2009). Our research shows that both JNK and NF-κB pathways are activated by UV-B. However, cis-UCA suppresses solely the JNK pathway, not NF-κB. In response to UV-B stress, HCE-2 cells showed decreased proliferation and increased LDH release, implying cell death, which could be alleviated by cis-UCA. Consistently with an earlier study with epidermal cells (Zhang et al. 2005), the inhibition of JNK pathway seems to be a critical target in the regulation of apoptosis also in human corneal epithelial cells. While cis- UCA was present in the culture medium during UV-B irradiation of the cells, it was inferred from previous experience (Viiri et al. 2009) that the cis-UCA concentration used in the current experiment does not appreciably block the transmission of UV-B photons.

Although acute and chronic damage and inflammation caused by UV radiation to the epithelial cells of the cornea are well known ophthalmologic diseases (e.g., photokeratitis and CDK), their precise mechanisms are still unclear. Current clinical therapy for ocular surface inflammation consists of anti-inflammatory agents that do not offer any protection against UV radiation-induced damage (Pflugfelder 2004). The present in vitro data show that cis-UCA regulates the JNK signaling pathway and it has at both anti-inflammatory and cytoprotective capacity against UV radiation on the corneal epithelial cells. Our results are supported by previous observations (Matsui, Tashiro 1967). cis-UCA may be useful also in other inflammatory conditions of the cornea (Cook 2004, Luo et al. 2004). It would be worthwhile to examine cis-UCA effect on these diseases as well. Therefore, further in vivo studies are required.

Acknowledgments

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6 Topical Cis-Urocanic Acid Prevents Ocular Surface Irritation in Both IgE -independent and -mediated Rat Model

ABSTRACT

Purpose: 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 to dexamethasone, ketotifen and olopatadine. In 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 OA model Evans Blue stain concentration of conjunctival tissue was used to evaluate vascular leakage due to allergic reaction.

Results: 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 6 hour 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 comparison between the active drug treatments, only ketotifen showed a significant difference ($p=0.023$) to cis-UCA treatment at the 1 hour 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 OA model, cis-UCA 0.5% did not inhibit the vascular leakage of conjunctiva, whereas cis-UCA 2.5% 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.

6.1 INTRODUCTION

Allergic conjunctivitis (AC) is a common and complex ocular inflammatory response that usually coincides with rhinoconjunctivitis, rhinosinuitis, asthma, urticarial and eczema tissue (Bielory 2011). 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 (Irkec, Bozkurt 2012). Infiltrating neutrophils, eosinophils and lymphocytes may also be involved in the responses of acute reactions. In type IV hypersensitivity chronic conjunctival inflammation, 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 (Pauly et al. 2011, Erdinest, Solomon 2014, Syed, Kumar & Bielory 2014). In severe cases, topical corticosteroids may be prescribed with caution, since their long-term use can cause sight-threatening side effects (Erdinest, Solomon 2014). 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 (Bjorklund et al. 2014) and a photo protective agent (Barresi et al. 2011), the cis isomer (cis-UCA) is recognized as a modulator of cell-mediated (Lauerma, Aioi & Maibach 1995, Ross et al. 1986, Dahl, McEwen & Katz 2010) and innate immunity (Rinaldi et al. 2006, Bannerman et al. 2009). More recently, cis-UCA has been found to improve skin barrier function and to suppress local cutaneous inflammation (Peltonen et al. 2014).

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 (Viiri et al. 2009). 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 (Viiri et al. 2009, Jauhonen et al. 2011). The activation of SAPK/JNK has been linked to ocular inflammatory conditions such as the dry eye disease (Pflugfelder et al. 2005). 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).

6.2 MATERIALS AND METHODS

6.2.1 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 (6 animals in each group). In the OA-induced allergic ocular inflammation model, male Sprague Dawley rats aged 10 weeks were used:7 animals in immunized placebo group, 6 animals in cis-UCA 2,5% group, 6 animals in cis-UCA 0,5% group, 8 animals in olopatadine group and 5 animals in 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 hr) with free access to food and water.

6.2.2 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 13C 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 amebocyte 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. 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 1mg/ml (Opatanol, Alcon, UK) was used as a positive comparator and immunized, vehicle-treated (saline) and non-immunized, vehicle-treated as negative controls.

6.2.3 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 (Tiligada et al. 2000) 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 light microscope. The rest of the animals were killed after the final evaluation at 24 h.

6.2.4 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 aluminium 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 (10μl 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 anaesthesia, 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 sulphate 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.

6.2.5 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-test was 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.

6.3 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 hour 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 hour time point, otherwise there were no statistically significant differences between the active drugs (Figure 20 and Table 1).

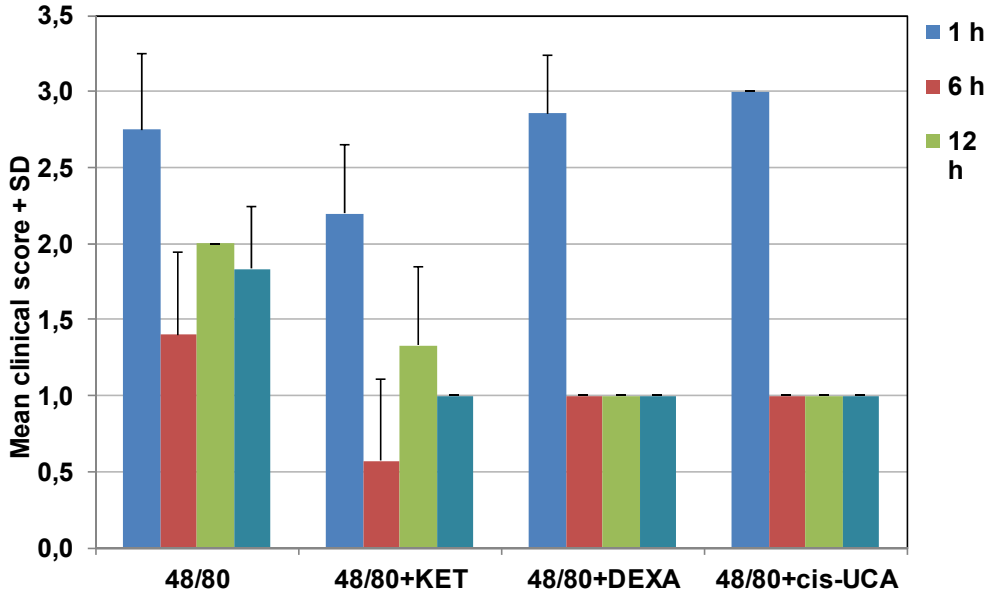

Figure 20. Conjunctival hyperemia in response to topical C 48/80 1000µg drop or combined with anti-allergic drugs applications. The eyes were treated with eye drops containing ketotifen (KET), dexamethasone (DEXA) or 0.5% cis-UCA at the time points of 0.5, 4 and 12 hours after the application of C 48/80. Severity scoring was performed from photographs taken at the time points of 1h, 6h, 12h and 24h. Severity scoring: none=0, mild=1, moderate=2; severe=3; mean + SD; n=6

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

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

Immunohistochemical analysis of the conjunctiva from three animals in the control group killed at 6 hours 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 (Figure 21). There were no statistically significant differences between the active treatment groups (Kruskall-Wallis test). Caspase-3 activity did not show statistically significant difference between any of the groups either (Kruskall-Wallis test, data not shown).

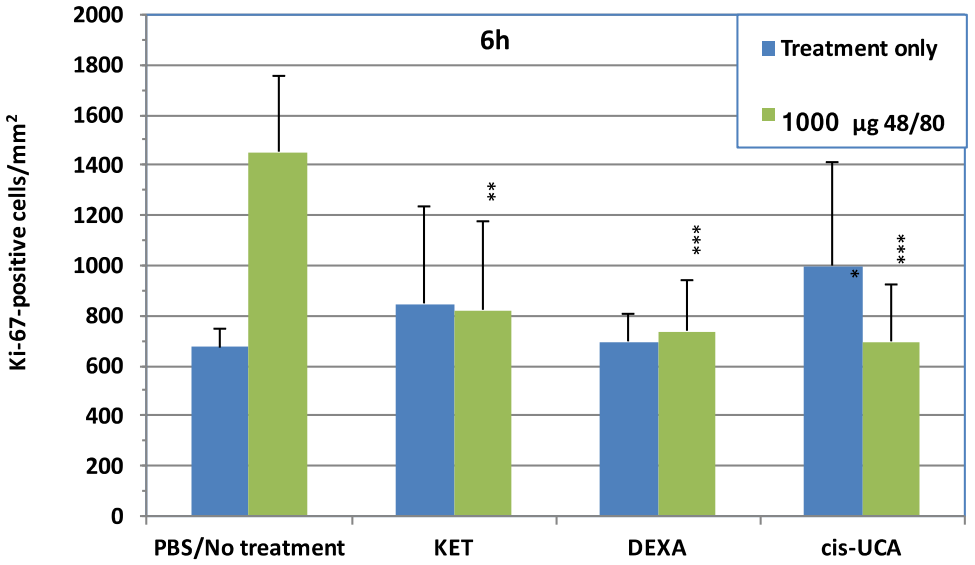

Figure 21. Ki-67 staining on the conjunctival epithelium in response to vehicle only (PBS), topical C48/80 drop (no treatment) or combined with anti-allergic drug applications. The eyes were treated with eye drops containing ketotifen (KET), dexamethasone (DEXA) or 0.5% cis-UCA at the time points of 0.5 and 4 hours 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, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

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 (Figure 22). 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).

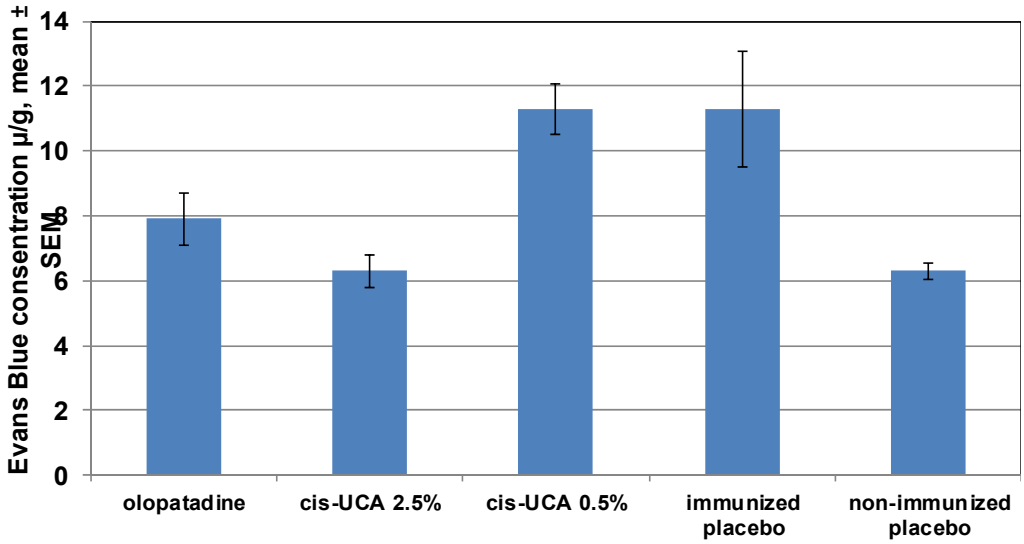

Figure 22. Effect of cis-UCA eye drops on conjunctival vascular leakage in OA sensitized rats. The eyes were treated with saline (immunized placebo and non-immunized placebo), 0.5 % or 2.5 % cis-UCA eye drops or the antihistamine, olopatadine. Vascular leakage was measured by Evans Blue accumulation 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**	
	p-value	bonferoni corrected	p-value	bonferoni corrected	p-value	bonferoni corrected	p-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

6.4 DISCUSSION

The C48/80 model of AO is an IgE-independent ocular irritation model that acts by a non-immunologic mechanism (Calonge, Siemasko & Stern 2003). C48/80 strongly induces histamine release from mast cells and modulates cell proliferation (Sheldon, Bauer 1960, Dartt, Masli 2014), resembling hypersensitivity type I reaction taking place in AC (Sheldon, Bauer 1960, Allansmith et al. 1989, Groneberg et al. 2003). Khosravi and co-workers (Khosravi, Elena & Hariton 1995) 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 (Scholzen, Gerdes 2000). The Ki-67 labeling index has been used for evaluation of histopathology and malignancy in ocular surface neoplasia (Ohara et al. 2004). 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 (Abu El-Asrar et al. 2006). 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 (Viiri et al. 2009). Cell proliferation is strongly regulated by JNK/SAPK and AP-1 signalling cascades, found to be suppressed in ocular cell cultures by cis-UCA (Jauhonen et al. 2011).

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 (Calonge et al. 1990, Calonge et al. 1996). In an ovalbumin model in the guinea pig (Sanchis-Merino et al. 2008), 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 (Tamura 2012). 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 (Tamura 2012). 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.

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 (Jauhonen et al. 2015). 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 (Viiri et al. 2009, Jauhonen et al. 2011, Jauhonen et al. 2015). 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.

Conflict of Interest

Hanna-Mari Jauhonen, Olli Oksala, Johanna Viiri, Reijo Sironen, Päivi Alajuuma and Kai Kaarniranta do not report any conflicts of interest. Lasse Leino and Jarmo Laihia were employees, stock holders and patent inventors for BioCis Pharma Ltd at the time the study was conducted.

7 A Randomized Phase I Clinical Study of Cis-Urocanic Acid Eye Drops in Healthy Adult Subjects

ABSTRACT

Purpose: To evaluate safety, ocular tolerability and pharmacokinetics of 0.5% and 2.5% cis-urocanic acid (cis-UCA) eye drops.

Methods: In this phase I, double-blinded, placebo-controlled trial, 37 healthy volunteers were randomized to three treatment arms: 0.5% cis-UCA (12 subjects), 2.5% cis-UCA (12 subjects) and placebo eye drops (13 subjects). In the first part, the subjects were dosed topically on a randomized eye with one drop three times at 7 ± 1 h intervals during one day. In the second part, the subjects self-administered three daily drops at 7 ± 1 h intervals on both eyes for 14 days. Physical examination of the eyes was performed seven times during the study. Tolerability of cis-UCA was assessed by ocular comfort rating questionnaire. Pharmacokinetic blood and urine samples were analyzed under good laboratory practice (GLP).

Results: All subjects completed both parts of the study. There were no significant adverse events (AEs). The most common treatment-related ocular AE was eye irritation (62.2% of subjects). Cis-UCA concentrations in plasma remained below the limit of quantification (0.195 μg/ml) in all but two subjects. The fraction of the administered drug excreted into urine over the total collection period ranged from 3.2% to 61.6% of the last dose and from 1.1% to 20.5% of the daily dose.

Conclusions: Topical ocular administration of cis-UCA solution is safe and apart from mild and short lasting eye irritation after administration well tolerated in healthy adult subjects. Topical ocular dosing leads to transient systemic exposure to cis-UCA that does not cause systemic AEs.

Key words: cis-UCA, ocular topical treatment, inflammatory ocular surface disease, clinical study

7.1 INTRODUCTION

Urocanic acid (UCA) is a major ultraviolet radiation (UV)-absorbing chromophore in the epidermis and has been suggested to act as one of the initiators of UV-induced immunosuppression (Gibbs, Tye & Norval 2008). The trans-isomer of UCA is formed by the deamination of histidine and it is isomerized to cis-UCA on UV-B exposure in a dose-dependent manner. Cis-UCA has been implicated in the down regulation of various immune functions such as contact sensitization and delayed-type hypersensitivity responses, reduction in natural killer cell activity, inhibition in the antigen presenting function of spleen cells and epidermal cells, and suppression of the activation of neutrophils in vitro (Kurimoto, Streilein 1992a). Cis-UCA may act by inducing synthesis of tumor necrosis factor (TNF)-α from epidermal cells, interleukin (IL)-6 from keratinocytes, and IL-10 from activated CD4+ T cells (Holan et al. 1998), although its mechanism of action still remains controversial.

It has been proposed that topically applied cis-UCA could represent a novel treatment modality in local inflammation. In a recent study, locally administered cis-UCA has been shown to attenuate clinical signs of inflammation in acute and subacute experimental mouse models of skin inflammation (Laihia et al. 2012). These findings were further elaborated in a clinical study where patients with mild to moderate atopic dermatitis were treated with cis-UCA containing emulsion cream, which was shown to suppress inflammation in the human skin (Peltonen et al. 2014).

In our previous in vitro studies we have shown that cis-UCA suppresses cell cytotoxicity and IL-6 and IL-8 secretion, alleviates apoptosis as well as proliferative decline, and inhibits stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) in the human corneal and conjunctival cell cultures after UV-B exposure (Viiri et al. 2009, Jauhonen et al. 2011). Moreover, our preliminary results in the compound 48/80 induced eye irritation model in the rat suggest that cis-UCA may reduce acute ocular inflammation (Jauhonen et al. 2012).

Therefore, we assume cis-UCA to be a promising anti-inflammatory treatment for ocular inflammatory diseases. In this phase I study, we wanted to investigate the safety, tolerability and pharmacokinetics of topical ocular administration of cis-UCA in healthy adult volunteers.

7.2 SUBJECTS AND METHODS

This clinical study was conducted in accordance with current Good Clinical Practice (GCP) guidelines and the ethical standards of the Declaration of Helsinki. The Ethics Committee of the North Savo Hospital District approved the study protocol. All volunteers gave their written informed consent before any screening procedure.

7.2.1 Study design

The study was a phase I single center, double-blinded, placebo-controlled, single and multiple dose study to evaluate safety, ocular tolerability and pharmacokinetics of 0.5% (w/v) and 2.5% (w/v) cis-UCA eye drops (Laurantis Pharma, Turku, Finland) in healthy adult volunteers. The study was double masked with regard to study personnel and subjects. Topical, one drop three times a day doses of 0.5% and 2.5% cis-UCA eye drops was selected both according to the principles for dose-response studies and based on the results obtained in preclinical studies (Viiri et al. 2009, Jauhonen et al. 2011) (Jauhonen et al. 2012). The ocular as well as systemic safety and tolerability of cis-UCA in preclinical studies have been very good, and the non-clinical toxicology data justified the use of cis-UCA with the planned doses and dosing regimens.

The study was composed of two parts (Figure 23). In the first part, the subjects were dosed topically with three daily drops of 0.5% and 2.5% cis-UCA or with placebo eye drops at 7 ± 1 h intervals on the randomized eyes of the subjects in a randomized right-left order in a double-blinded fashion on Day 1, while the other eye served as a control. Blood samples were drawn and urine collected before and after the first dosing for single-dose pharmacokinetics. The subjects were followed until one hour after the last study drug administration in the study center. Subjects with no treatment-emergent safety concerns in the first part and willing to continue were included in to the second part that lasted for 14 days from Day 9 to Day 22 (Day 22 inclusive). Altogether, the total exposure time for cis-UCA or placebo was 15 days. During the second part, the subjects self-administered three daily drops of 0.5% and 2.5% cis-UCA or placebo eye drops at 7 ± 1 h intervals on both eyes, except on Day 22. On Day 22 (the pharmacokinetic day), only one dose (i.e., the morning dose) was administered at the study site. At home, the study subjects filled in the diary (AEs, concomitant medications, other deviations, dose applications) starting from the screening visit until the end-of-study visit.

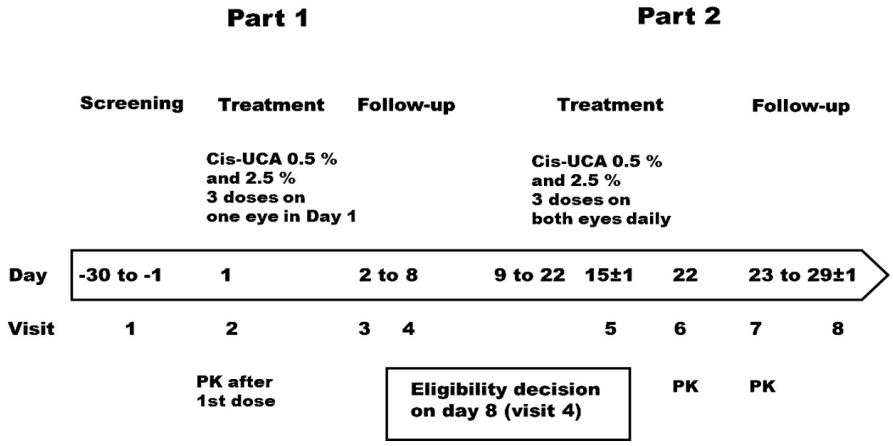

Figure 23. Flow diagram of the progress of trial. PK= pharmacokinetic sampling day.

The study was conducted in Finland during period from November to March, when exposure to natural UV-light radiation is low. In addition, the subjects were instructed to avoid any strong ocular exposure to UV-light such as solarium during the study from screening until end-of-study visit.

7.2.2 Investigational products

All eye drop products contained water for injection, sodium chloride, and polyvinyl alcohol, which was used to increase slightly the viscosity of the ophthalmic solution. No preservatives were used. The pH was adjusted to target 6.5−6.7; however, for the placebo product the pH spontaneously decreased at-release to pH 5.4. The viscosity was same in all products, i.e. about 6.4 mPa*s (shear velocity 20/s) and 5.5 mPa*s (shear velocity 100/s). The at-release osmolality was 344 mOsm/kg, 519 mOsm/kg and 302 mOsm/kg for 0.5% cis-UCA, 2.5% cis-UCA and placebo eye drops, respectively. The drop volume is about 0.040 ml for each product. The eye drops were sterile filtered and filled aseptically in white plastic non-transparent eye drop bottles. All products were kept unopened and protected from light at ambient room temperature until use. A fresh eye drop bottle was opened every day for each subject. The shelf-life of the eye drops products was 12 months at ambient room temperature based on previous stability studies conducted under Good Manufacture Practice (GMP), which covered the whole study period. GMP stability study was performed also for these clinical batches according to the protocol included to the Clinical Trial Application (CTA).

7.2.3 Subjects

The subjects were recruited using advertisements on bulletin boards and in the Internet. In total, 46 adult healthy Finnish volunteers were screened of which 37 (21 males and 16 females) were included in the study. The mean age of the included subjects was 25.8 years (range 19–54 years). The mean body weight was 74.0 kg (range 47–114 kg) and the mean BMI was 24.0 kg/m2 (range 18–36 kg/m2). The main inclusion criteria required volunteers to be healthy, 18–65 years of age, to show visual acuity (VA) score of ≥0.1 logMAR (logarithm of the minimal angle of resolution) in each eye, intraocular pressure (IOP) ≤21 mmHg with difference between eyes <4 mmHg, and with no history of a significant eye disease or any current eye disease that could affect the pharmacokinetics of cis-UCA. Women in childbearing age were included if they had a negative pregnancy test at screening and used a reliable contraception method throughout the study. Volunteers were excluded if they had used any ocular agents within the past month day, participated in another clinical drug or device study within 2 months prior to the first dosing or were current smokers.

7.2.4 Assessments

The subjects who met the enrolment criteria were randomized to the three treatment arms: 0.5% cis-UCA group (12 subjects, 6 female/6 male), 2.5% cis-UCA group (12, 6F/6M) and placebo group (13, 4F/9M). A randomization expert performed the randomization using SAS® System for Windows, version 9.3 (SAS Institute Inc., Cary, NC, USA). Investigational products were coded with subject numbers, which were given in consecutive order once enrolment had been confirmed.

Safety laboratory tests were taken and physical examination was performed at screening visit and at the end-of-study visit (on Day 29 ± 1). The safety laboratory tests were same at screening and at the end of study (Table 3) except urine drug screen and pregnancy test, HIV antibodies and hepatitis serology that were done only at screening. Physical examination of the eyes was performed by an ophthalmologist in the first part at screening and before the first dose on Day 1, Days 2 and 8 (end-of-study visit for those not continuing to the second phase), and in the second part on Days 8, 15 ± 1, 23 and 29 ± 1 (end of study). The examination included combinations of the following tests: the best corrected VA (ETDRS table, logMAR scale), assessment of bulbar conjunctival redness (BCR), upper lid tarsal conjunctival redness and corneal staining type (CST) and extent (CSE) scored using IER (Institute of Eye Research) grading scales (Table 4) (Schulze, Hutchings & Simpson 2009), anterior chamber cells and flare, intraocular pressure, lens evaluation, fundus examination for macula, peripheral retina and papilla and anaesthetized Schirmer's test.

Table 3. Screening laboratory tests.

Haematology	**Plasma chemistry**	**Urinalysis**
Haemoglobin Haematocrit Red blood cell (RBC) count Mean corpuscular volume of RBC (MCV) Mean corpuscular haemoglobin of RBC (MCH) White blood cells (WBC) with differential count Blood platelet count (thrombocytes)	Creatinine Alanine aminotransferase (ALT) Alkaline phosphatase (ALP) Glutamyl transferase (GT)	pH Protein Glucose Ketones Blood Nitrites Leukocytes Further examinations if indicated
Immunology	**Urine pregnancy test**	**Urine drug screen**
Serum HIV antigen and anti-bodies (S-HIV-AgAb) Serum hepatitis B virus surface antigen (S-HBsAg) Serum hepatitis C virus antibodies (S-HCVAb)	For premenopausal females (females are regarded as post-menopausal if 2 years at the minimum has elapsed since last menstrual bleeding)	Methadone Benzodiazepines Cocaine Amphetamine/meth-amphetamine Opiates Tetrahydrocannabinol

Table 4. Scores for bulbar and lid redness, corneal staining type and extent (IER grading scale).

Score	Bulbar redness/ Lid redness	Corneal staining type	Corneal staining extent,%
1	Very slight	Micropunctate	1-15
2	Slight	Macropunctate	16-30
3	Moderate	Coalescent macropunctate	31-45
4	Severe	Patch	>45

Ocular and systemic adverse events were monitored and recorded throughout the study on every visit. Tolerability of cis-UCA was assessed by ocular comfort ratings on each visit (screening, Days 1, 2, 8, 15 ± 1, 23, and 29 ± 1) by using a questionnaire which covered the following symptoms: sensitiveness to light, sensation of a piece of sand or

foreign body in the eyes, sore or painful eyes, burning of the eyes and blurring of vision. The symptoms were scored from 1 to 4 according to frequency of occurrence since the previous visit (all the time=4, most of the time=3, some of the time=2, not at all=1).

7.2.5 Pharmacokinetic analyses

Blood samples for the determination of cis-UCA in plasma were collected on Day 1 (first part) and on Day 22 (second part) as follows: within 1 h prior to the first dose and at 3 min, 6 min, 15 min, 0.5 h, 1 h, 2 h, and 4 h after dosing. In the second part, blood samples were collected also at 8 h and 24 h after the dosing.

On Day 1 (first part) and on Day 22 (second part), urine fractions were collected for the determination of cis-UCA as follows: In the first part, a sample within 1 hour prior to the morning dose, fractions 0–1 h, 1–2 h, and 2–4 h after the dosing; in the second part, also fractions 4–6 h and 6–24 h after the dosing were collected.

Pharmacokinetic blood and urine samples were analyzed by using a validated LC-MS/MS method under GLP. The LC column used in the analyses was Hilic Spherisorb S5 NH2, 4.6 x 250 mm (Waters) at 40 °C, and the elution solvent was 70% acetonitrile/30% water with 0.1% acetic acid for plasma samples and 90% acetonitrile/10% water with 0.1% acetic acid for urine samples. Plasma samples were pretreated with acetonitrile before analysis, whereas urine samples were just diluted with the mobile phase before LC-MS/MS. 13C3-labeled cis-UCA was used as an internal standard in each run.

7.2.6 Statistical methods and determination of sample size

Descriptive statistics by treatment group and study day were provided to summarize the study results. All statistical analysis were done using SAS® System for Windows. Descriptive statistics and tabulations were used for demographic and baseline characteristics and safety and tolerability data. Wilcoxon rank sum test was used to analyze ocular tolerability ratings, bulbar conjunctival redness and corneal staining with p=0.05 as a level of significant difference. Ocular AEs were reported with frequencies and number of subjects reporting AEs and CMH (Cochran-Mantel-Haenzel) test was used to test difference between three most common AE. Pharmacokinetic variables were listed by subjects and time points and no calculations were made on plasma concentrations because only in two subjects the result was over detection limit. Individual cis-UCA concentration curves were made based on urine results from three and seven out of 12 subjects who had quantifiable urine concentrations after 0.5% and 2.5% cis-UCA eye drops administration. The number of subjects planned to be included in the study was based on clinical considerations only. Formal sample size calculations were therefore not performed.

7.3 RESULTS

7.3.1 Adverse event evaluation

All 37 enrolled and randomized subjects completed both parts of the study and their compliance was good. Twelve received 0.5% cis-UCA eye drops, 12 2.5% cis-UCA eye

drops, and 13 placebo eye drops. The study treatments were at least moderately tolerated and safe. There were no deaths, serious or other significant AEs.

Altogether 25 ocular AEs were reported in 11 subjects receiving placebo eye drops, 48 in 12 subjects receiving 0.5% cis-UCA and 36 in 11 subjects receiving 2.5% cis-UCA eye drops. The most common treatment-related ocular AE was eye irritation, which was reported three times by three subjects in placebo group, 16 times by 11 subjects in 0.5% cis-UCA group and 17 times by nine subjects in 2.5% cis-UCA group. Eye discharge was reported eight times in eight subjects in placebo group, seven times by six subjects in 0.5% cis-UCA group and four times by four subjects in 2.5% cis-UCA group. All ocular AEs are presented in Table 5. Two of the ocular AEs in two subjects in 2.5% cis-UCA group were moderate, one case of eye irritation lasting for a minute, and one case of burning of the eye lasting for less than one minute. The other eye-related AEs were mild. Only the eye irritation had statistically significant difference compared with placebo drops in both 0.5% cis-UCA and 2.5% cis-UCA groups (Figure 24).

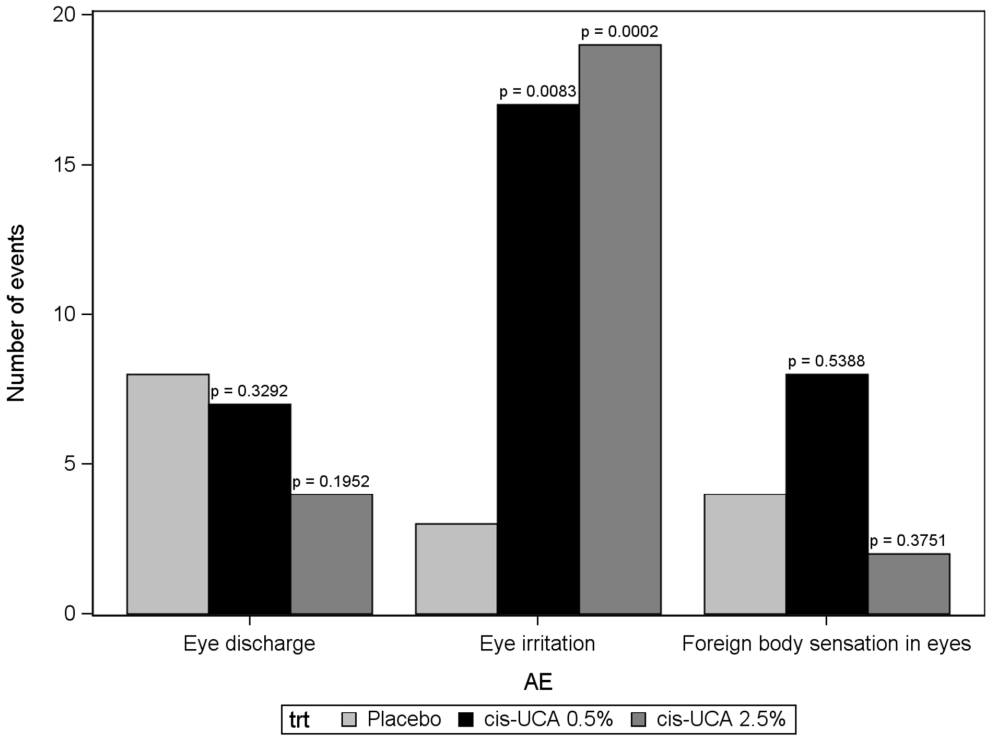

Figure 24. Three most frequent ocular AEs. P values show the difference between placebo and cis-UCA treatment groups. Only eye irritation shows statistically significant difference between placebo group and both 0.5% and 2.5% cis-UCA treatment groups, CHM test.

Table 5. Incidence of all reported ocular adverse events (incidence, number of subjects reporting AE, fraction of all subjects in the group (%).

Treatments	**0.5% cis-UCA** (N = 12)			**2.5% cis-UCA** (N = 12)			**Placebo** (N = 13)			**Total** (N = 37)		
Ocular AE	f	n	(%)	f	n	(%)	f	n	(%)	f	n	(%)
Total	47	12	(100.0)	32	11	(91.7)	23	11	(84.6)	102	34	(91.9)
Abnormal sensation in eye	1	1	(8.3)	1	1	(8.3)	0			2	2	(5.4)
Conjunctival haemorrhage	0			0			1	1	(7.7)	1	1	(2.7)
Conjunctivitis	0			1	1	(8.3)	0			1	1	(2.7)
Dry eye	3	3	(25.8)	2	2	(16.7)	0			5	5	(13.5)
Erythema of eyelid	1	1	(8.3)	0			0			1	1	(2.7)
Eye discharge	7	6	(50.0)	4	4	(33.3)	8	8	(61.5)	19	18	(48.6)
Eye irritation	17	11	(91.7)	19	9	(75.0)	3	3	(23.1)	39	23	(62.2)
Eye pain	1	1	(8.3)	0			1	1	(7.7)	2	2	(5.4)
Eye pruritus	1	1	(8.3)	0			4	3	(23.1)	5	4	(10.8)
Eye swelling	0			0			1	1	(7.7)	1	1	(2.7)
Eyelid irritation	1	1	(8.3)	0			0			1	1	(2.7)
Foreign body sensation in eyes	8	5	(41.7)	2	1	(8.3)	4	3	(23.1)	14	9	(24.3)
Lacrimation increased	1	1	(8.3)	1	1	(8.3)	0			2	2	(5.4)
Meibomian gland dysfunction	1	1	(8.3)	0			0			1	1	(2.7)
Ocular hyperaemia	2	2	(16.7)	1	1	(8.3)	0			3	3	(8.1)
Photophobia	2	1	(8.3)	1	1	(2.5)	1	1	(7.7)	4	3	(8.1)
Vision blurred	1	1	(8.3)	0			0			1	1	(2.7)

The most common non-ocular AE was headache, which was reported 11 times by seven subjects in placebo group, six times by three subjects in 0.5% cis-UCA group and three times by three subjects in 2.5% cis-UCA group. Altogether four non-ocular AEs in four subjects were moderate; including one case of headache, one case of flu, one case of pharyngeal pain, and one case of stomach pain. All the other non-ocular AEs were mild. They were considered not treatment related. One case of dysgeusia in 2.5% cis-UCA group was regarded as treatment related all others non-ocular AEs were considered not related to treatment.

7.3.2 Ocular tolerability and comfort

Ocular comfort was rated by questionnaires filled in at all of the eight visits during the study. Only values '1 = not at all' and '2 = some of the time' (overall range 1–4) were given for all parameters (Figure 25). "Burning of the eye" was the only parameter which showed statistically significant increase with 2.5% cis-UCA vs. placebo at Day 15 ($p = 0.004$, Wilcoxon rank sum test) and at Day 23 ($p = 0.011$) and with 0.5% cis-UCA vs. placebo at day 15 ($p = 0.030$) and at Day 23 $p = 0.004$) (Figure 26). Still, the study treatments were considered at least moderately tolerated because the symptoms were very short-term in nature (seconds) and were graded mild, in average less than 2 on scale 1 to 4.

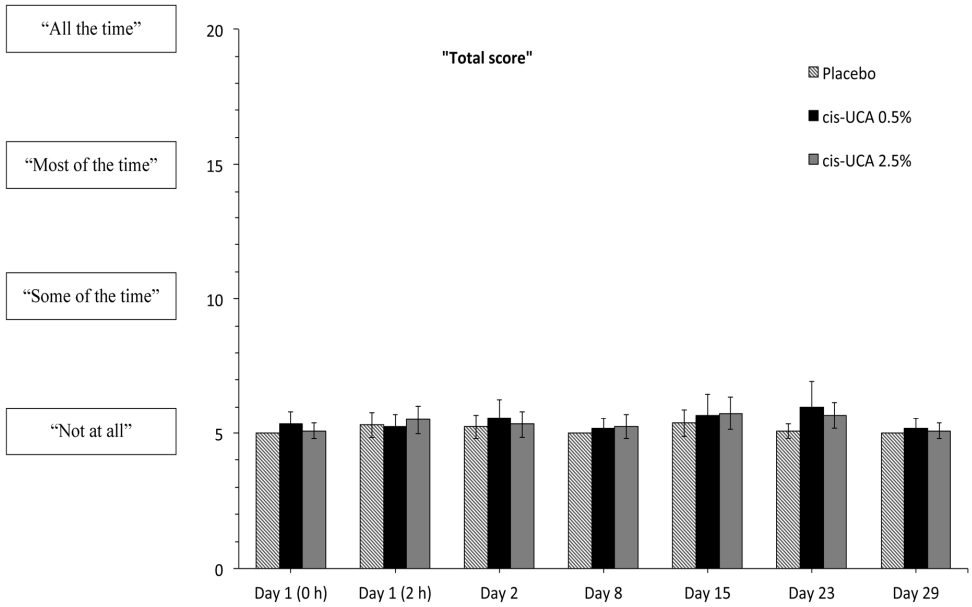

Figure 25. Total symptom score in ocular comfort rating questionnaire. There were no statistically significant differences between the groups.

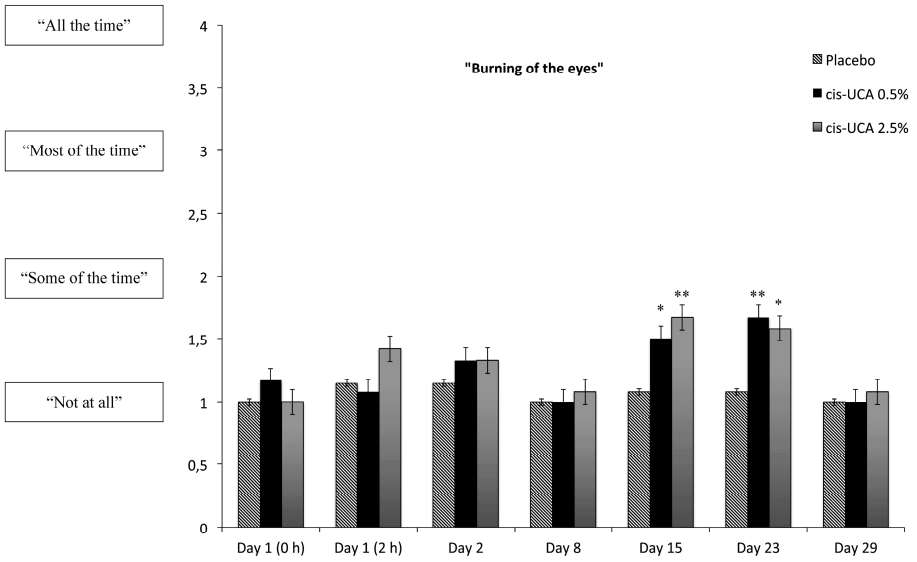

Figure 26. Burning of the eyes symptom score in ocular comfort rating questionnaire. Differences were statistically significant between 2.5% cis-UCA versus placebo at day 15 (p=0.004, **) and at day 23(p=0.011,*) and between 0.5% cis-UCA versus placebo at day 15 (p=0.030, *) and at day 23 (p=0.004, **), Wilcoxon rank-sum test.

7.3.3 Physical examination of the eyes

An ophthalmologist performed physical examination of the eyes on seven study days for each participant. BCR, lid redness, CST and CSE were evaluated using IER grading scales (Table 4). BCR scores between eyes on the same day or between each study day and the day immediately before or after were only ± 1 (Table 6). CST evaluation showed micropunctate (value 1) staining in four eye evaluations in two subjects in the placebo group, in two eye evaluations in one subject in the 0.5% cis-UCA group and in seven eye evaluations in two subjects in 2.5% cis-UCA group (Table 7). Macropunctate staining was observed only in one subject of the 2.5% cis-UCA group on Day 15. CSE in all observed cases was between 1% and 15% (Table 7). There was no statistically significant difference between the placebo and 0.5 % or 2.5% cis-UCA group at any time point for BCR or CST or CSE (Table 8).

There were no differences in VA between the study groups at any time point or between screening and end-of-study visits. There were no unexpected safety observations or changes on biomicroscopy evaluations of the anterior chamber, lens transparency or fundus examination (papilla, macula, and peripheral retina).

Also IOP values (i.e., 8–19 mmHg) remained normal in all 37 subjects throughout the study. Schirmer's test at screening, on Day 15 and at the end-of-study visit gave results in the range of 3–35 mm. The values less than 5 mm are regarded as insufficient, and 35 is the highest possible result. In five eyes of five subjects, the value was 3–4 mm; three low values (<5 mm) were reported in placebo-treated subjects and a single low value in both of the active treatment groups (Table 9).

Table 6. Bulbar conjunctival redness Day 2, Day8, Day 15 and Day 23*.*

		Total				**Cis-UCA 0,5%**				**Cis-UCA 2,5%**				**Plasebo**			
		Treated eye		Non-treated eye		Treated eye		Non-treated eye		Treated eye		Non-treated eye		Treated eye		Non-treated eye	
PART I	Category	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Screening	Very slight	17	45,9	15	40,5	6	50,0	5	41,7	5	41,7	3	25,0	6	46,2	7	53,8
	Slight	18	48,6	20	54,1	6	50,0	7	58,3	6	50,0	8	66,7	6	46,2	5	38,5
	Moderate	2	5,4	2	5,4	0	0	0	0	1	8,3	1	8,3	1		1	7,7
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	Very slight	6	16,2	7	18,9	3	25,0	3	25,0	2	16,7	1	8,3	1	7,7	3	23,1
	Slight	26	70,3	24	64,9	9	75,0	9	75,0	8	66,7	8	66,7	9	69,2	7	53,8
	Moderate	5	13,5	96	16,2	0	0	0	0	2	16,7	3	25,0	3	23,1	3	23,1
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 8	Very slight	9	24,3	11	29,7	4	33,3	4	33,3	2	16,7	2	16,7	3	23,1	5	38,5
	Slight	21	56,8	21	56,8	8	66,7	8	66,7	7	58,3	8	66,7	6	46,2	5	38,5
	Moderate	7	18,9	5	13,5	0	0	0	0	3	30,8	2	16,7	4	30,8	3	23,1
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Right eye		Left eye		Right eye		Left eye		Right eye		Left eye		Right eye		Left eye	
PART II	Category	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Day 15	Very slight	10	27,0	11	29,7	5	41,7	5	41,7	1	8,3	2	16,7	4	30,8	4	30,8
	Slight	24	64,9	21	56,8	7	58,3	7	58,3	10	83,3	8	66,7	7	53,8	6	46,2
	Moderate	3	8,1	4	10,8	0	0	0	0	1	8,3	1	8,3	2	15,4	3	23,1
	Severe	0	0	1	2,7	0	0	0	0	0	0	1	8,3	0	0	0	0
Day 23	Very slight	13	35,1	11	29,7	6	50,0	5	41,7	2	16,7	2	16,7	5	38,5	4	30,8
	Slight	23	62,2	24	64,9	6	50,0	7	58,3	9	75,0	8	66,7	8	61,5	9	69,2
	Moderate	1	2,7	2	5,4	0	0	0	0	1	8,3	2	16,7	0	0	0	0
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 29	Very slight	10	27,0	8	21,6	4	33,3	4	33,3	1	8,3	0	0	5	38,5	4	30,8
	Slight	26	70,3	27	73,0	8	66,7	8	66,7	10	83,3	11	91,7	8	61,5	8	61,5
	Moderate	1	2,7	2	5,4	0	0	0	0	1	8,3	1	8,3	0	0	1	7,7
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 7. Corneal staining type and extent on Day1, Day8, Day 15 and Day 23.

		Total				**Cis-UCA 0,5%**				**Cis-UCA 2,5%**				**Plasebo**			
		Treated eye		Non-treated eye		Treated eye		Non-treated eye		Treated eye		Non-treated eye		Treated eye		Non-treated eye	
PART I	Category	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Screening	Not existing	37	100,0	37	100,0	12	100,0	12	100	12	100,0	12	100,0	13	100,0	13	100,0
	Micropunctate; extent 1-15%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Macropunctate;extent 1-15%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	Not existing	34	91,9	35	94,6	11	91,7	11	91,7	11	91,7	11	91,7	12	92,3	13	100,0
	Micropunctate; extent 1-15%	3	8,1	2	5,4	1	8,3	1	8,3	1	8,3	1	8,3	1	7,7	0	0
	Macropunctate;extent 1-15%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 8	Not existing	36	97,3	36	97,3	12	100,0	12	100,0	11	91,7	11	91,7	13	100,0	13	100,0
	Micropunctate; extent 1-15%	1	2,7	1	2,7	0	0	0	0	1	8,3	1	8,3	0	0	0	0
	Macropunctate;extent 1-15%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Right eye		Left eye		Right eye		Left eye		Right eye		Left eye		Right eye		Left eye	
PART II	Category	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Day 15	Not existing	37	100,0	34	91,9	12	100,0	12	100,0	12	100,0	10	83,3	13	100,0	12	92,3
	Micropunctate; extent 1-15%	0	0	2	5,4	0	0	0	0	0	0	1	8,3	0	0	1	7,7
	Macropunctate;extent 1-15%	0	0	1	2,7	0	0	0	0	0	0	1	8,3	0	0	0	0
Day 23	Not existing	36	97,3	36	29,7	12	100,0	12	100,0	11	91,7	11	91,7	13	100,0	13	100,0
	Micropunctate; extent 1-15%	1	2,7	1	2,7	0	0	0	0	1	8,3	1	8,3	0	0	0	0
	Macropunctate;extent 1-15%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 29	Not existing	36	97,3	36	97,3	12	100,0	12	100,0	12	100,0	12	100,0	12	97,3	12	97,3
	Micropunctate; extent 1-15%	1	2,7	1	2,7	0	0	0	0	0	0	0	0	1	2,7	1	2,7
	Macropunctate;extent 1-15%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 8. p-values (Wilcoxon rank-sum test) between the groups.

Bulbar conjunctival redness				
Visit	cis-UCA 0.5% vs. placebo		cis-UCA 2.5% vs. placebo	
Part I	Treated eye		Treated eye	
screening	0.7348		0.8557	
Day 2	0.0676		0.5300	
Day 8	0.1529		1.0000	
Part II	Right eye	Left eye	Right eye	Left eye
Day 15	0.3551	0.2642	0.4876	0.7165
Day 23	0.5910	0.6010	0.1714	0.1958
Day 29	0.8190	0.7218	0.0609	0.1089
Corneal staining type				
Visit	cis-UCA 0.5% vs. placebo		cis-UCA 2.5% vs. placebo	
Part I	Treated eye		Treated eye	
screening	1.0000		1.0000	
Day 2	1.0000		1.0000	
Day 8	1.0000		0.3367	
Part II	Right eye	Left eye	Right eye	Left eye
Day 15	1.0000	0.3785	1.0000	0.4999
Day 23	1.0000	1.0000	0.3367	0.3367
Day 29	0.3785	0.3785	0.3785	0.3785
Corneal staining extent				
Visit	cis-UCA 0.5% vs. placebo		cis-UCA 2.5% vs. placebo	
Part I	Treated eye		Treated eye	
screening	1.0000		1.0000	
Day 2	1.0000		1.0000	
Day 8	1.0000		0.3367	
Part II	Right eye	Left eye	Right eye	Left eye
Day 15	1.0000	0.3785	1.0000	0.5302
Day 23	1.0000	1.0000	0.3367	0.3367
Day 29	0.3785	0.3785	0.3785	0.3785

Table 9. Schirmer's test results (mean, standard deviation, standard error, minimum, median and maximum); cis-UCA, cis-urocanic acid.

		Left eye							Right eye						
Visit day	**Treatment group**	**n**	**Mean**	**Std**	**SE**	**Min**	**Med**	**Max**	**n**	**Mean**	**Std**	**SE**	**Min**	**Med**	**Max**
Screening	Cis-UCA 2,5%	12	18,67	9,838	2,840	3	18,5	35	12	18,67	9,159	2,644	5	16,5	35
	Cis-UCA 0,5%	12	16,75	11,60	3,349	3	14,0	35	12	17,92	11,50	3,320	3	16,5	35
	Placebo	13	24	10,46	2,900	9	27,0	35	13	21,23	10,44	2,896	5	25,0	35
Day 15	Cis-UCA 2,5%	12	17	8,158	2,355	5	14,0	33	12	17,67	9,238	2,667	3	16,5	35
	Cis-UCA 0,5%	12	18,08	10,13	2,924	6	15,0	35	12	18,08	10,41	3,006	6	15,5	31
	Placebo	13	21,15	11,10	3,078	4	18,0	35	13	19,77	9,722	2,697	6	21,0	35
Day 29	Cis-UCA 2,5%	12	17,58	9,346	2,698	8	15,5	35	12	19,5	8,028	2,318	7	18,5	34
	Cis-UCA 0,5%	12	16	11,15	3,1219	4	12,0	35	12	17,58	10,66	3,078	6	17,5	33
	Placebo	13	19,08	11,65	3,231	3	17,0	35	13	22,08	11,98	3,321	3	18,0	35

7.3.4 Pharmacokinetic results

Cis-UCA concentrations in the plasma remained below the lower limit of quantification (LLQ, 0.195 μg/ml) in all but two subjects. Both concentrations were measured on Day 22, one after 0.5% cis-UCA (0.283 μg/ml in the pre-dose sample), and the other after 2.5% cis-UCA administration (0.459 μg/ml in the 15 minutes' sample). Thus, the plasma concentrations of cis-UCA were negligible after the single dose (Day 1) and repeated dose (Day 22) instillation of 0.5% and 2.5% cis-UCA eye drops, and consequently, no further pharmacokinetic calculations could be performed.

Only two concentrations of cis-UCA above the LLQ (1.95 μg/ml) were seen in the urine samples after the single dose on Day 1. Both concentrations were measured after the administration of 2.5% cis-UCA eye drops (2.73 μg/ml and 2.77 μg/ml, both in the 1–2 h post-dose fraction).

On Day 22, three (25%) and seven (58%) out of the 12 subjects had quantifiable urine concentrations in the 0.5% and 2.5% cis-UCA groups, respectively. The concentrations seen with 0.5% cis-UCA were low (2.44 μg/ml in the pre-dose sample and 3.85 μg/ml in the 1–2 h post-dose sample of one subject, 4.54 μg/ml and 2.57 μg/ml in the 1–2 h post-dose samples of two subjects), whereas the concentrations seen with the 2.5% cis-UCA were higher and detectable from pre-dose up to 6 h in six subjects. For the 2.5% cis-UCA, the maximum concentration (10.95 μg/ml) was seen in the 1–2 h post-dose samples of two subjects who also had the only quantifiable concentrations in the

corresponding urine fraction collected on Day 1. For the subjects with complete urine volume data (N = 6), the amount of cis-UCA excreted in urine over the total collection period ranged from 64.46 to 1229.45 μg. Accordingly, the fraction of the administered drug excreted into urine over the total collection period ranged from 3.2% to 61.6% of the last dose and from 1.1 to 20.5% of the daily dose.

7.4 DISCUSSION

The primary objective of the randomized, double-blinded and placebo-controlled clinical trial was to obtain data on the safety and tolerability of topical ocular cis-UCA treatment in healthy volunteers. Apart from mild and short lasting eye irritation after administration the 0.5% and 2.5% cis-UCA eye drops solutions were well tolerated and safe. Although the total number of ocular AEs was slightly higher in the cis-UCA treated groups than in the placebo group, there was no concentration-dependent trend. The most common ocular AEs were rapidly resolving eye irritation, eye discharge and foreign body sensation in eyes, all of which were classified as mild or moderate. Of these symptoms, the two latter ones were more common in the placebo group than in the high-dose cis-UCA group. The lower pH of placebo eye drop solutions may have influenced these symptoms. Only short-term eye irritation had statistically significant difference compared with placebo drops in both 0.5% cis-UCA and 2.5% cis-UCA groups ($p=0.0083$and $p=0.0002$ respectively).

Of the non-ocular, systemic AEs, the most common was mild headache, which was reported over two times more frequently in the placebo group than in the cis-UCA groups. The only treatment related non-ocular adverse event, as judged by the investigator, which occurred in the cis-UCA group (2.5%), was a case of dysgeusia (transient distortion of the sense of taste).

Subjective ocular comfort rating assessments were in line with the safety evaluation data. The overall rating showed no difference between the study treatments. However, it seems that cis-UCA may induce a sensation of burning of the eyes as this symptom had a significantly higher rate after repeated dosing. Nevertheless, also this symptom appears to be mild and short-term in nature as it had an average value less than 2 on a scale of 1 to 4. Moreover, it showed no concentration dependence. Extensive physical examination of the eyes by the ophthalmologist revealed no harmful effects of cis-UCA on the eyes compared to placebo. IOP remained unchanged and anaesthetized Schirmer's test showed no acute impact on tear secretion. Taken together, the present data indicate that repeated topical ocular administration of cis-UCA ophthalmic solution up to 2.5% concentration is safe at least for 2 weeks in healthy adult subjects. This observation is supported by a recent study on topical dermal administration of 5% cis-UCA in 45 adult subjects, including 13 patients with mild to moderate atopic dermatitis (AD); cis-UCA was well tolerated both locally on the skin and systemically even after twice a day dosing for 28 days (Peltonen et al. 2014).

Pharmacokinetic analysis of plasma samples showed negligible systemic exposure to cis-UCA after single or repeated ocular dosing of the ophthalmic products. Again, this is well in line with our previous dermal study where 0.7 mg cis-UCA kg-1 day-1 was

applied on the skin of AD patients for 10 days without detectable cis-UCA in plasma samples (Peltonen et al. 2014). However, in contrast to topical dermal application, topical ocular cis-UCA administration resulted in transient accumulation of cis-UCA in urine in about half of the high-dose subjects on Day 22, i.e., after repeated dosing. Although the urine cis-UCA concentrations were in general low (at μg/ml level), we observed that a significant fraction (up to about 60%) of a single cis-UCA dose may be absorbed quickly from the conjunctiva and systemic exposure may occur. On the other hand, the circulating amount of cis-UCA was rapidly eliminated in the urine and did not cause any systemic adverse effects or show systemic accumulation at the dose levels investigated in this study.

Cis-UCA has established anti-inflammatory properties both in vitro and in vivo. We have shown that in the human corneal and conjunctival cell cultures cis-UCA suppresses UV-B induced IL-6 and IL-8 secretion and cytotoxicity (Viiri et al. 2009). Moreover, cis-UCA restored the metabolic activity of the UV-B-irradiated cells. These effects may be mediated by inhibition of the SAPK/JNK, which play an important role in cellular responses to external stress factors, including inflammation (Jauhonen et al. 2011). Interestingly, activation of SAPK/JNK has been linked to the inflammatory processes of the dry eye disease (DED) (Pflugfelder et al. 2005). A wide range of inflammatory molecules are found in the ocular tissues and tears in DED. Interleukins (e.g. IL-1α, IL-6, and IL-8), chemokines (e.g. CXCL9, CXCL10, CXCL11) and TNF-α are elevated in DED tears and the conjunctival epithelium (Na et al. 2012). Some of these markers correlate with clinical signs of disease severity (Lam et al. 2009). It can be therefore hypothesized that cis-UCA represents a promising candidate to control the inflammatory responses seen in DED.

In conclusion, the present report shows that topical ocular administration of cis-UCA eye drops for at least 14 days three times a day is safe and apart from mild and short lasting eye irritation after administration well tolerated both locally and systemically, in healthy adult subjects. Topical ocular dosing leads to transient systemic exposure to cis-UCA that does not cause systemic AEs. To our knowledge, this is the first study where cis-UCA has been administered on the human eye. Additional clinical trials are needed in appropriate patient populations to evaluate the benefit of cis-UCA in inflammatory ocular diseases.

8 General Discussion

8.1 SUMMARY

Anterior ocular inflammatory diseases are common; for example, in the United States they affect over 40% of the population (Bielory, Syed 2013). The cases of infectious bacterial and viral conjunctivitis are exceeded by AC and DED when measured by numbers of patients. Environmental factors are known to exacerbate symptoms and findings in both of the latter mentioned diseases. The role of UV light, especially UVB, which is able to induce anterior ocular inflammatory reaction is significant since even short term irradiation may cause morphological changes and cell death (Cejka et al. 2010). Because of the depletion of the ozone layer, an increasing proportion of UV-radiation now reaches the Earth. This may increase the susceptibility of anterior ocular structures to inflammatory and oxidative stress mediated diseases.

In publications I and II, the effects of cis-UCA were studied in human ocular surface epithelial cell cultures after UV-B exposure. In publication I, both human conjunctival epithelial cells (HCEC) and corneal epithelial cells (HCE) were used to assess the toxicity of cis-UCA. The MTT assay revealed that a concentration of 5000μg/ml was toxic, whereas a much lower concentration, 100μg/ml, evoked favourable effects on viability of both cell types as measured by MTT assay without and even after UV-B exposure. In addition, the 100μg/ml concentration of cis-UCA suppressed the secretions of IL-6 and IL-8 after UV-B exposure. Similarly, cis-UCA 100μg/ml prevented UV-B induced apoptosis in the both the MTT assay and in the activity of caspase-3. In publication II, the effects of cis-UCA were examined in HCE cell cultures after UV-B exposure. Cis-UCA was shown to inhibit the AP-1 pathway by decreasing the binding of two AP-1 subunits, c-Jun and c-Fos, to the corresponding promoter area. Furthermore, it was demonstrated that cis-UCA was able to inhibit phosphorylation of c-Jun and SAPK/JNK which are the key steps in the AP-1 mediated inflammatory response.

There are no previous studies of cis-UCA effects on ocular surface cells. Since the cis-UCA effects are cell specific, these findings are both novel and relevant. Previous studies revealed that cis-UCA increased the production of two cytokines, IL-6 and IL-8, in a dose-dependent manner and triggered apoptic cell death in human keratinocytes (Kaneko et al. 2008, Kaneko et al. 2011). In contrast, in our ocular cell cultures, cis-UCA suppressed UV-B induced cytokine production.

AP-1 is a major regulator of many inflammatory cascades on the ocular surface and IL-6 and IL-8 are both upreglated in ocular allergic conjunctivitis (Leonardi et al. 2006). In publication III, cis-UCA treatment was evaluated for the first time in two experimental animal models of allergic conjunctivitis. In the IgE- independent C48/80 EAC model, cis-UCA 0.5% eye drops were well tolerated and showed at least equal efficacy in the inhibition of clinical signs of ocular irritation as dexamethasone 1 mg/ml and ketotifen 0.25 mg/ml administrations. Moreover, in publication III, nuclear Ki-67

expression was used to quantify the proliferation of conjunctival epithelial cells with caspase-3 being used as a marker of apoptosis. The data emerging from this study indicate that cis-UCA has anti-proliferative and cytoprotective effects that are consistent with the outcomes of the in vitro studies described in publications I and II. Recently, the drug development for AC treatments has undergone a shift toward more sophisticated immunobiologicals, which are targeted to modify the inflammatory process (Bielory et al. 2016, Bron et al. 2014); from this viewpoint, the effects of cis-UCA in these two experimental animal studies are promising.

Finally, as a continuum of the cis-UCA drug development process on ocular surface, in publication IV, cis-UCA 0.5% and 2.5% eye drops were evaluated in a phase I clinical study in healthy subjects with respect to safety, tolerability and pharmacokinetics in a double-blinded, placebo-controlled setting. The topical administration of cis-UCA was safe and well tolerated in our healthy subjects, inducing only short term and mild ocular irritation. Topical ocular dosing caused transient systemic exposure to cis-UCA, which did not cause any systemic adverse events and was well tolerated in this setting. Even with two weeks' continuous administration, cis-UCA did not evoke any severe adverse effects either systemically or in the ocular structures.

8.2 LIMITATIONS OF PRESENT STUDY

This study is the first to have exploited ocular surface epithelial cells as well as experimental ocular allergy models to evaluate effects of cis-UCA. Since the mechanisms of action of cis-UCA seem to be cell-dependent, the comparison of the results in publications I, II and III to experiments conducted with cis-UCA in other types of cell culture studies and other experimental allergy models must be done with caution. In addition, the differences in experiment settings limit the validity of comparisons with previous studies. The UV-B exposure used in cell culture studies in publications I and II evokes inflammatory reactions, which are not strictly comparable to DED or AC inflammatory reactions (Leonardi et al. 2006, Black et al. 2011, Lam et al. 2009, Yoon et al. 2007). Thus, the experiments using UV-B exposure cell cultures should be repeated in ocular allergy and DED cell culture models (Sakai et al. 2013).

Experimental animal models never fully mimic human clinical disease. Our EAC model using C48/80 is an ocular irritation model rather than ocular allergy model, because the mast cell degranulation occurs without antigen presenting cell (APC) mediated IgE production and thus, actually mimics better contact blepharoconjunctivitis, which is encountered in the toxic reactions to topically administered ocular drugs (Guzman et al. 2014, Ayaki, Iwasawa & Niwano 2012). The evaluation method of clinical effects was performed from photographs which are always somewhat subjective, but nonetheless do provide a good indication of effects. Moreover, only the cytoprotective effect of cis-UCA was evaluated in histological samples (via Ki-67 and caspase-3 activity) which are more or less indicative of apoptosis; this meant that other effects of cis-UCA on cellular reactions of ocular allergy were unresolved. In addition, our groups were relatively small (6 animals per group), which restricts the statistical power of the study. In the OA experiment, group sizes were also small and the only end point used was conjunctival vessel leakage.

In the drug development process, both cell culture and experimental animal experiments are mandatory and necessary before embarking on phase I clinical trials in human subjects; these were reported for the first time using cis-UCA eye drops in publication IV. The tolerability of cis-UCA concentrations was good and all the subjects completed both parts of the study. The duration of the daily ocular administration was limited to two weeks, whereas the need for treatment in ocular surface diseases typically ranges from months to continuous use. The accumulation of topically administered cis-UCA to cornea or penetration to the anterior chamber fluid was not evaluated, since that would require invasive measurement procedures.

8.3 FUTURE DIRECTIONS

Environmental change and ozone depletion mean that increasing amounts of UV radiation are reaching the Earth's surface. This may add to the harmful effects of UV radiation on the ocular surface as well as in the inner parts of eye (Cejkova et al. 2004, Lofgren 2017, Taylor, Davies 1987, Zigman 1977, Zuclich 1989). Sunglasses, contact lenses and ordinary glasses constitute some level of protection against UV radiation, but still light scattering and reflecting radiation reach the ocular surface, predisposing it to inflammatory reactions and cell damage. Cis-UCA functions like a natural "sunscreen" in the skin. As shown, it exerts anti-inflammatory and cytoprotective effects on ocular surface cells when they are exposed to UV-B radiation. Thus, it might be worthwhile investigating the effect of cis-UCA in the prevention and treatment of UV radiation evoked inflammation and cell damage present in photokeratitis and climatic droplet keratopathy. Moreover, cis-UCA's protodynamic action (Laihia et al. 2009, Peuhu et al. 2010, Laihia et al. 2010) might be beneficial in the treatment of ocular surface squamous neoplasia (OSSN) which is strongly associated with solar UV radiation's ability to induce mutations in tumor-suppressor gene p53 (Gichuhi et al. 2014). One of the current treatment modalities of OSSN is interferon alpha-2b (Fernandes et al. 2014) which shares some characteristics with cis-UCA. One can postulate that other ocular surface neoplasias like corneo-conjunctival intra-epithelial neoplasia (CCIN) might also be suitable targets for cis-UCA protodynamic treatment.

Since the illness handicap and the economic burden of ocular allergy and DED are significant (Bielory, Syed 2013), it is important that the immunopathology of these diseases is clarified and more targeted treatments are devised. Cis-UCA displays promising anti-inflammatory and cytoprotective effects in cell cultures and is well tolerated. However, there is an unarguable need for further experiments both in cell cultures and in EAC models in order to understand the mechanisms of action of cis-UCA in ocular surface inflammation. Since the tear cytokines and chemokines are promising bio-markers of anterior ocular inflammatory diseases (Cocho et al. 2016, Hagan, Tomlinson 2013, Leonardi 2013) they should be measured both in cell cultures and EAC models as well as in the clinical trials to evaluate cis-UCA's anti-inflammatory spectrum in greater detail. In addition, the penetration of cis-UCA into the anterior chamber fluid and the vitreous should be measured in experimental animal models to

evaluate possible side effects or beneficial anti-inflammatory treatment potential in the intra-ocular space.

Inevitably, longer exposure of cis-UCA in topical ocular administration will be needed to evaluate long-term clinical and adverse effects. A natural continuation of this drug development process will be to conduct phase II clinical studies recruiting patients suffering from AC or DED, to evaluate the pharmacokinetics after administration on diseased conjunctiva and cornea; this would help to clarify cis-UCA's tolerability and effects on clinical signs and symptoms. The emerging therapies for ocular surface diseases involve different immunobiological modulators (Bielory et al. 2016, Syed, Kumar & Bielory 2014). Cis-UCA may provide a novel treatment alternative for ocular surface inflammatory disease either on its own or in combination therapies.

9 Conclusions

I Cis-UCA possesses promising anti-inflammatory and cytoprotective properties to prevent inflammation in human corneal and conjunctival cell cultures evoked by UV-B exposure.

II Cis-UCA's anti-inflammatory effects after UV-B exposure are proposed to be mediated by inhibition of the JNK signaling pathway.

III Cis-UCA displays good tolerability and anti-allergic effects both in IgE-independent and IgE-mediated experimental animal models indicating promising anti-allergic potency both against immediate and delayed-type allergic reactions in the eye.

IV The cis-UCA solution, when administered to healthy volunteers as eye drops three times per day for two weeks, was well tolerated and safe. One side effect of the topical delivery was ocular irritation but this was mild and short-lasting; topical administration of cis-UCA did not evoke any systemic adverse events.

10 References

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The ocular surface is constantly exposed to environmental challenges. Anterior ocular inflammatory diseases e.g. ocular allergy and dry eye disease are common and more targeted treatments are needed for these diseases. This study examined cis-UCA's effects on the ocular surface. It showed promising anti-inflammatory and anti-allergic properties. Cis-UCA was well tolerated and safe and topical administration did not evoke any systemic adverse events.

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