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INVITED REVIEW

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Review of Preclinical Outcomes of a Topical Cationic Emulsion of Cyclosporine A for the Treatment of Ocular Surface Diseases

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ABSTRACT

Background: Cyclosporine A (CsA) has been used as a topical treatment for various ocular surface diseases including dry eye disease (DED). Several CsA formulations are available as solutions or emulsions.
Purpose: This review describes the development and the preclinical testing of a cationic oil-in-water emulsion of CsA (CE-CsA) in terms of pharmacodynamics, pharmacokinetics, and ocular tolerance. Due to the cationic charge, CE electrostatically interacts with the negatively-charged ocular surface, improving its residence time. Compared to other CsA formulations, CE-CsA and CE itself were found to reduce the signs and symptoms of DED, by restoring tear film stability and properties, and inhibiting the expression and secretion of pro-inflammatory factors. No delay in wound healing nor ocular toxicity were observed using CE formulations.
Conclusion: these findings indicate that the type of vehicle can significantly affect the performance of eye drops and play an ancillary role in DED treatment. CE appears as a promising strategy to deliver drugs to the ocular surface while maintaining its homeostasis.

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The ocular surface encompasses a variety of structures, such as the cornea, conjunctiva, lacrimal and meibomian glands, tears, connective tissues, eyelids, eyelashes and nasolacrimal duct, all of which are connected with the trigeminal pathway and the immune system, and play a part in maintaining the homeostasis of the eye.[1] Dry eye disease (DED), also called keratoconjunctivitis sicca, is one of the most common ocular surface disease with a prevalence ranging from 5% to 50% worldwide.[2] DED is a multi-factorial and often chronic disease, characterized by multiple underlying pathophysiological mechanisms including tear film instability and hyperosmolarity, neurosensory abnormalities, ocular surface inflammation and damage, leading to loss of homeostasis.[2,3] These combined mechanisms result in a "vicious circle" of immunopathogenesis, which can be particularly challenging to treat.[4]

Cyclosporin A (CsA) is an immunomodulatory agent discovered in the 1970s. Since then, it has been marketed for various medical applications such as psoriasis, Crohn's disease and organ transplants.[5–8] In ophthalmology, CsA was initially investigated to prevent graft versus host disease after transplantation of donor corneal tissues.[9] Nowadays, this medication is used for the treatment of ocular surface diseases associated with inflammation such as DED,[10] seasonal allergic conjunctivitis[11] and vernal keratoconjunctivitis (VKC), a severe and chronic form of ocular pediatric allergies.[12,13] The efficacy of CsA comes from its ability to inhibit the activity of T cells and the production of pro-inflammatory cytokines, both responsible for hyper-inflammation,[14] however, its large molecular weight and hydrophobicity result in low ocular bioavailability.[15,16] Topical solutions and emulsions represent the most common commercial formulations of CsA currently available.[17] Papilock mini® (marketed since 2005 in Japan by Santen Pharmaceutical Co. Ltd.), Modusik-A Ofteno® (marketed since 2003 in South America by Laboratorios Sophia), and TJ Cyporin® (marketed since 2003 in South Korea by Taejoon Pharm Co., Ldt.) are the main CsA solutions available on the market. In 2018, Sun Pharma Global marketed Cequa®, a new generation of CsA solutions based on a nanomicelle technology,[18] in the US and in Australia. Despite a general ease of preparation, CsA solutions require the use of surfactants and co-solvents to stabilize the formulation and prevent CsA precipitation. Unfortunately, repeated instillations of high surfactants concentrations on the ocular surface can induce ocular irritation.[17,19,20] This limitation has led to the development of new drug delivery systems (DDS) to improve CsA retention on, and penetration through, the ocular surface.[17]

Oil-in-water emulsions are well suited to solubilize lipophilic CsA as they limit the concentration of surfactants needed.[17] Moreover, oily excipients also tend to prevent ocular surface desiccation by helping to restore the lipid layer of the tear film.[21,22] Restasis® (AE-CsA, Allergan) and Ikervis® (CE-CsA, Santen) are both CsA emulsions approved by the FDA in the US and by the EMA in Europe, respectively. While AE-CsA is composed of anionic components, CE-CsA is composed of cationic ones. The advantage of cationic DDS (i.e. positively charged) is their ability to interact with the negatively-charged

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mucin layer of the tear film, increasing the residence time of CsA on the ocular surface.[15,23,24]

In this review, we will give an overview of the development of cationic emulsions (CE) as well as its main preclinical outcomes collected over the last decade. We will compare these features with the other marketed CsA formulations and hospital-compounded preparations, in term of pharmacodynamics, pharmacokinetics, ocular tolerance and toxicity, as is recommended by health authorities for the approval of new medical products.[25] Finally, we will discuss the translation of these preclinical outcomes to patients with DED.

Development of a cationic emulsion of cyclosporine A

The concept of CE was first introduced in 1996 for the oral administration of progesterone.[26] A few years later, this technology was assessed in ophthalmology for the first time, to deliver indomethacin to the ocular surface.[27] In this section, we will describe the three main components of the CE-CsA (CsA, an oil-in-water emulsion and a cationic agent) as well as their interaction with the ocular surface.

Cyclosporine A

The immunosuppressive activity of CsA was first reported in 1976 by Borel et al.[28] Since then, mechanistic studies have shown that CsA inhibits phosphatase calcineurin, preventing nuclear factor of activated T-cell (NFAT) activation and subsequent gene expression of interleukin-2 (IL-2) in activated T cells.[29,30] In addition to the calcineurin/NAFT pathway, other mechanisms of action of CsA have been hypothesized over the last few decades.[29,31] It has been shown that CsA also may inhibit T cell activation by blocking JNK and p38 signaling pathway.[29] More recently, Liddicoat et al. described that CsA may also impact innate immune dendritic cells (DC) using the similar calcineurin/NAFT pathway, which can influence adaptive immune responses via regulatory T cells induction, T helper ($CD4^+$) cell polarization and humoral responses.[31] CsA may also impact other types of innate immune cells, such as macrophages and neutrophils,[31,32] however, more studies are needed to better understand the underlying mechanisms of action. In ophthalmology, CsA has been assessed to prevent corneal graft rejection[9] and rapidly gained interest for ocular surface diseases. In addition, CsA was shown to significantly reduce the presence of $CD4^+$ T cells on the ocular surface of patients with Sjögren's syndrome,[33] while in patients with VKC, CsA also decreased the number of antigen-presenting cells (APC) cells, $CD4^+$ T cells and B cells.[34] Another study showed that CsA increased the number conjunctival goblet cells and decreased the epithelial turnover, which can be beneficial for the maintenance of ocular surface homeostasis.[35]

Oil-in-water emulsion

Oil-in-water emulsions are biphasic formulations containing oily droplets dispersed in a continuous water phase. They have been used as delivery systems for hydrophobic drugs due to their ability to incorporate water insoluble molecules. To create these emulsions, various oils and emulsifiers (i.e., surfactants) have been used as ocular DDS.[36] The choice of these components as well as their concentrations (i.e. oil/surfactant ratio) represent an important factor in the safety profile of emulsions on the ocular surface, as is described in the section 5. In CE-CsA, medium-chain triglycerides are used as the oily phase to solubilize CsA. To stabilize the oil nano-droplets (<200 nm) of CsA in the continuous phase, two nonionic surfactants are used: tyloxapol (hydrophobic) and poloxamer 188 (hydrophilic) (Figure 1a). These surfactants are already used in eye drop formulations, tyloxapol can exhibit anti-inflammatory effects in some instances,[37,38] and both are known to be well tolerated in the human eye.[39] Glycerol can also be used in the continuous phase in order to protect the ocular surface from the hyperosmolarity induced by DED.[40] The

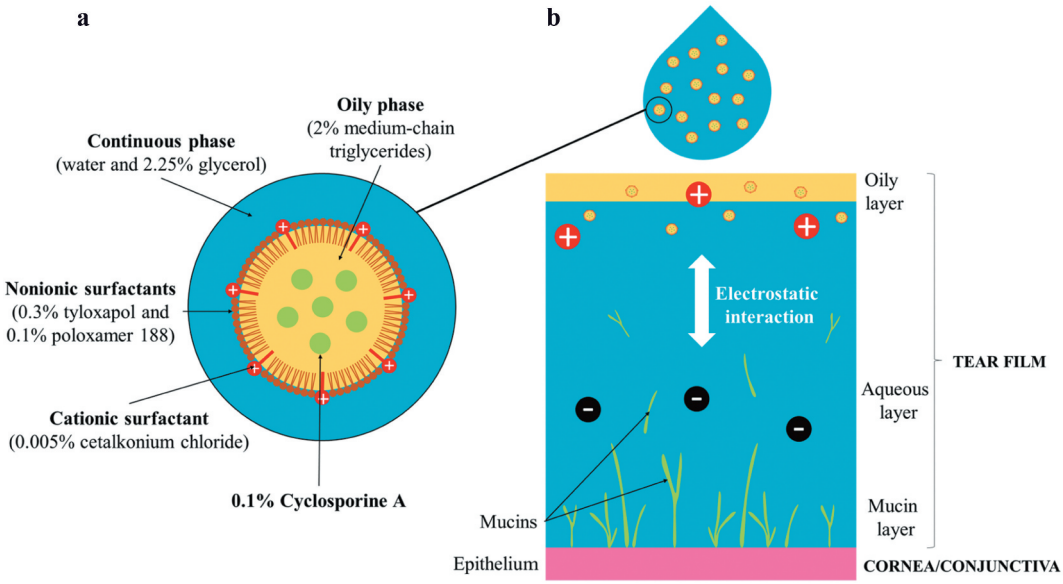


Figure 1. Principle of the use of cationic emulsion of CsA (CE-CsA) on the ocular surface. (a) Schematic structure of the CE-CsA and (b) interaction with the ocular surface.

tear film of the eye comprises 3 layers: the outer oily layer secreted by Meibomian glands, the intermediate aqueous layer produced by the lachrymal glands, and the inner mucin layer (Figure 1b). Clinically, DED is characterized by a loss of volume in all tear film layers.[3] Because of the similarities in composition between emulsions and the tear film (both oily and aqueous phases), emulsions can act as a tear film substitute, effectively reducing symptoms of DED.[41]

Cationic and polar agents

The mucin layer of the tear film plays a significant role in protecting the ocular surface. Mucins are high-molecular weight glycoproteins composed of carboxyl and sulfate groups that are negatively charged.[42] Thus, the use of cationic agents in emulsions can induce electrostatic interaction with the anionic mucin layer and improve drug bioavailability. Moreover, cationic agents cause electrostatic repulsions between nano-droplets which help to stabilize the emulsions.[43] CE have demonstrated additional benefits over nonionic or anionic emulsions in drug delivery for various biomedical applications.[44] CE-CsA contains a cationic agent called cetalkonium chloride (CKC) a homolog of benzalkonium chloride (BAK). BAK is a quaternary ammonium compound currently used as a preservative at concentrations up to 0.02% in many eye drops formulations due to its bactericidal and microbicidal properties at high concentrations. Nevertheless, several side effects have been observed in patients using formulations containing BAK, such as dry eye exacerbation[45] and corneal cell damage.[46,47] While BAK contains 12 or 14 carbons in its alkyl chains, CKC contains 16 carbons resulting in a much higher hydrophobicity and increased polarity of the molecule. As opposed to the shorter C12 and C14 alkyl chains, all the CKC is incorporated at the oil interface increasing the stability of oil nano-droplets and most importantly, reducing the amount of free molecules in the water phase that are responsible for ocular side effects.[48] In addition, *in vitro* and *in vivo* studies revealed that CE increased tear film elasticity and thickness[49] and can potentially compensate for Meibum gland dysfunction, a common clinical sign of DED.

The originality of CE-CsA comes from the combination of CsA with emulsion and the non-conventional use CKC as cationic agent and polar lipid. Concentrations of the nonpolar and polar components have been selected in order to mimic the composition of the tear fluid lipid layer (Table 1) in terms of both quantities and ratios. In particular, the limited concentration of CKC used in the CE allows to bring cationic charges as well as surfactant effects without inducing any toxic effect as it is described in section 5. Therefore, each of these components provides specific properties to the final emulsion and tear-film supplementation, which helps to break the vicious circle and restore homeostasis of a multifactorial disease such as DED. Moreover, the oils contained in CE can diffuse in the tear film lipid layer which can then act as a drug reservoir for the active molecules (eg., CsA) solubilized in the oils.[53]

Table 1. Similarities between the composition of the tear film and a cationic emulsion.

	Function	Healthy tear film composition	Cationic emulsion composition
Lipid phase	To prevent desiccation of the ocular surface.	<1%*	2.005%#
Polar lipids in the lipid phase	To stabilize the lipid phase	Up to 8%[50–52]	0.25%

*40-90 nm of lipid layer thickness for an aqueous layer thickness of up to 4000 nm[51]; #2% medium-chain triglycerides + 0.005% CKC.

Pharmacodynamics profile

In the last decade, many preclinical studies have been published about the pharmacodynamics of CE-CsA and its vehicle using *in vitro* and *in vivo* models (Table 2). Several outcomes have been emphasized in these studies including anti-inflammatory potency, effect on DED symptoms, effect on wound healing, as well as the modulation of the gene expression profile.

Anti-inflammatory potency

Ocular immunological diseases, such as DED, are usually associated with self-perpetuating inflammation, resulting in a chronic disease state.[4] *In vitro* studies assessed the anti-inflammatory effect of both CE-CsA and CE alone. One such study compared different formulations of CE containing two different concentrations of CKC (0.002% and 0.005%) or with tyloxapol surfactant alone.[55] Both CKC concentrations decreased the *in vitro* secretion of several proinflammatory factors in several cell types with several types of induced stress (Figure 2a). The study also revealed that emulsions containing only tyloxapol reduced the secretion of IL-6 and IL-8 from human corneal epithelial cells (HCECs) stressed using lipopolysaccharide.[55] Interestingly, a recent study has shown the anti-inflammatory activity of cationic lipids through the Protein Kinase C (PKC) pathway[66] and similarly, CKC has been shown to act as an inhibitor of the PKC pathway, which downregulates proinflammatory factors,[55] and parallels the observation by Chen and collaborators with PKC alpha knock-out mice. Therefore, CE has higher anti-inflammatory effects compared to AE, independently of the CsA action, suggesting that CE alone has anti-inflammatory potency. These results are confirmed by another study assessing the effect of CE-CsA and AE-CsA with the same CsA concentration on an *in vitro* desiccation stress model of HCECs.[54] Results showed that levels of the proinflammatory factors (phospho Nuclear Factor kappa B (*p*-NF-κB p65) and phospho Inhibitor of kappa B alpha (*p*-IκBα)) and a proinflammatory cytokine (Tumor Necrosis Factor (TNF-α)) were found to be lower when HCECs were treated with CE-CsA compared to AE-CsA.[54]

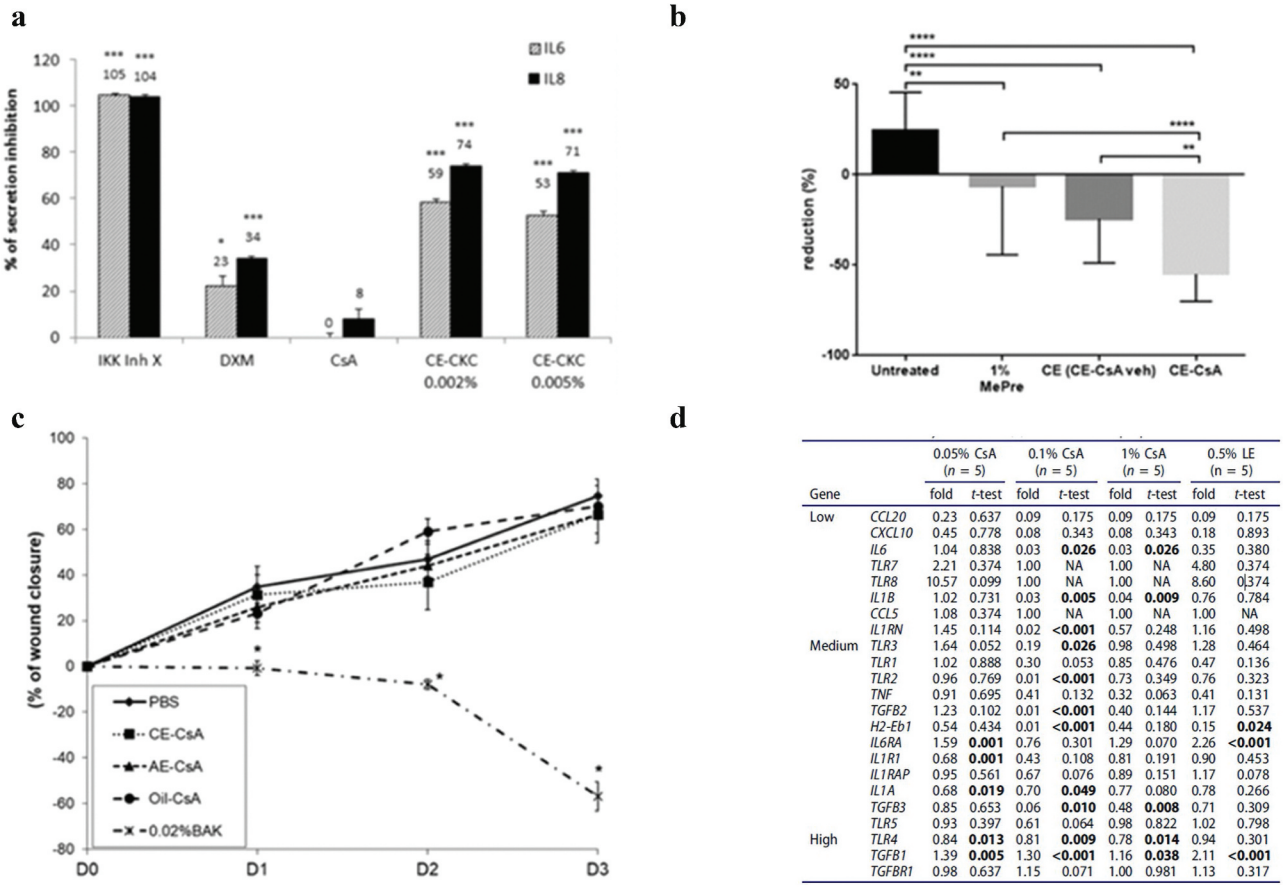
Effect on the DED signs

Effects of CE-CsA and CE alone on signs of DED have also been tested using *in vivo* mice models.[59–64] DED was induced in mice using a controlled environment room with low humidity, as previously described in the literature.[67] Clinically, two

Table 2. Overview of the pharmacodynamics studies realized on the CE-CsA.

Study types	Cell/Tissue/Animal Model	Assessments	Main results	Ref.
In vitro studies	Human corneal epithelial cell line with induced of desiccating stress.	Quantification of inflammatory factors (*p*-NF-kB p65, *p*-IkBa and TNF-α).	Decrease of the inflammatory factors for CE-CsA compared to AE-CsA.	54
	Peripheral blood mononuclear cells, CD4 + T lymphocytes and human corneal epithelial cell line.	Quantification of inflammatory factors after stress exposure to stimulation with anti-CD3/anti-CD28 or lipopolysaccharide (LPS).	Decreased inflammatory gene expression (IFN-γ, IL-17A, CXCL-9 and TNF-α, THBS1 and CCL-2) in presence of CE. Decreased secretion of IL-17, TNFα, IFN-γ, IL-2, IL-6 and IL-8 in presence of CE.	55
	Human corneal epithelial cell line with scrapping area	Wound healing analysis (fluorescence microscopy)	Similar behavior of wound healing for CE-CsA, AE-CsA, and PBS.	56,57
In vivo studies	Mice with spontaneous development of Sjögren's syndrome (NOD.B10.H2^b model)	Tear volume (PRT lacrimation test), corneal staining (lissamine), conjunctival goblet cell density and IL1-β quantification	Significant increase of tear volume at days 30 and 45 for CE-CsA and nanomicelle-CsA (Cequa®) groups compared to AE-CsA group. No significant difference of corneal staining, goblet cell density and IL-1β concentration between CE-CsA, nanomicelle-CsA, and AE-CsA groups.	58
	C57BL/6 N mice with DED induced in a controlled environment room	Tear volume (PRT lacrimation test), corneal staining (fluorescein), histological analysis of the ocular surface (HES staining).	Increased tear volume for both CE-CsA and AE-CsA groups. Reduced CFS scores for CE-CsA group compared to AE-CsA group. Only 2 out 10 mice presented ocular lesions for CE-CsA group compared to 5 mice for AE-CsA group.	59
	C57BL/6 N mice with DED induced in a controlled environment room	Corneal staining (fluorescein), measurement of the expression profile of inflammatory genes	After 7 days of treatment, CFS scores were reduced by 21% and 31% with AE-CsA and CE-CsA, respectively. Decreased gene expression of IL-1α and TLR-4 for both AE-CsA and CE-CsA groups. Decreased gene expression of H2-Eb1, IL-1β, IL-1RN, IL-6, TGF-β2, TGF-β3, TLR-2 and TLR-3, only for CE-CsA group.	60
	C57BL/6 N mice with DED induced in a controlled environment room	Corneal staining (fluorescein), measurement of the expression profile of inflammatory genes	Reduced CFS scores (vs. dry eye baseline) by 9.2, 18.5 and 22.8% with lifitegrast solution (Xiidra®), CE and CE-CsA, respectively. Difference in gene expression (IL-6, CXCL-1, CXCL-10, CCL-19 and TNF) between lifitegrast solution, CE and CE-CsA groups.	61
	C57BL/6 N mice with DED induced in a controlled environment room	Tear volume (PRT lacrimation test), corneal staining (fluorescein)	Reduced CFS scores (vs. dry eye baseline) by 28, 36 and 59% with 1% methylprednisolone, CE and CE-CsA, respectively.	62,63
	C57BL/6 N mice with DED induced in a controlled environment room	Tear volume (PRT lacrimation test), corneal staining (fluorescein).	Significant increased tear volume only for CE-CsA. Reduced CFS scores for both AE-CsA and CE-CsA groups.	64
	Rats with corneal scraping	Quantification of corneal wound healing and inflammatory cell infiltration by IVCM	5 days after scrapping, IVCM scores and cell infiltration were found lower for CE-CsA group compared to CE, 0.2% BAK solution or NaCl.	65

Abbreviations: AE = Anionic Emulsion; BAK = benzalkonium chloride; CCL = C-C motif Ligand; CE = Cationic Emulsion; CFS = Corneal Fluorescein Staining; CsA = Cyclosporine A; CXCL = C-X-C motif Ligand; DED = Dry Eye Disease; H2-Eb1 = H-2 class II histocompatibility antigen; HES = Hematoxylin/Eosin/Safran; IFN = Interferon; IL = Interleukin; IVCM = In Vivo Confocal Microscopy; PBS = Phosphate Buffered Solution; PRT = Phenol Red Thread; TGF = Tumor Growth Factor; TLR = Toll-like Receptor; THBS1 = Thrombospondin.



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		0.05% CsA (n = 5)		0.1% CsA (n = 5)		1% CsA (n = 5)		0.5% LE (n = 5)	
Gene		fold	t-test	fold	t-test	fold	t-test	fold	t-test
Low	*CCL20*	0.23	0.637	0.09	0.175	0.09	0.175	0.09	0.175
	CXCL10	0.45	0.778	0.08	0.343	0.08	0.343	0.18	0.893
	IL6	1.04	0.838	0.03	**0.026**	0.03	**0.026**	0.35	0.380
	TLR7	2.21	0.374	1.00	NA	1.00	NA	4.80	0.374
	TLR8	10.57	0.099	1.00	NA	1.00	NA	8.60	0.374
	IL1B	1.02	0.731	0.03	**0.005**	0.04	**0.009**	0.76	0.784
	CCL5	1.08	0.374	1.00	NA	1.00	NA	1.00	NA
	IL1RN	1.45	0.114	0.02	**<0.001**	0.57	0.248	1.16	0.498
Medium	*TLR3*	1.64	0.052	0.19	**0.026**	0.98	0.498	1.28	0.464
	TLR1	1.02	0.888	0.30	0.053	0.85	0.476	0.47	0.136
	TLR2	0.96	0.769	0.01	**<0.001**	0.73	0.349	0.76	0.323
	TNF	0.91	0.695	0.41	0.132	0.32	0.063	0.41	0.131
	TGFB2	1.23	0.102	0.01	**<0.001**	0.40	0.144	1.17	0.537
	H2-Eb1	0.54	0.434	0.01	**<0.001**	0.44	0.180	0.15	**0.024**
	IL6RA	1.59	**0.001**	0.76	0.301	1.29	0.070	2.26	**<0.001**
	IL1R1	0.68	**0.001**	0.43	0.108	0.81	0.191	0.90	0.453
	IL1RAP	0.95	0.561	0.67	0.076	0.89	0.151	1.17	0.078
	IL1A	0.68	**0.019**	0.70	**0.049**	0.77	0.080	0.78	0.266
	TGFB3	0.85	0.653	0.06	**0.010**	0.48	**0.008**	0.71	0.309
	TLR5	0.93	0.397	0.61	0.064	0.98	0.822	1.02	0.798
High	*TLR4*	0.84	**0.013**	0.81	**0.009**	0.78	**0.014**	0.94	0.301
	TGFB1	1.39	**0.005**	1.30	**<0.001**	1.16	**0.038**	2.11	**<0.001**
	TGFBR1	0.98	0.637	1.15	0.071	1.00	0.981	1.13	0.317

Figure 2. Main outcomes regarding pharmacodynamics of CsA cationic emulsions. (a) Effects of the cationic emulsions of cetalkonium chloride (CE-CKC) emulsions, IKK Inhibitor X (IKK Inh X), dexamethasone (DXM), and cyclosporine A (CsA) on IL-6 and IL-8 release by HCE-2 cells following lipopolysaccharide (LPS) stimulation. *p < .05; **p < .01; ***p < .001.[55] (Adapted from reference [55]). (b) Percentage of CFS score reduction after 10 days-treatment using 1% methylprednisolone (MePre), CE (CE-CsA veh) and CE-CsA on an *in vivo* mouse dry eye model. **p < .01; ***p < .001; ****p < .0001.[62] (Adapted from reference [62]). (c) Wound closure from day 1 to day 3 after the different treatments. * $p < .0001–0.02$ compared to the other four groups at the corresponding times.[56] (Adapted from reference [56]). (d) Fold changes and t-test values (vs. DED untreated) for the 23 genes detected among the 34 genes followed in this study in the DED (± treatment) mice corneas. (LE, loteprednol etabonate).[60] (Adapted from reference [60]).

common signs of DED are a loss of tear fluid volume and keratitis.[3] A phenol red thread (PRT) lacrimation test and corneal fluorescein staining (CFS) were performed to assess the tear fluid volume and corneal abrasion, respectively. Results showed that the CFS scores of mice with induced DED were reduced by 36% for the group treated with CE alone (CE-CsA vehicle) and by 59% for the group treated with CE-CsA when compared to the untreated group[62] (Figure 2b), however, only CE-CsA was found to significantly increase tear fluid volume of mice.[64] CE-CsA also exhibited a greater reduction of CFS scores compared to other corticosteroids including 1% methylprednisolone[62] and 0.5% loteprednol etabonate (LE, Lotemax®, Baush+Lomb)[60] as well as 5% lifitegrast solution (Xiidra®, Shire), a lymphocyte function-associated antigen-1 (LFA-1) antagonist.[61] Interestingly, CE alone was found to be more efficient at reducing CFS scores compared to 5% lifitegrast solution.[61]

Compared to AE-CsA, treatment of DED with CE-CsA induced lower CFS scores, but a significant difference was not achieved in all studies.[59,60,64] However, CE-CsA treatment did reduce the occurrence of ocular lesions due to DED, compared to AE-CsA. These results highlight that CE itself can reduce keratitis induced by DED, which can be explained by its anti-inflammatory activity as well as its improved electrostatic interactions with the ocular surface and tear film lipid layer, previously described. A recent study tested different CsA formulations on mice with spontaneous development of Sjögren's syndrome.[58] The results revealed that one instillation a day (QD) of CE-CsA induced a higher tear fluid volume than two instillations a day (BID) of AE-CsA. More interestingly, when comparing CE-CsA (QD) and nanomicelle-CsA (BID),[58] no significant difference in tear fluid volume (except at day 60) was found. Overall, these studies proved that the synergic effects of CE and CsA significantly reduced signs of DED in *in vivo* models using a single instillation a day.

Effect on wound healing

Some topical anti-inflammatory medications, such as prednisolone or dexamethasone, are known to delay corneal wound healing.[68,69] *In vitro* and *in vivo* studies have been performed

with CE-CsA to assess its effect on wound healing. HCECs were used to assess the cytotoxicity of different CsA formulations, as well as their effects on cell migration and proliferation.[56,57] Results showed that all CsA formulations, including CE-CsA, maintained an *in vitro* wound healing rate similar to phosphate buffered saline (PBS) (Figure 2c).[56,57] Effect on wound healing has also been evaluated *in vivo* using a rat scrapping assay.[65] Rat corneas treated with CE-CsA or CE were found to heal in 5 days, a similar timeframe to corneas treated with the control (NaCl). Moreover, the number of inflammatory cells after 5-days healing was found to be lower for corneas treated with CE-CsA, compared to control group.[65] It is worth noting that, contrary to BAK, CKC contained in CE-CsA showed no sign of cytotoxicity nor delay of cell proliferation.

Modulation of the gene expression profile

Several studies revealed that the use of CE-CsA or CE itself can modulate the expression profile of inflammatory genes. It has been shown that the presence of CE on stress-induced cells *in vitro* decreased the expression of several inflammatory genes, such as interferon (IFN)-γ, IL-17A, C-X-C motif ligand (CXCL)-9 and TNF-α, Thrombospondin (THBS)-1 and C-C motif ligand (CCL)-2.[55] This modulation of gene expression profile has also been observed *in vivo* in mice models.[60] While both CE-CsA and AE-CsA reduced expression of IL-1α and toll-like receptor (TLR)-4, CE-CsA was the only one able to reduce the expression of numerous other inflammatory genes including 2-Eb1, IL-1β, IL-1RN, IL-6, transforming growth factor (TGF)-β2, TGF-β3, TLR-2 and TLR-3 (Figure 2d).[60]

Several compelling pharmacodynamics outcomes have been highlighted in the different *in vitro* and *in vivo* studies performed during the preclinical development of CE-CsA. The anti-inflammatory properties, provided by both CsA and the vehicle, CE, indicate that CE-CsA is very effective at treating the symptoms of DED, especially as CE-CsA demonstrates significant reductions in ocular lesions while also facilitating the restoration of tear fluid volume. Moreover, in comparison to other topical treatments, no delay in wound healing was observed using CE-CsA, while the differences achieved in corneal gene expression show how the formulation can modulate ocular inflammation. Although these *in vitro* and *in vivo* findings present strong data for the efficacy of CE-CsA alone, they have additionally been confirmed clinically, as patients with severe DED have reported reductions in the signs and symptoms of their DED after using CE-CsA.[70,71]

Pharmacokinetics outcomes

Several studies assessing the pharmacokinetics profile of CE-CsA have been published over the last decade (Table 3).

It has been shown that after a single dose of either 0.05% or 0.1% CE-CsA, the CE formulation was found to deliver a higher CsA concentration (C_{max}) to the rabbit corneas as well a better exposition (area under the curve (AUC)) compared to a single dose of 0.05% AE-CsA (Figure 3a).[74] CsA concentration was measured in ocular and non-ocular tissue after 10 days of treatment using 0.05% CE-CsA (QD), 0.1% CE-CsA (QD) or 0.05% AE-CsA (BID). Results showed no significant difference in CsA concentration between the three groups.[74] Therefore, we hypothesize that CE-CsA acts as a drug reservoir for the sustained release of CsA, resulting in a lower dose regimen requirement (QD vs. BID for AE-CsA). CE-CsA has also been compared to CsA compounded formulations prepared in hospital pharmacies. It was found that $AUC_{(0.5-24h)}$ was 5.4 and 3.9 times higher for CE-CsA compared to CsA hospital-compounded preparations (CsA-HP), in the cornea and the conjunctiva, respectively (Figure 3b).

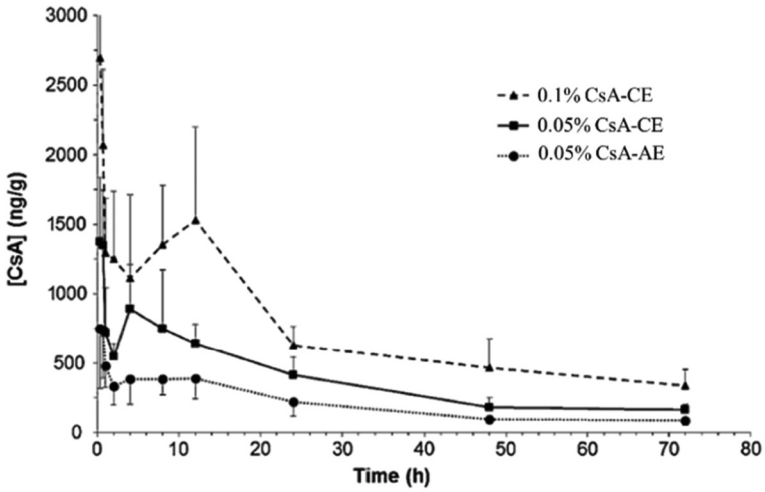
These results suggest the superiority of CE-CsA in term of ocular bioavailability compared to other formulations (AE-CsA or CsA-HP). This higher performance can be explained by the unique electrostatic interaction of CE with the tear film, as reported in section 2.2. More recently, two *ex vivo* studies have been published about the pharmacokinetics profile of new CsA DDS in academic research development. Results obtained to date demonstrate a slight increase of CsA in *ex vivo* corneas using these DDS compared to CE-CsA, but otherwise are largely comparable.[72,73] However, significant investigations regarding the *in vivo* pharmacokinetics and pharmacodynamics of these new formulations still need to be performed, to evaluate the real benefit of these technologies for the treatment of ocular surface diseases.

Table 3. Overview of the pharmacokinetics studies realized on the CE-CsA.

Study types	Cell/Tissue/ Animal Model	Assessments	Main results	Ref.
Ex vivo studies	Porcine eyeballs	CsA quantification in the corneas 1 h after eye drops instillation (HPLC analysis in homogenized tissues).	Increased CsA concentration in the corneas treated with CE-CsA compared to AE- CsA. Decreased CsA concentration in the corneas treated with semifluorinated alkane-based eye drops compared to CE-CsA.	[72]
	Porcine eyeballs	CsA quantification in the corneas after eye drops instillation and washing (HPLC analysis in homogenized tissues).	No significant difference in the CsA concentrations for the corneas treated with CE-CsA compared to Poloxamer 407/TPGS mixed micelles.	[73]
In vivo studies	Pigmented rabbits	Quantification of CsA in conjunctiva and cornea at different time points.	$AUC_{(0.5-24h)}$ 5.4 and 3.9 times higher for CE-CsA compared to CsA hospital-compounded preparations, in the cornea and the conjunctiva, respectively.	Data on file
	Pigmented rabbits	Quantification of CsA concentrations in ocular tissues at different time points.	Higher Cmax and AUC to the cornea and conjunctiva for CE-CsA 0.1% and 0.05% compared to AE-CsA.	[74]

Abbreviations: AE = Anionic Emulsion; AUC = Area Under the Curve; CE = Cationic Emulsion; C_{max} = Maximal Concentration; HPCL = High Performance Liquid Chromatography.

a



b

	Cornea		Conjunctiva	
	CsA-CE	CsA-HP	CsA-CE	CsA-HP
C_{max}(ng/g)	695	229	239	488
T_{max} (hours)	1	0.5	1	0.5
$AUC_{(0.5\text{-}24h)}$ (ng/g)	7377	1374	1484	382

Figure 3. Main outcomes regarding pharmacokinetics of CE-CsA. (a) Changes in CsA concentration with time after a single unilateral topical administration in the cornea of pigmented rabbits.[74] (Adapted from reference [74]). (b) Pharmacokinetics profile of CsA delivery after a single unilateral topical administration of CE-CsA or CsA hospital-compounded preparations (CsA-HP) in the cornea and conjunctiva of pigmented rabbits.

Table 4. Overview of the ocular toxicity and tolerance studies realized on the CE-CsA.

Study types	Cell/Tissue/ Animal Model	Assessments	Main results	Ref.
In vivo studies	Mice	Non-radioactive local nymph node assay (LLNA) using 5-BrdU incorporation.	CE-CsA did not induce delayed contact hypersensitivity.	Data on file
	Mice	Phototoxicity and photosensitizing (photoallergic) skin tests.	No phototoxic or photosensitizing (photoallergic) potential observed for CE-CsA or CE.	Data on file
	New Zealand albino rabbits	28-day tolerance study. Eye irritation test (Draize test). Histological analysis	CE-CsA was found safe and well tolerated following 4 and 6 daily instillations over 28 days.	Data on file
	Rabbits	Clinical observation (IVCM), eye irritation test (Draize test)	Lowest toxicity similar to PBS for CE-CsA group.	56,57
	New Zealand albino rabbits	Conjunctiva changes using slit-lamp examination, flow cytometry and impression cytology. Corneal changes using IVCM. Histological analysis	After 15 instillations at 5 min intervals, highest toxicity induced by BAK-solution. Moderate toxicity for BAK-CE and CKC-solution. Low toxicity similar to PBS for CKC-CE. Lower CD45+ cell infiltration and apoptotic cells for BAK-CE and CKC-CE compared to BAK- or CKC-solutions.	75

Abbreviations: AE = Anionic Emulsion; BrdU = Bromodeoxycytidine; CE = Cationic Emulsion; IVCM = In Vivo Confocal Microscopy.

Ocular toxicity and tolerance

Exploratory and regulatory (according to the Good Laboratory Practices) studies of ocular toxicity and tolerance have been performed to assess the safety of CE-CsA (Table 4).

The first study focused on the assessment of the safety of CE without an active molecule. For that, repeated instillations (15 times at 5-min interval) of 0.002% CKC versus 0.02% BAK in solution (CKC-sol, BAK-sol) or as a CE were tested on rabbits.[75] Results showed that BAK-sol induced the

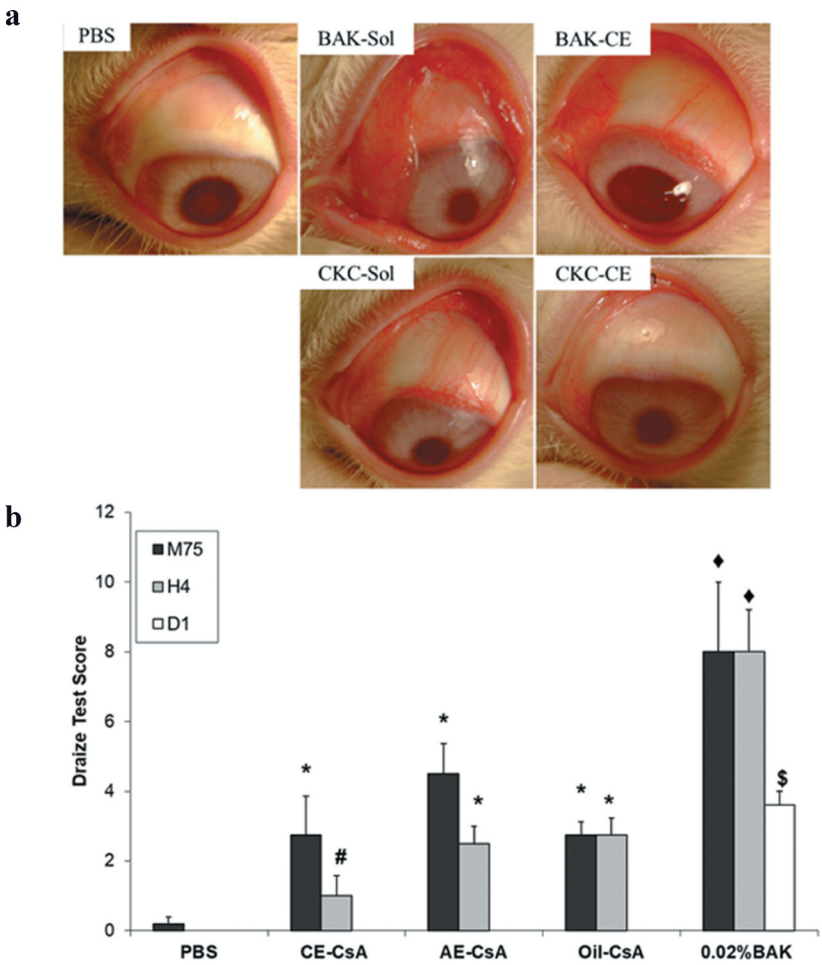


Figure 4. Main outcomes regarding ocular toxicity and tolerance of CE-CsA. (a) Microphotographs of typical clinical features of rabbit eyes after 15 instillations at 5-min interval of 0.002% CKC or 0.02% BAK containing in a solution (sol) or a CE.[75] (b) Draize test score calculated at different time points (75 min, 4h and 1 day) after 15 instillations of CE-CsA, AE-CsA, Oil-CsA or 0.02% BAK. * $p < .02$ compared to PBS and $p < .004$ compared to 0.02% BAK. # $p < .01$ compared to AE-CsA, Oil-CsA, and 0.02% BAK. ♦ $p < .0001$ compared to PBS. $ $p = .0003$ compared to PBS, CE-CsA, AE-CsA, and Oil-CsA groups.[56] (Adapted from reference [56]).

Table 5. Similarities between preclinical outcomes and results of clinical studies.

	Preclinical outcomes	Results of clinical studies
Ocular inflammation	Reduced expression and secretion of pro-inflammatory factors[55,60]	Lower expression of a cell surface inflammatory marker (HLA-DR) for the group treated with CE-CsA and CE.[70,71,76]
Tear film volume	Increased tear fluid volume (PRT: Phenol Red Thread Test)[59]	72.4% and 58.2% improvement in Schirmer tear test observed with CE-CsA and CE treatment, respectively.[76]
Tear fluid stability	Increased tear fluid elasticity and thickness[49]	40.5% and 22.2% improvement in TBUT observed with CE-CsA and CE, respectively.[76]
Corneal damage	Decreased CFS	Change over time (vs baseline) and CFS score reduction with both CE-CsA and CE. Ref 74 Greater improvement with CE-CsA over vehicle in CFS change after 6-months treatment.[71,76,77]

CE = Cationic Emulsion; CFS = Corneal Fluorescein Score; HLA-DR = Human leukocyte antigen DR; TBUT = tear breakup time.

highest corneal epithelial damage, inflammatory infiltration as well as clinical signs of eye inflammation (hyperemia, chemosis and purulent secretions) (Figure 4a). In contrast, CKC-CE exhibited the lowest toxicity, similar to the control group (PBS). Both BAK-CE and CKC-sol were found to induce moderate toxicity.[75] Contrary to the 0.02% BAK usually used in many marketed eye drops as preservative, a concentration of CKC of 0.002% in CKC-CE is well-tolerated on the eye without inducing any side effects. The second study assessed the toxicity of three formulations of CsA including CE-CsA, AE-CsA and CsA-oil using a Draize test (eye irritation test).[56] CE-CsA showed a lower Draize test score, indicating less ocular irritation, 4 hours after instillation compared to both CE-CsA and CsA-oil (Figure 4b). Following these two successful exploratory studies, 28-day (GLP) local tolerance studies (Table 4) with 0.002% and 0.005% CKC in CKC-CEs were performed before pivotal phase III studies were initiated, as recommended for new drug approval. Results of these GLP regulatory studies showed that CE-CsA containing 0.005% CKC was safe and well-tolerated following 4 to 6 daily installations over 28 days. Additionally, no delayed contact hypersensitivity was found

using a local lymph node assay on mice (data on file). Finally, CE-CsA or CE did not show any phototoxic or photosensitizing (photoallergic) potential (data on file).

These studies indicate that the excipients (i.e., its concentration) of the vehicle and its formulation (solution vs emulsion) used to deliver CsA to the ocular surface can significantly impact the ocular tolerance of the formulation. The use of emulsion containing 0.002% or 0.005% CKC induced the lowest toxicity compared to the other CsA formulations tested, showing that CE using low CKC concentrations is well-tolerated by the eye. These findings can also be explained by the high anti-inflammatory potency of CE described in section 3.1.

Translation of preclinical outcomes to patients with DED

The previous sections described the main outcomes observed through the preclinical development of a CE-CsA. The promising results obtained during this phase allowed testing of CE-CsA on human patients via clinical trials (SANSIKA and SICCANOVE for DED and VEKTIS for VKC). Very interestingly, the clinical studies showed clinical outcomes that can be explained with the preclinical findings in term of therapeutic performance on patients with DED signs and symptoms (Table 5).

First, a significant decrease of HLA-DR, a cell surface inflammatory marker, was observed on patients with DED after treatment with CE-CsA or CE alone,[70,71,76] further confirming the anti-inflammatory potency observed during the preclinical testing.[55,60] Interestingly, HLA-DR was shown to be well correlated with some inflammatory markers that are observed in both dry eye patients and dry eye mice model using transcriptomics and gene profiling.[60,78]

Tear breakup time (TBUT) was found to be higher for patients after treatment with both CE-CsA or CE.[76] This result corroborates the increased tear fluid elasticity and thickness observed after the same treatment in animal models.[49] In addition, the tear fluid volume was found to increase using CE-CsA and CE alone, in both preclinical and clinical studies[76] with phenol red thread (PRT) and Schirmer tests respectively. Finally, a decrease in corneal epithelial damage, similar to that observed during preclinical development, has also been well observed in clinical studies via a significant improvement in CFS after treatment with CE-CsA and CE.[71,76]

Conclusion

The multiple benefits of using CsA to treat ocular surface diseases, such as DED, are widely accepted, and position CsA formulations among the armamentarium of anti-inflammatory drugs used for DED management. Many alternative CsA formulations are currently used in clinical settings, the most common being solutions, AE, CE and hospital-compounded preparations. In this review, we discussed the mechanisms of action and preclinical outcomes of a sophisticated CE-CsA. The use of CE as vehicle provides unique properties to the CsA formulation: (1) reduced secretion of pro-inflammatory factors promoted by the components composing the oil-in-water emulsion, (2) stabilization and restoration of tear fluid surface and (3) high precorneal residence time of CsA, due to the innovative and non-conventional use of CKC. These combined modes of action result in a improved reduction of the signs and symptoms of DED compared to the other CsA formulations. Very interestingly, CE alone was found to modulate the expression of many pro-inflammatory genes, and more studies should be performed in gene expression profile to better understand the underlying mechanisms of action of CE on the ocular surface. Additionally, although the use of quaternary ammonium in eye drops is contested due to well-known BAK ocular toxicity, no signs of cytotoxicity, ocular toxicity nor delay of wound healing was observed using CE-CsA containing CKC. The safety of CKC can likely be explained by its chemical properties (high hydrophobicity and polarity), allowing for a better incorporation of CKC at the surface of the oil nanodroplets and thus resulting in a very low concentration of free CKC molecules in the aqueous phase from where they can contribute to ocular toxicity.

The good ocular tolerance and efficiency observed during the preclinical and clinical testing of CE-CsA lead to the successful approval of this technology by Santen for the treatment of DED in Europe, Asia and Australia, and for the treatment of VKC in Europe and Canada. The higher performance of CE-CsA over AE-CsA, led to a decreased posology requirement (QD vs BID), while still significantly reducing the signs and symptoms of DED in patients. While two instillations of AE-CsA are required per day, only one instillation of CE-CsA is needed. This lower dose regimen may result in better patient compliance and treatment experience. With regards to hospital preparations, in addition to the higher *in vivo* efficacy, CE-CsA presents other major advantages including well-defined product information, a standardized manufacturing process and quality controls which include adherence to strict regulations and pharmacovigilance.

CE appears to be an optimal vehicle by which hydrophobic drugs, such as CsA, can be delivered. This delivery system is additionally able to protect the ocular surface, which is often altered or damaged during both acute and chronic diseases. Therefore, we believe that CE could represent a promising drug delivery solution for many other types of ocular surface diseases.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. JSG and PD are employees of Santen SAS.

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