Anti-inflammatory activity of CKC-containing cationic emulsion eye drop vehicles

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Purpose: Preservative-free cationic emulsion-based artificial tears (ATs) or drug vehicles are innovative eye drop formulations with tear film stabilization and drug delivery properties, and valuable in vivo anti-inflammatory and wound healing properties. These ATs have recently reached the market as ATs for the management of dry eye disease (DED) symptoms (i.e., Cationorm) or as a drug vehicle for cyclosporine (Ikervis). The aim of the present study was to explore the mechanism of action underlying the intrinsic anti-inflammatory and wound-healing efficacies harbored by the cationic emulsions of cetalkonium chloride (CE-CKC).
Methods: The anti-inflammatory activity of two CE-CKC (0.002% and 0.005% CKC) emulsions was evaluated by assessing the expression of proinflammatory genes and the secretion of various markers in the following human cell types stressed by different agents: peripheral blood mononuclear cells (PBMCs; stimulation with anti-CD3/anti-CD28 or lipopolysaccharide (LPS)), CD4+ T lymphocytes (TCD4; stimulation with anti-CD3/anti-CD28), and a human corneal epithelial cell line (HCE-2; stimulation with LPS). The cells were incubated for 30 min with a 10% dilution of CE-CKC emulsions and then cultured without the emulsions for 24 h or 72 h in the presence of the various challenging agents. The supernatant was collected, and the secreted markers quantitated with flow cytometry or an enzyme-linked immunosorbent assay (ELISA). Gene expression of inflammatory markers was evaluated only in the PBMCs and HCE-2 cells stimulated with LPS. The in vitro protein kinase C (PKC) binding assay for IC_{50} determination was performed using standard procedures.
Results: The CE-CKC emulsions decreased inflammatory gene expression in LPS-stimulated PBMCs (*IFN-γ*, *IL-17A*, *CXCL-9*, and *TNFα*) and LPS-stimulated HCE-2 cells (*THBS1* and *CCL2*). Both CE-CKC emulsions inhibited the secretion of IL-17 (from anti-CD3/anti-CD28-stimulated TCD4), TNFα, IFN-γ, and IL-2 (from anti-CD3-/anti-CD28-stimulated PBMCs), and IL-6 and IL-8 (from LPS-stimulated HCE-2). The in vitro PKC binding assay revealed that CKC, the cationic agent, is a specific PKCα inhibitor. In addition, tyloxapol, another excipient, showed some anti-inflammatory activity on IL-6 and IL-8 in the LPS-stimulated HCE-2 cells.
Conclusions: This study indicates that the CE-CKC emulsions are able to directly modulate the secretion and expression of proinflammatory cytokines and chemokines. The results also suggest that CKC and tyloxapol are pharmacologically active excipients with potentially beneficial effects in vivo. These data shed new light on the efficacy observed on the DED signs of these CE-CKC emulsions in clinical trials.

Dry eye disease (DED) is a complex multifactorial disease that affects the ocular surface and is characterized by symptoms of discomfort and blurred vision (DEWS II definition) [1]. To alleviate the symptoms and clinical signs of DED, numerous artificial tears (ATs) have been developed to either replenish the water phase of the tear (e.g., aqueous-based ATs) or stabilize the tear film (TF), for example, by improving the TF lipid layer (TFLL) stabilization with oil-based (i.e., lipid-containing) ATs [2-7]. Cationic oil-in-water emulsions are new ATs based on the Novasorb® technology [8] that deliver nonpolar and polar lipids (i.e., surfactant) to

the ocular surface to restore and stabilize the deficient TFLL found in patients with DED [9]. Nonpolar oils are either light and heavy mineral oils or medium-chain triglycerides (MCTs) whose function is to thicken the nonpolar phase of the TFLL [10,11]. The polar lipid used in the Novasorb® technology cationic emulsions (CEs) is cetalkonium chloride (CKC), a highly lipophilic lipid with an aliphatic chain containing 16 carbons and a positive charge harbored by a stable quaternary ammonium, which positions itself at the oil/aqueous phase interface in the emulsions and in the TF [12,13]. Through this mechanical stabilization of the TFLL/aqueous phase interface [10], the cationic emulsion AT Cationorm® was demonstrated to improve TF breakup time (TFBUT) and alleviate DED symptoms in patients [6,14]. Parallel to this improvement in the TF mechanical properties, Cationorm® is also able to rapidly induce a significant reduction of the corneal fluorescein staining (CFS) score in patients with mild

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to moderate DED [6]. Moreover, Ikervis® vehicle, which is a cationic emulsion of CKC (CE-CKC, 0.005% CKC), is able to significantly improve the CFS score in a population of patients with severe DED [15].

During the development process of CE-CKC emulsions in vivo data in different animal models strongly suggest that the cationic emulsion ATs, or cationic emulsion vehicles, with CKC as the cationic agent, possessed anti-inflammatory and epithelial healing properties. CE-CKC emulsions are able to improve the CFS score (i.e., improve wound healing) in a mouse model of DED better than 1% methylprednisolone [16], promote the corneal epithelium healing process in a rat corneal epithelium debridement model [17], inhibit the recruitment of inflammatory cells in rabbit conjunctival associated lymphoid tissue (CALT) [18], and decrease the number of inflammatory cells in the stroma of rat corneas subjected to experimental corneal epithelium debridement [17,19].

Preliminary in vitro experiments also suggested that CE-CKC emulsions support the wound-healing process [17] and decrease the secretion of interleukins and chemokines [19,20]. The aim of the present study was to confirm that CE-CKC emulsions have a direct ancillary anti-inflammatory activity and elucidate the mechanism—i.e., identify the contributor(s)—responsible for the anti-inflammatory activity of CE-CKC emulsions, because it is evident that the mechanical properties of the CE-CKC emulsions (for which these emulsions were designed) cannot solely explain all the in vivo beneficial properties observed of the CE-CKC emulsions.

METHODS

Material and cell lines: This study followed the tenets of the Declaration of Helsinki and adhered to the ARVO statement on human subjects. Total peripheral blood was obtained from the French Blood Institute (cession agreement reference: CA-PLER-2016 012). Patient gave written consent for the use of the blood. Peripheral blood mononuclear cells (PBMCs) and CD4+ T lymphocytes (TCD4) were collected from a 52-year-old female donor. PBMCs were isolated from total peripheral blood following a standard Ficoll® procedure, and TCD4 were further purified by magnetic cell separation using human CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Human corneal epithelial cells immortalized with SV40 (HCE-2 cells) were from the ATCC (Molsheim, France). Reference compounds IKK Inhibitor X and protein kinase C (PKC) Inhibitor (Bisindolylmaleimide I) were from Merck Chemical (Fontenay sous Bois, France), and dexamethasone (DXM) and cyclosporine A (CsA) were from Sigma (Saint Quentin Fallavier, France).

HCE-2 cell line authentication: Cell line authentication was performed by the ATCC cell line authentication service (Manassas, VA) using short tandem repeat (STR) analysis as described in 2012 in the ANSI Standard (ASN-0002) authentication of human cell lines: standardization of STR profiling by the ATCC Standards Development Organization (SDO). Briefly, 17 STR loci plus the gender-determining locus, amelogenin, were amplified using the commercially available PowerPlex® 18D kit from Promega (Madison, WI). The cell line sample was processed using the ABI Prism 3500xl Genetic Analyzer (Foster City, CA). Data were analyzed using GeneMapper® ID-X v1.2 software (Applied Biosystems, Foster City, CA). Appropriate positive and negative controls were run and confirmed for each sample submitted [21]. The comparative output (Table 1) of the STR analysis confirmed the authentication of the HCE-2 cell line used in this study (Appendix 1).

Cell culture conditions and challenges: Eye drops, once applied on the ocular surface, are rapidly diluted by the continuous flow of tears (approximately 1 μl/min [22]) and eliminated from the eye surface with a 14.5%/min turnover of the TF aqueous phase [23]. Thus, to mimic in vitro the high turnover rate of tears, which decreases the exposure time of the eye drops applied, and the eye drops' rapid dilution and elimination (due to the constant tear flow), which reduces the concentration of the eye drops instilled on the ocular surface, the CE-CKC emulsions tested were applied to the cells at a 10% dilution and for only a 30-min time period. This is identified as preincubation in Figure 1. The subsequent 24- to 72-h incubation contained the stimulating agents and the positive controls or the reference compounds but no CE-CKC emulsions. Table 2 summarizes the experimental conditions used for the different cell types.

TCD4 and PBMCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% inactivated fetal bovine serum (FBS), 2 mM glutamine, and penicillin/streptomycin and incubated at 37 °C under 95% humidity and 5% CO_2. The cells were incubated for 30 min with medium containing the CE-CKC emulsions at a 1/10 dilution and reference compounds (dexamethasone at 10^{-7} M, CsA at 10^{-6} M). After this 30-min preincubation period, the medium was replaced with fresh medium, without CE-CKC emulsions, containing or not (unstimulated control) the inducer (1 μg/ml LPS or 1 μg/ml anti-CD3 (BioLegend, San Diego, CA) in combination with 1 μg/ml anti-CD28 (Diaclone, Besançon, France). The reference compounds and the cells were cultured for another 24 h (gene expression assay and quantification of marker in cell supernatants) or 72 h (for IL-17 release only). After incubation, the cell culture

TABLE 1. SHORT TANDEM REPEAT (**STR**) ANALYSIS RESULTS.

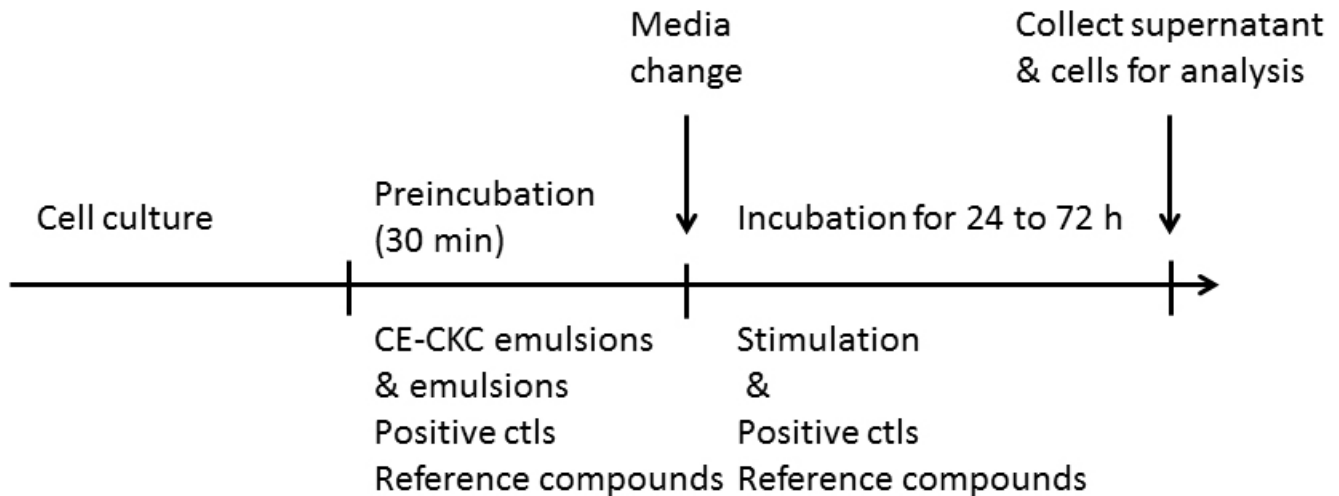| Designation	Locus										% Match with
	D5S818	D13S317	D7S820	D16S539	vWA	THO1	AMEL	TPOX	CSF1PO		reference
CRL-1135 HCE-2 (50.B1) reference	11, 12	8, 11	8, 10	9, 11	15, 18	6, 9	X, Y	10, 11	10, 13		/
STRA7150 (HCE-2 used in this study)	11, 12	8, 11	8, 10	11	15, 18	6, 9	X, Y	10, 11	10, 13		94

Figure 1. Summary of the experimental conditions used in the in vitro assays.

supernatants were collected, and the markers of interest were quantified using flow cytometry or an enzyme-linked immunosorbent assay (ELISA). For gene expression analysis, the cells were washed with PBS (1X; 137.9 mM NaCl, 2.67 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2) and stored at −80 °C until RNA extraction.

HCE-2 cells were cultured in fibronectin-/gelatin-coated wells in KBM-Gold medium (Lonza, Levallois, France) supplemented with bovine pituitary extract, epidermal growth factor, hydrocortisone, transferrin, epinephrine, insulin, and

gentamycin-amphotericin, and incubated at 37 °C under 95% humidity and 5% CO_2. The cells were incubated for 30 min with medium containing the CE-CKC emulsions at a 1/10 dilution and the reference compounds (IKK Inhibitor X, PKC Inhibitor and dexamethasone). After this 30-min preincubation period, the medium was replaced with fresh medium, without CE-CKC emulsions, containing or not (unstimulated control) the inducer LPS (1 µg/ml) and the reference compounds, and the cells were further cultured for another 24 h. After incubation, the cell culture supernatants were collected, and the markers of interest were quantified using

TABLE 2. SUMMARY OF THE EXPERIMENTAL CONDITIONS USED FOR THE SECRETION INHIBITION IN VITRO ASSAYS.

Cell type	Stimulation (dose)	Positive ctls (dose)	Incubation time	Markers	Marker quantification method
PBMC	LPS	DXM	24h	IL-6	FC: BD™ CBA kit (558,276)
				IL-8	FC: BD™ CBA kit (558,277)
				IL-12	FC: BD™ CBA kit (560,154)
				TNFα	FC: BD™ CBA kit (558,273)
PBMC	Anti CD3 & anti CD28	CsA	24h	IL-2	FC: BD™ CBA kit (558,270)
				IL-10	FC: BD™ CBA kit (558,274)
				INFγ	FC: BD™ CBA kit (558,269)
				TNFα	FC: BD™ CBA kit (558,273)
T CD4+	Anti CD3 & anti CD28	CsA	24h	INFγ	ELISA: DIACLONE (851,560,005)
				TNFα	ELISA: DIACLONE (851,570,015)
			72h	IL-17	ELISA: R&D system (DY208)
HCE-2	LPS	IKK inhX	24h	IL-6	ELISA: R&D system (DY206)
				IL-8	ELISA: R&D system (DY208)

Abbreviations: CsA, cyclosporine A; ctls, controls; DXM, dexamethasone; FC, flow cytometry; IKK inhX, IKK inhibitor X.

ELISA. For gene expression analysis, the cells were washed with PBS and stored at −80 °C until RNA extraction.

Quantification of released markers in cell supernatants: The markers released by PBMCs were quantitated using flow cytometry with the appropriate BD™ CBA kit (Table 2) according to the manufacturer's standard procedure (BD Biosciences, Le Pont de Claix, France). The markers of interest released by the TCD4 and HCE-2 cells were quantified using ELISA kits from Diaclone (Besançon, France) or R&D Systems (Lille, France) according to the manufacturers' standard procedures.

Gene expression determination in PBMCs and HCE-2 cells: Marker expression was quantified using real-time quantitative PCR (RT-qPCR). Briefly, total RNA were extracted from cells with the TriPure Isolation Reagent® according to the manufacturer's protocol (Sigma), and their quantity and quality assessed with capillary electrophoresis (Bioanalyzer 2100, Agilent, Santa Clara, CA). RT-qPCR was performed using the LightCycler-FastStart DNA MasterPlus SYBR Green I kit (Roche, Meylan, France) on LightCycler 480 (Roche, Meylan, France). The standard qPCR conditions included 5 min at 95 °C followed by 50 cycles of extension at 95 °C for 20 s ,15 s at 64 °C and 72 °C for 20 s. Relative gene expression levels were normalized to GAPDH levels and calculated using the comparative ($\Delta\Delta Ct$) method as previously described. A detailed list of the primers is available on request. [24]. Briefly, C_T is the cycle number for which the detected fluorescence exceeds the threshold value and ΔC_T the difference between the target gene of interest and the housekeeping control gene. $\Delta\Delta C_T$ is the difference between the ΔC_T of the sample analyzed and the control sample. The results were then expressed as a percentage of the control condition (set at 100%).

IC_{50} calculation of cationic surfactants of various alkyl chain lengths on PKCs: Protein kinase C is a family of kinases that contains ten different isoenzyme members grouped in conventional, novel, and atypical isoforms. They seem to be implicated in inflammatory mechanisms, and PKC inhibitors possess anti-inflammatory properties in vitro and in vivo [25]. Cationic lipids, i.e., molecules harboring a positive charge associated with an aliphatic chain, were described as having an anti-inflammatory activity via the modulation of PKC-associated pathways [26]. Alkyl benzyl derivatives of the quaternary ammonium family are positively charged cationic lipids. The efficacy of alkyl benzyl quaternary ammonium derivatives containing eight to 18 carbons in their aliphatic chain at inhibiting PKCα, PKCβ$_1$, PKCβ$_2$, and PKCδ was performed at Cerep Inc. (Celle l'Evescault, France) according to established methods [27]. Briefly,

purified human recombinant PKC isoforms were incubated at room temperature for 15 min (PKCα and PKCβ$_2$) to 30 min (PKC β$_1$ and PKCδ) with ATP and the different specific substrates at concentrations from 25 to 400 nM in the presence of increasing concentrations of the various alkyl benzyl quaternary ammonium derivatives. Detection used the time-resolved fluorescence resonance energy transfer (TR-FRET) method with the LANCE® (Perkin Elmer, Waltham, MA) and HTRF® (Cisbio, Codolet, France) kits. The results were expressed as percent inhibition of control-specific activity. The IC_{50} values (concentration causing half-maximal inhibition of control-specific binding) and the Hill coefficient (nH) were determined with nonlinear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting. This analysis was performed using the Hill software (Cerep) and validated by comparison with data generated by the commercial software Sigma Plot® 4.0 for Windows.

Statistical analysis: All experiments and cultures were performed in triplicate. Gene expression experiments (RT-qPCR) were performed in duplicate. Inter-group comparison was performed using an unpaired Student *t* test, and a p value of less than 0.05 was considered statistically significant.

RESULTS

In vitro anti-inflammatory effects of the CE-CKC emulsions: The effects of CE-CKC emulsions on the release of proinflammatory chemokines and cytokines were evaluated on human immune cells (PBMCs and TCD4) and on human corneal epithelial cells (HCE-2 cells). Both CE-CKC emulsions, containing either 0.002% or 0.005% CKC, were effective at reducing the lipopolysaccharide (LPS)- / anti-CD3 + anti-CD28-stimulated secretion of proinflammatory cytokines and chemokines (Table 3). In some cases, the CE-CKC emulsions were almost as effective as the positive controls. Interestingly, in the HCE-2 cells, the CE-CKC emulsions were more effective than DXM or CsA on the inhibition of LPS-induced interleukin (IL)-6 and IL-8 secretion (Figure 2).

Modulation of gene expression by CE-CKC emulsions in stimulated PBMCs and HCE-2 cells: Further to the efficacy of the CE-CKC emulsions at inhibiting the secretion of chemokines and cytokines, the modulation of proinflammatory genes at the expression level was evaluated. In the PBMCs, LPS stimulation markedly increased the expression of the chemokine (C-C motif) ligand 3 (*CCL3,* OMIM 182283), *CCL4* (OMIM 182284), and Interleukin 8 (*IL8,* OMIM 146930), and of the cytokine interferon (*IFN*) γ (*IFNG,* OMIM 147570), interleukin 17A (*IL17A*, OMIM 603149), and Interleukin 6 (*IL6,* OMIM 147620; Figure 3). The addition of the positive

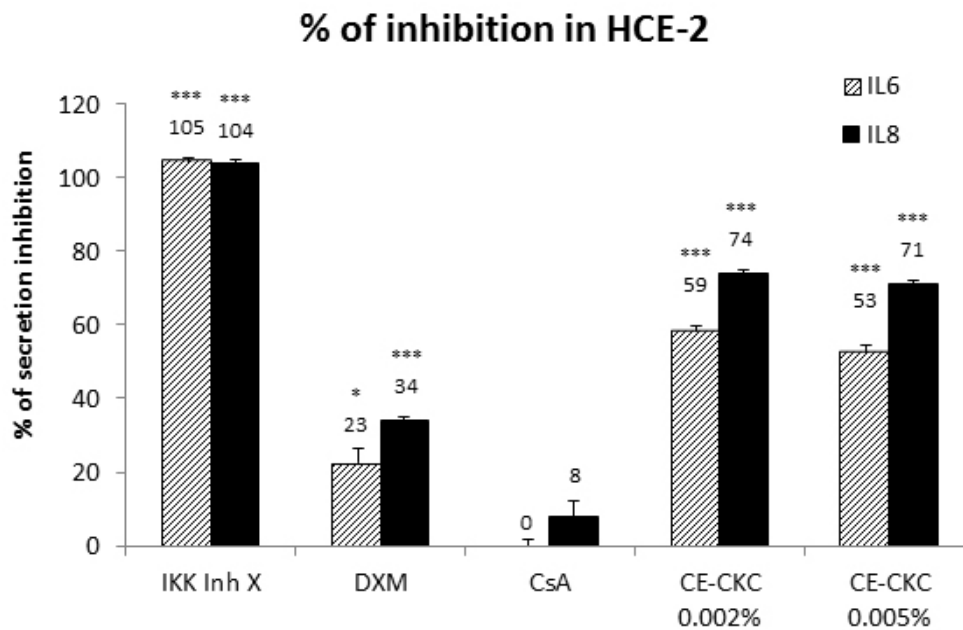## % of inhibition in HCE-2

Figure 2. 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. Results are expressed as mean ± standard error of the mean (SEM) of the percentage of inhibition versus LPS-stimulated cells. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Experiments were performed in triplicates.

TABLE 3. EFFECTS OF CE-CKC EMULSIONS ON MARKER SECRETION BY INFLAMMATORY OR CORNEAL EPITHELIAL CELLS UPON STIMULATION.

Cell type (*Stimulation*)	Marker	% Of marker secretion inhibition		
		CE-CKC 0.002%	CE-CKC 0.005%	Reference compound
				CsA
PBMCs (*anti-CD3/28*)	IL2	22±2	37±5*	79±14**
	IL10	8±7	44±13	>100***
	IFNγ	42±1*	55±6**	99***
	TNFα	49±6***	62±7***	90±1***
				DXM
PBMCs (*LPS*)	IL6	8±2	63±7**	72±1*
	IL8	11±6	64±10**	68±2***
	IL12	5±5	61±8**	97±1***
	TNFα	8±11	59±5*	85±1**
				CsA
T cells CD4+ (*anti-CD3/28*)	IFNγ	12±2	15±5	>96***
	TNFα	0±7	2±2	>88***
	IL17	65±3**	98±1**	100***
				IKK inhX
HCE-2 (*LPS*)	IL6	59±1**	53±2***	105±0***
	IL8	74±1***	71±1***	104±1***

Results are expressed as mean ± SEM of % of inhibition versus stimulated cells. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Abbreviations: CsA, cyclosporine A; DXM, dexamethasone; IKK inhX, IKK inhibitor X.
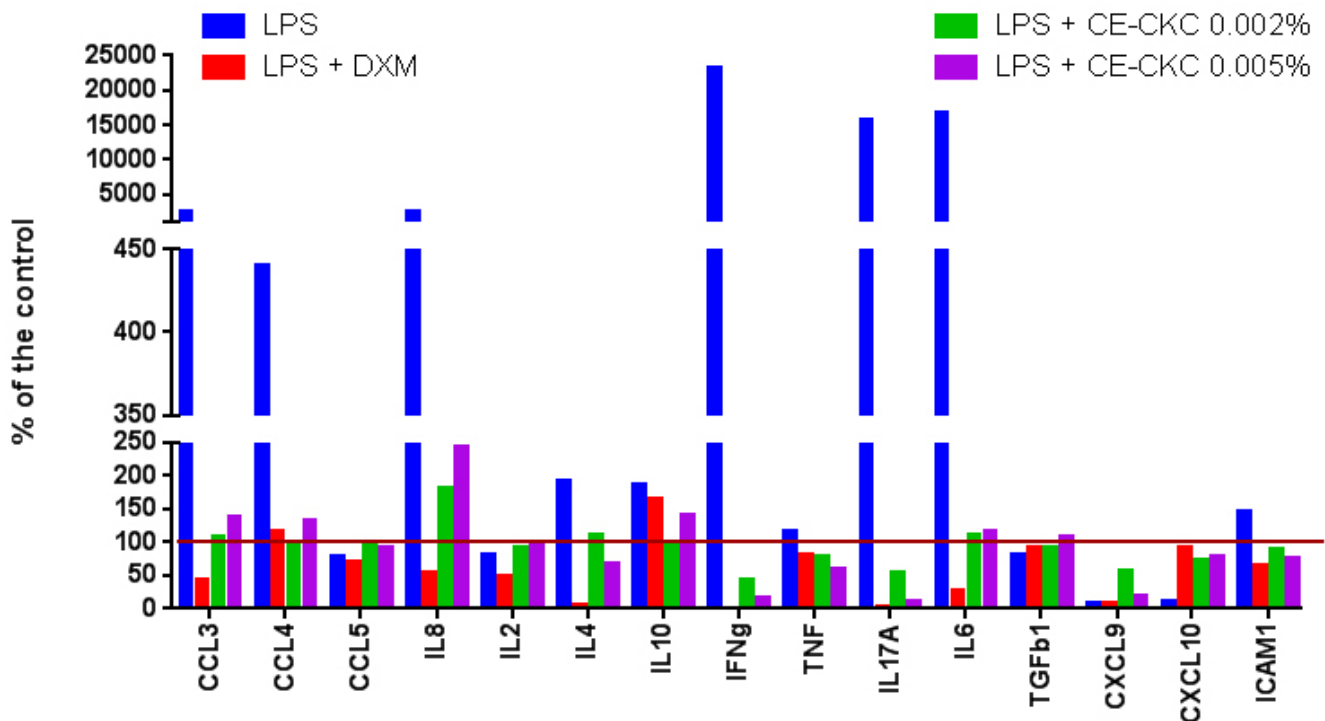
Figure 3. Effects of CE-CKC emulsions and DXM on gene expression in PBMCs following LPS stimulation. Peripheral blood mononuclear cells (PBMCs) were preincubated for 30 min with cationic emulsions of cetalkonium chloride (CE-CKC) or dexamethasone (DXM). The cells were then stimulated with lipopolysaccharide (LPS) for 24 h. Emulsions were omitted during LPS stimulation, whereas DXM was present. Results are expressed as a percentage of the unstimulated control (set at 100%). Experiments were performed in duplicates.

control DXM (0.1 µM) suppressed LPS-induced stimulation of all the genes analyzed. In the same way, CE-CKC emulsions also displayed an inhibitory effect against LPS-induced expression of inflammatory genes. In HCE-2 cells, LPS stimulation resulted in the overexpression of mRNA coding for *IL-8*, *CCL2* (OMIM 158105), *IL1A* (OMIM 147760), *IL6*, tumor necrosis factor α (*TNFA*, OMIM 191160), and matrix metalloproteinase 9 (*MMP9*, OMIM 120361; Figure 4). CsA was able to decrease LPS-induced overexpression of these genes. The effects of the CE-CKC emulsions appeared to be less than in the PBMCs, but the CE-CKC emulsions were still able to normalize LPS-induced overexpression except *MMP-9*.

Exploration of the role of CKC and tyloxapol on the anti-inflammatory effects of CE-CKC emulsions: HCE-2 cells and LPS stimulation were used to explore the effect of two of the excipients present in the CE-CKC emulsions for which previously published data indicated that they might have anti-inflammatory properties: tyloxapol [28-31] and the cationic lipid surfactant, CKC [26]. For this purpose, the effect of the

CE-CKC 0.005% emulsion was compared to emulsions made only from oil (MCT) and one surfactant: tyloxapol (Tylo Em) or CKC (CKC Em). These simpler emulsions were able to statistically significantly reduce the LPS-induced secretion of IL-6 and IL-8 (Figure 5), but to levels smaller than those observed for the CE-CKC 0.005% emulsion. A nonspecific pan-PKC inhibitor (Bisindolylmaleimide I, 3 µM) was used as reference in the test, and the data showed that the pan-PKC inhibitor and the CKC Em were almost as effective at inhibiting LPS-induced IL-6 secretion.

CKC is a specific inhibitor of PKCα: The inhibition potential of alkyl benzyl derivatives of the quaternary ammonium family on conventional PKCα, PKCβ$_1$, PKCβ$_2$, and novel PKCδ was evaluated in an in vitro binding assay. The data demonstrate that CKC, the alkyl benzyl derivatives with 16 carbons in its aliphatic chain, was a specific inhibitor of PKCα (Table 4), with an IC$_{50}$ of 4.3 µM. Interestingly, the inhibitory activity, as well as the selectivity for PKCα, increased with the lengthening of the alkyl chain, with a maximum reached for a 16-carbon alkyl chain.

DISCUSSION

DED is a complex multifactorial disease of the ocular surface in which inflammation plays an etiological role [1]. Inflammation was long considered the core mechanism in DED [32] and is implicated in the worsening of the DED condition. Most of the new DED treatments contain anti-inflammatory active principles, such as glucocorticoids (e.g., fluorometholone or loteprednol etabonate) [33,34], CsA [15,35,36], or lifitegrast [37,38], emphasizing the role of inflammation in DED physiopathology. Artificial tears, although are designed to restore the homeostasis of the tear film from a mechanical point of view, may possess some ancillary low anti-inflammatory activities that might contribute to the overall efficacy of the ATs [19,39]. The aim of the present study was to further characterize the anti-inflammatory activity observed in vivo with CE-CKC emulsions [16-18] by directly exploring the effects of a 10% dilution of the CE-CKC emulsions on human inflammatory and corneal epithelial cells.

The CE-CKC emulsions contain only commonly used excipients, such as MCT or mineral oils (as the oily components of the emulsion), and surfactants (tyloxapol, poloxamer 188, and CKC) at low concentrations (e.g., from 0.002 to 0.005% for CKC) to avoid any risk of ocular toxicity while stabilizing the oil/water phase interface of the emulsions [8]. When applied to PBMCs or HCE-2 cells, with a procedure that mimics the ocular instillation exposure time of eye drops on the ocular surface, the CE-CKC emulsions were able to statistically significantly modulate the secretion of either anti-CD3 + anti-CD28- or LPS-induced chemokine and cytokine secretion (Table 3). Moreover, it seems that some kind of dose-ranging effect exists with the cationic emulsions, with the CE-CKC 0.005% emulsion being seemingly more effective than the CE-CKC 0.002% emulsion. In addition, for some markers the 10% dilution of the CE-CKC 0.005% is almost as effective as the positive controls at inhibiting their secretion (for IL-17 in TCD4 cells stimulated with LPS, and for IL-6 and IL-8 in PBMCs stimulated with LPS). Upon dry eye stress (such as hyperosmolarity), corneal epithelial cells
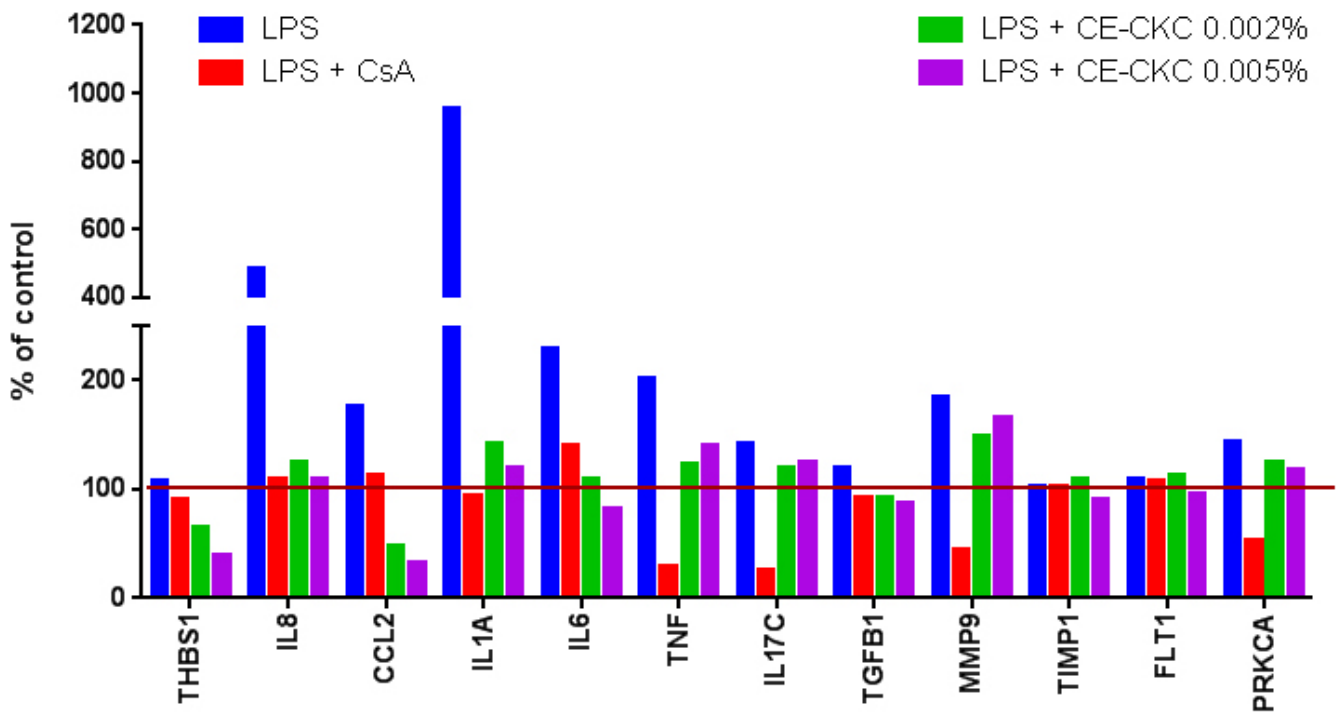

Figure 4. Effects of CE-CKC emulsions and cyclosporine A (CsA) on gene expression in HCE-2 cells following LPS stimulation. HCE-2 cells were preincubated for 30 min with cationic emulsions of cetalkonium chloride (CE-CKC) or dexamethasone (DXM). The cells were then stimulated with lipopolysaccharide (LPS) for 24 h. Emulsions were omitted during LPS stimulation, whereas CsA was present. Results are expressed as percentage of the unstimulated control (set at 100%). Experiments were performed in duplicates.
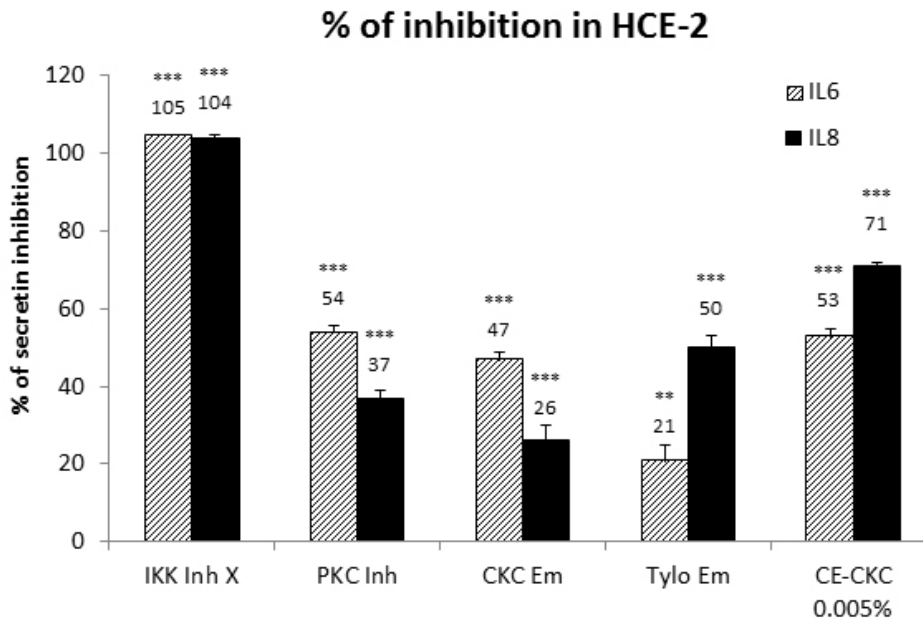
Figure 5. Effects of CE-CKC 0.005% emulsion and emulsion excipients (CKC Em and Tylo Em) on IL-6 and IL-8 secretion inhibition following lipopolysaccharide (LPS) stimulation in HCE-2 cells. Results are expressed as mean ± standard error of the mean (SEM) of percentage of inhibition versus LPS-stimulated cells. *, p<0.05; **, p<0.01; ***, p<0.001. Tylo, tyloxapol. Experiment were performed in triplicates.

release IL-6 or IL-8 [40-43], and it was shown that toll-like receptor (TLR) 4, which is the receptor for LPS, is expressed by cornea epithelial cells in a mouse model of dry eye [44]. In a model of allergy, the use of a neutralizing monoclonal antibody against human TLR4 could block the expression and the release of IL-33 by corneal cells induced by short ragweed, thus confirming the presence and role of TLR4 in ocular pathology, whereas skin keratinocytes, for example, do not express IL-33 and do not respond to TLR4-mediated inflammation [45]. Corticosteroids and CsA are generally used for the management of DED signs and symptoms; however, in the HCE-2 cells stimulated by LPS, DXM and CsA only marginally reduced the LPS-induced secretion of IL-6 and IL-8, while both CE-CKC emulsions (diluted at 10%) statistically significantly reduced IL-6- and IL-8-induced secretion (Figure 2). Although DXM and CsA are more effective on

inflammatory cells, it seems that the CE-CKC emulsions are more effective at controlling the release of inflammatory cytokines and chemokines from cornea epithelial cells. Modulation of inflammatory chemokines and cytokines by the CE-CKC emulsions was also visible at the expression level. A 10% dilution of the CE-CKC emulsions reduced *IL8*, *IL6*, *IL17A*, *CCL3*, and *CCL4* mRNA overexpression induced by LPS in the PBMCs (Figure 3), and in a manner similar to that of *IL1A*, *IL6*, *IL8*, *CCL2*, and *THBS1* (OMIM 188060) mRNA in LPS-stimulated HCE-2 cells (Figure 4), thus confirming the effect observed at the secretion level.

The question then was to explore which one of the "non-pharmacologically" active excipients present in the CE-CKC emulsion is responsible for this anti-inflammatory activity. It has previously been reported that tyloxapol (a nonionic surfactant) was able to modulate inflammation

TABLE 4. EFFECTS OF THE ALKYL CHAIN LENGTH ON PKC INHIBITION OF VARIOUS ALKYL BENZYL DERIVATIVES.

Alkyl chain length	IC$_{50}$ (µM)			
	PKCα	PKCβ$_1$	PKCβ$_2$	PKCδ
C8	-	-	-	-
C10	>128.2	-	-	>128.2
C12	22.6	58.8	58.8	>117.6
C14	5.4	10.1	14.1	>108.7
C16 (CKC)	4.3	-	-	-
C18	11.7	74.6	39.3	-

Abbreviations: IC$_{50}$, half maximal inhibitory concentrations; PKC, protein kinase C.

in a NF-KB-dependent manner [29,30]. Because the same amount of tyloxapol (0.3%) is in the CE-CKC 0.002% and CE-CKC 0.005% emulsions, it is unlikely that tyloxapol can explain the CE-CKC dose effect observed in the PBMCs and TCD4 (Table 3). In contrast, CKC is the only excipient (apart from oil) that is present at a different concentration in the two CE-CKC emulsions (0.002% versus 0.005%). CKC is a lipophilic polar lipid of the quaternary ammonium family, which is used at these low concentrations to bring a positive charge to the oil nanodroplets present in the cationic emulsions [8]. It has been described that cationic lipids have an anti-inflammatory activity [26] via the modulation of PKC; however, the cationic lipids described by Filion and Philips (1997) [27] are not structurally related to CKC. The effect of a 10% dilution of the CE-CKC 0.005% emulsion on inhibiting LPS-induced secretion of IL-6 and IL-8 in HCE-2 cells was then compared to simpler emulsions containing the oil phase stabilized only by either tyloxapol or CKC at the concentration present in the final CE-CKC 0.005% emulsion to assess the role of each excipient. A pan-PKC inhibitor was also used for comparison. It appeared that the CKC Em emulsion was as effective as the CE-CKC 0.005% emulsion at inhibiting LPS-induced IL-6 secretion (Figure 5). CKC Em emulsion was almost as effective as the pan-PKC inhibitor, suggesting that CKC might be responsible for the dose-ranging efficacy of the CE-CKC emulsions via PKC modulation. It was then interesting to determine among the PKC protein family members which PKC isoform(s) was/were modulated by CKC. An inhibitory binding assay demonstrated that CKC was a selective inhibitor of PKCα. Interestingly, varying the aliphatic chain length on the alkyl benzyl derivatives modifies the affinity and selectivity of the molecules (Table 4). The IC_{50} of the alkyl benzyl derivatives for PKCα decreased with the lengthening of the aliphatic chain to reach a low at 4.3 μM with the 16-carbon aliphatic chain molecule (i.e., CKC). Increasing the length to 18 carbons resulted in a poorer IC_{50} value and selectivity for PKCα.

Now that CKC has been shown to be a PKCα inhibitor, can it explain the in vivo effects observed for the CE-CKC emulsions? CE-CKC emulsions were demonstrated to improve the CFS score (i.e., wound healing) in a mouse model of dry eye [16], improve the reepithelization process in a rat model of corneal epithelium scraping [17,19], inhibit the recruitment of inflammatory cells in the CALT of rabbits [18], and modulate the infiltration of inflammatory cells in the corneal stroma [17,19]. In a recent paper, Chen et al. used a PKCα knockout mouse model to demonstrate that PKCα−/− mice have "more rapid corneal epithelial wound healing, perhaps due to a decreased neutrophil infiltration"

when placed in a dry environment [46]. Based on the present study data and the data from Chen et al., it is reasonable to hypothesize that the presence of CKC at low concentrations in the CE-CKC emulsion might be the reason for, at least in part, the beneficial effects of the CE-CKC emulsions (and its 10% dilution) on inflammation and the wound-healing process observed in the in vitro and in vivo models, as well as in clinical settings.

In conclusion, the CE-CKC emulsions appear to have a dual mechanism of action: (1) a mechanical action via the replenishment of a thick TFLL (by bringing oil to the ocular surface) and the stabilization of its interface with the TF aqueous phase (via CKC and tyloxapol, as polar lipid and nonionic surfactant, respectively) [10,13] and (2) an ancillary direct anti-inflammatory activity that contributes to the improvement of wound healing by decreasing DED-associated inflammation in cornea epithelial cells and inflammatory cell infiltration in the diseased tissues. The excipients tyloxapol and CKC (as a specific inhibitor of PKCα) might contribute to the direct anti-inflammatory effects observed for the CE-CKC emulsions.

APPENDIX 1. STR ANALYSIS.

To access the data, click or select the words "Appendix 1"

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