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Research paper

In vivo characterisation of a novel water-soluble Cyclosporine A prodrug for the treatment of dry eye disease

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ABSTRACT

Cyclosporine A (CsA) has been demonstrated to be effective for the treatment of a variety of ophthalmological conditions, including ocular surface disorders such as the dry eye disease (DED). Since CsA is characterised by its low water solubility, the development of a topical ophthalmic formulation represents an interesting pharmaceutical question. In the present study, two different strategies to address this challenge were studied and compared: (i) a water-soluble CsA prodrug formulated within an aqueous solution and (ii) a CsA oil-in-water emulsion (Restasis®, Allergan Inc., Irvine, CA). First, the prodrug formulation was shown to have an excellent ocular tolerance as well as no influence on the basal tear production; maintaining the ocular surface properties remained unchanged. Then, in order to allow in vivo investigations, a specific analytical method based on ultra high pressure liquid chromatography coupled with triple quadrupole mass spectrometer (UHPLC–MS/MS) was developed and optimised to quantify CsA in ocular tissues and fluids. The CsA ocular kinetics in lachrymal fluid for both formulations were found to be similar between 15 min and 48 h. The CsA ocular distribution study evidenced the ability of the prodrug formulation to penetrate into the eye, achieving therapeutically active CsA levels in tissues of both the anterior and posterior segments. In addition, the detailed analysis of the in vivo data using a bicompartmental model pointed out a higher bioavailability and lower elimination rate for CsA when it is generated from the prodrug than after direct application as an emulsion. The interesting in vivo properties displayed by the prodrug solution make it a safe and suitable option for the treatment of DED.

1. Introduction

The dry eye disease (DED) includes a broad spectrum of signs and symptoms that makes its definition and classification highly complex. That is one of the reasons why there are various other terms associated with this condition, such as dry eye syndrome, chronic dry eye disease, dysfunctional tear syndrome, keratoconjunctivitis sicca or keratitis sicca [1–4]. The lack of consent regarding the definition and diagnosis of the disorder contributes to the difficulty of evaluating its prevalence and impact on the patients' life. Nevertheless, a study showed that the impact of the DED on patients' quality of life could be compared with the one of a moderate angina [5], evidencing the importance of developing an appropriate treatment. As a general concept, it has been agreed that DED affects the ocular surface and has an unmistakable correlation with local inflammatory processes, in which the immune T cells and the lachrymal functional unit are highly involved. The inflammatory component of DED is assumed to play a key role in its pathology. It has been reported that some patients continue complaining about eye irritation even after adequate aqueous enhancement treatment [3]. Therefore, the underlying inflammatory process needs serious consideration for effective treatment, putting CsA in the spotlight for this application.

Cyclosporine A (CsA) is an undecapeptide produced by the microorganism *Tolypocladium inflatum Gams* that presents very useful properties for ophthalmic applications [6,7]. CsA has very interesting mechanisms of action that can be valuable in the treatment of DED. For example, CsA binds to cyclophilins and causes an

Abbreviations: CsA, Cyclosporine A; DED, dry eye disease; CLSO, confocal laser scanning ophthalmoscope; UHPLC–MS/MS, ultra high pressure liquid chromatography coupled with triple quadrupole mass spectrometer; d_{12}CsA, deuterated CsA; IS, internal standard; PBS, phosphate buffer solution; CS, calibration standard; STT, Schirmer tear test; bid, *bis in die*.

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inhibition of the calcineurin pathway that results in a suppression of the expression of cytokines genes; this leads to a non-activation of the T cells [8]. CsA is also known to take part in the inhibition of the c-Jun N-terminal kinases (JNK) and p38 signalling pathways involved in the activation of T cells [9]. In addition, CsA participates in the stimulation of apoptosis of the T cells that are already in an activated state [10], and it contributes to the enhancement of the release of a neurotransmitter, substance P, from the sensory nerves directing a stimulating interaction with the parasympathetic nerves [11,12]. Once in contact with the eye, CsA leads to a decrease in inflammation and an increase of the lachrymal production, both effects being highly beneficial for the treatment of DED.

DED is an ocular surface condition; therefore, it would be appropriate to treat the pathological process on a local ocular level by applying a topical ophthalmic formulation. This strategy helps to avoid some severe side effects caused by CsA when applied systemically such as nephrotoxicity, hepatotoxicity or hypertension [13].

Nevertheless, the use of CsA for ocular application represents a challenge regarding its pharmaceutical formulation. CsA is a highly hydrophobic molecule "practically insoluble" in water [14] that is difficult to be formulated in a totally hydrophilic vehicle, which makes its topical delivery into the eye a complicated matter.

Despite many attempts to address this question, only one formulation has been approved by the United States Food and Drug Administration (FDA) for the treatment of DED in humans to date; it is a CsA oil-in-water emulsion commercialised under the name of Restasis® (Allergan Inc., Irvine, CA).

In the present work, the selected procedure for the formulation of CsA into a topical ocular form was a chemical modification of the active molecule to increase its hydrophilicity. This prodrug approach is based on an ester of CsA carrying a phosphate group as a solubilising moiety, already described in previous studies [15–19]. The chemical structures of CsA and the prodrug are illustrated in Fig. 1. The prodrug, OPPH 088, exhibited a solubility that was approximately 25,000 times higher than CsA in isotonic phosphate buffer solution (PBS) at pH 7 [18]. In addition, OPPH 088 has the property to be quickly biotransformed into CsA once in contact with the ocular surface because of the esterase-like enzymatic activity of the tears [19].

Drug precorneal behaviour and ocular distribution are fundamental factors that strongly condition the bioavailability and efficacy of the formulations. Nevertheless, only little is known about CsA eyedrops' behaviour once in contact with the eye.

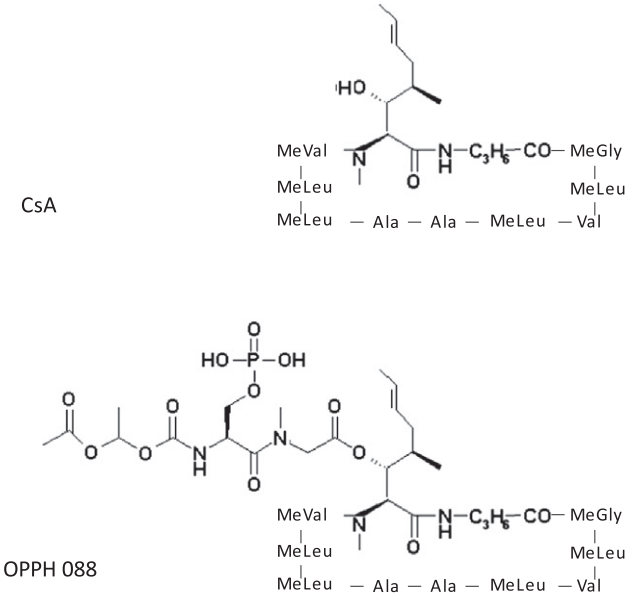

Fig. 1. Chemical structures of CsA and OPPH 088.

The aim of the present study was to characterise the in vivo behaviour of a novel formulation based on OPPH 088, and compare it to the commercial formulation, Restasis. The influence of the formulations on ocular surface properties was evaluated by measuring the corneal damage and basal tear production after their administration. Also, a dedicated UHPLC–MS/MS (ultra high pressure liquid chromatography coupled with triple quadrupole mass spectrometer) analytical method was developed and optimised in order to quantify CsA in ocular tissues and fluids. It allowed the determination of the ocular kinetics of OPPH 088 and Restasis as well as the CsA ocular distribution after prodrug administration. This information provided a basis for a model development and comparison of the formulations.

2. Materials and methods

2.1. Materials

OPPH 088 was synthesised at the Institute of Chemical Sciences and Engineering of the Ecole Polytechnique Fédérale de Lausanne (Switzerland) following the procedure described by Wenger et al. [15]. CsA was kindly provided by Dr. Wenger. Deuterated CsA (d_{12}CsA), used as an internal standard (IS), was a gift from Novartis (Basel, Switzerland), and mannitol was purchased from Acros Organics (Belgium). Water was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA). Acetic acid and methanol were of ULC/MS grade and were purchased from Biosolve (Valkenswaard, Netherlands). Isopropanol was provided by Sigma–Fluka (Buchs, Switzerland).

2.2. Methods

2.2.1. Preparation of the prodrug solution

A 0.066% w/v OPPH 088 aqueous formulation was prepared at a concentration equivalent to 0.050% w/v CsA using an aqueous 5% w/v mannitol solution as a vehicle. The pH was measured (Metrohm, Herisau, Switzerland) and adjusted to 7 with 1 N NaOH, and the isotonicity was verified (Vapour Pressure Osmometer 5500, Wescor, Logan, Utah, USA) prior to the experiment. The solution was filtered through a 0.22 μm polyvinylidene fluoride membrane (Millipore, Cork, Ireland) and kept in an appropriate eyedrop container until use.

2.2.2. Analytical method

2.2.2.1. UHPLC–MS/MS instrumentation. Analyses were performed on a Waters Acquity ultra performance liquid chromatograph (UPLC) system hyphenated with a Waters TQD triple quadrupole mass spectrometer fitted with a Z-spray electrospray ionisation source (Waters, Milford, MA, USA).

The chromatographic system included a binary solvent manager with a maximum delivery flow rate of 2 mL/min, a sample manager with an injection loop volume of 2 μL (full loop injection), and a column oven set at 60 °C. The chromatographic column was a Waters Acquity BEH C18 (50 × 2.1 mm I.D., 1.7 μm). Dwell volume of the UPLC-MS/MS configuration was estimated at 100 μL with a 2 μL injection loop. Chromatographic conditions for the separation were as follow: the analysis was carried out in the gradient mode at a flow rate of 600 μL/min (without splitting). The mobile phase consists of a mixture of water with 0.02% v/v acetic acid (A) and methanol with 0.02% v/v acetic acid (B). A linear gradient from 60% to 100% B was applied for 3 min, followed by a reequilibrating step of 1 min (corresponding to 5 column dead volumes). After each analysis, the injection system was washed in order to avoid

any CsA adsorption onto the surface; 1000 μL of pure isopropanol were used as strong wash, and 2000 μL of pure methanol as weak wash. The sample manager temperature was maintained at 4 °C during the time of analysis to avoid transformation and degradation.

The TQD instrument possessed an upper mass limit of *m*/*z* 2000. The ESCi® ionisation source was used in the ESI positive mode, and selected reaction monitoring (SRM) was performed, using the pseudo-molecular ion of each compound as the precursor ion and the most intense fragment. The use of an internal standard for the quantification of CsA in ocular tissues and fluids was highly beneficial to minimise the variability of the results. The selected molecule to be used as a reference was a 12 times deuterated CsA (d_{12}CsA) that has the same chromatographic behaviour than CsA with a higher m/z. The ratio between signals of CsA and d_{12}CsA was the basis for quantification. Collision energies and cone voltages were tuned by infusing each compound individually at 1 μg/mL using a flow rate of 600 μL/min. Optimal values were 65 V for the cone voltage and 63 eV for the collision energy. The pseudo-molecular parent ion and fragment corresponding to CsA have an *m*/*z* of 1224.7 and 1112.4, respectively; concerning d_{12}CsA, the parent ion has an *m*/*z* of 1236.7 and its daughter ion of 1124.4. Nitrogen was used as the drying gas and argon as the collision gas. The capillary voltage and the source extractor voltages were set at +4 kV and +3 V, respectively. The source temperature was maintained at 140 °C, the desolvation gas temperature and flow at 450 °C and 600 L/h, respectively, and the cone gas flow at 50 L/h. The collision gas flow was set to 0.2 mL/min of argon, and the entrance and exit potentials were adjusted to 1 and 0.5 V, respectively. Finally, the inter-channel delay was set to 5 ms, and dwell time was set to 200 ms for CsA and 20 ms for d_{12}CsA to maintain enough data points across the narrow peaks produced by the UHPLC–MS/MS instrument. The high mass resolution was adjusted to 10 to improve the sensitivity of the method. Data acquisition, data handling and instrument control were performed by Masslynx v4.1 Software (Waters, Milford, MA, USA).

2.2.2.2. Solutions for calibration. Calibration standards (CSs) included samples containing known concentrations of analytes. CSs were prepared in an independent way. A series of eleven concentration levels were selected corresponding to the wide range of concentrations expected in biological samples. CSs were replicated three times and independently prepared on different days.

For the precorneal kinetic study, tears from 8 rabbits were collected and pooled to obtain blank tear fluid that was subsequently spiked with 0.1 μg/mL d_{12}CsA as an internal standard, and the drug concentrations was varied at: 10, 5, 2, 1, 0.5, 0.2 and 0.1 μg/mL and 5, 2, 1 and 0.5 ng/mL. For the ocular penetration study, the same CsA and d_{12}CsA concentrations were used.

2.2.2.3. Analytical method for CsA quantification at kinetic time points after 24h. Two different analytical situations were defined in the determination of the kinetic profile according to the amount of CsA expected in the biological samples: during the kinetic experiment, the highest levels of CsA were expected for the time points taken earlier than 3 h after administration of the drug. As a consequence, the initially developed analytical method was modified to address the specific challenges for the quantification of the active molecule in the samples collected more than 3 h after the eyedrop administration.

The modifications focus on two parameters of the method: (i) dwell time for CsA was raised from 0.2 to 1 s, which allowed a higher sensitivity and (ii) calibration was performed with solutions containing 0.1 μg/mL of d_{12}CsA and lower concentrations of CsA: 0.2, 0.1, 0.05, 0.02 and 0.01 ng/mL. These results should be considered as pseudo-quantitative.

2.2.3. Animals

2.2.3.1. Rabbits for ocular tolerance evaluation, basal lachrymal production measurements and kinetic study. Female albinos New Zealand rabbits weighing approximately 4–5 kg (University Medical Center, Geneva, Switzerland) were used in this study. Animals were individually housed in stainless steel cages and maintained in a 12 h light/dark cycle at 19 ± 1 °C. They were allowed water and food ad libitum. All animals were healthy and free of clinically observable ocular abnormalities throughout the study. Animals were not sacrificed at the end of the experiments and remained healthy and with no observable ocular signs after the studies. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research and were approved by the local veterinary authority for animal experimentation.

2.2.3.2. Rats for ocular distribution study. Eight-week-old female Lewis rats weighing 150–200 g were used in this study. At the end of the study, rats were sacrificed using CO_2. All experiments were performed in accordance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research and were approved by the European Committee Directives for animal experimentation.

2.2.4. Ocular tolerance evaluation protocol

Twenty-five microlitres of the solution to be tested were instilled to the cornea of the right eye of a rabbit four times a day, for a period of 3 days and once on the fourth day just before observation of the cornea. After the last administration, rabbits were placed on a high-adjustable trolley in front of a camera head. A volume of 25 μL of a 0.5% w/v sodium fluorescein sterile isotonic solution was applied to allow the injured areas to be selectively marked. The eye was then rinsed for 1 min with 0.9% w/v sterile NaCl. Finally, the cornea was observed with a confocal laser scanning ophthalmoscope (CLSO® Zeiss, Oberkochen, Germany), which was modified by the addition of a set of lenses to view the cornea instead of the retina [20]. An argon ion laser operating at a 488 nm wavelength was used as the excitation light source. The fluorescence signal was detected by a photomultiplier. Images were obtained using an Epiplan-Neofluar 2.5×/0.075 NA objective lens (Zeiss, Oberkochen, Germany). Optical sectioning was performed parallel to the corneal surface at 16 equidistant focal planes; the focus shifting ranged from 0 to 470 μm covered the entire corneal thickness. The images were displayed on a digital video monitor. An image processing system (Analysis SIS, Münster, Germany) allowed the calculation of the total surface of the fluorescent zones, which indicated injured areas. Each formulation was tested on six rabbits.

Student's *t* test was used to compare the corneal damage. A *p* value of less than 0.05 was considered statistically significant.

2.2.5. Basal lachrymal production measurements

The Schirmer test was used to measure basal tear production. The evaluation was performed on 6 rabbits for each formulation; Restasis and the prodrug solution. A first sample was taken before drug administration to evaluate the tear basal production at time zero. After the administration of 25 μL of one or the other formulation, samples were collected at: 1, 2, 4, 6, 24 and 48 h. The sample collection was preceded by a topical anaesthesia with one drop of 0.4% oxybuprocaine solution; 5 min later, the Schirmer test strip was inserted into the cul-the-sac and left in place for 5 min. The height of absorbed lachrymal fluid was measured in mm and reported in STT (Schirmer tear test). Student's *t* test was used to compare the experimental results, and *p* values of less than 0.05 were considered statistically significant.

2.2.6. Ocular distribution study protocol

The ocular distribution of CsA was evaluated after the administration of the prodrug solution bid (twice a day) during 5 days on 6 rats. At the end of the experiment, animals were sacrificed with CO_2, eyes were enucleated and the cornea, conjunctiva, aqueous humour, iris-ciliary body, vitreous humour and retina were collected and stored at −80 °C in protected vial. Prior to analyses, the tissue samples were thawed at room temperature, weighted, manually grinded, introduced in a vial containing 200 μL of methanol along with 0.1 μg/mL d_{12}CsA and stirred over night. The day after, samples were centrifuged, and the supernatants were analysed by UHPLC–MS/MS.

2.2.7. Kinetic evaluation

Normal nasolachrymal drainage was verified 3 days before the experiment by applying one drop of fluorescein solution (0.5% w/v in phosphate buffered solution at pH 7). Rabbits were divided into two groups according to the tested eyedrop formulation: 6 rabbits for Restasis and others 6 rabbits for OPPH 088. The experiment began with the administration of 25 μL of the appropriate formulation in the right eye of six non-anaesthetised rabbits. Tear fluid samples were collected from the lower marginal strip using 2 μL disposable glass microcapillary tubes (Microcaps Drummond, Thomas Scientific, New Jersey). A first sample was taken before administering the drug to verify the absence of CsA before the start of the experiment. After the administration of the formulation, samples were collected at different times: 1, 3, 6, 8, 10, 12, 14, 16, 18, 20, 30, 60, 90, 120, 150, 180 min and at 24 and 48 h. After collection, the tear fluid sample was gently blown out of the capillary into a vial containing 50 μL of methanol along with 0.1 μg/mL d_{12}CsA. The vial was gently vortexed to allow the methanol to stop the conversion of the prodrug by denaturising the proteins involved in the reaction. The vials were analysed into the UHPLC–MS/MS system, and CsA concentrations were determined. The statistical non-parametrical Mann–Whitney *U*-test was chosen to compare the kinetics of the studied formulations. A bicompartment model was developed based on the experimental results.

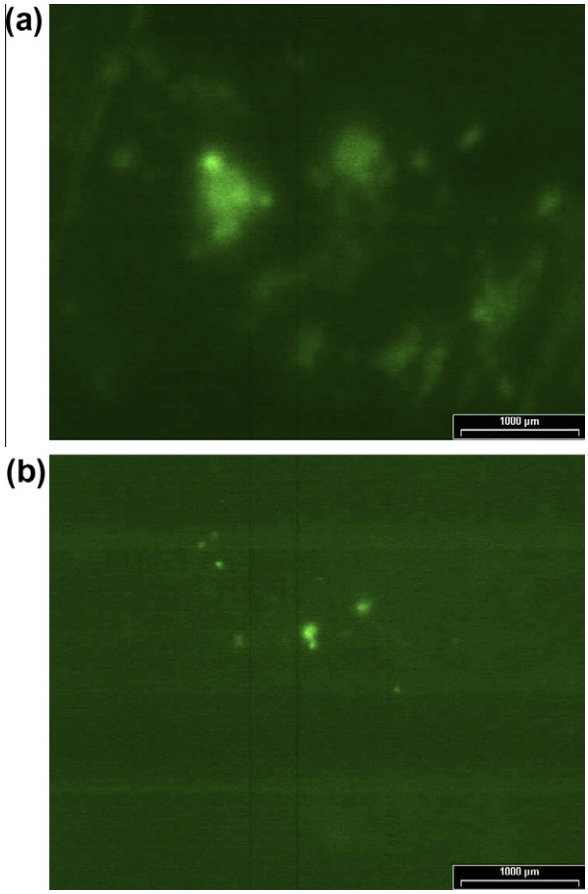

Fig. 3. Representative pictures of corneal injury caused by (a) Restasis and (b) OPPH 088. The damaged corneal cells are permeable to the fluorescein staining and are visible as fluorescent areas. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Ocular tolerance

The percentages of corneal damage experimentally observed after the administration of Restasis and OPPH 088 formulations are shown in Fig. 2 along with the value for an isotonic sterile saline solution determined during a previous study [20]. The corneal injury surface evaluated after the administration of OPPH 088 was 4.84 ± 2.72% of the observed area, while for Restasis, it was 18.20 ± 6.82%; the value for a sterile saline solution was 1.15 ± 0.35%.

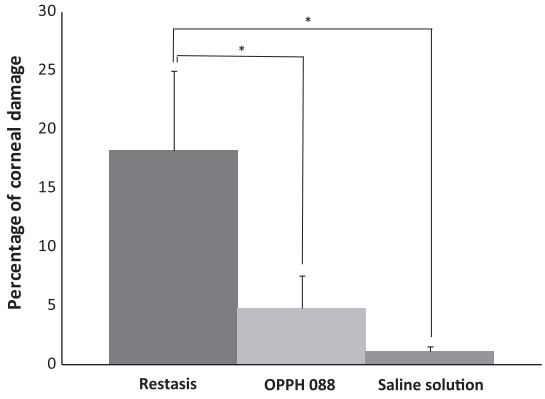

Fig. 2. Percentage of corneal damage after instillation of Restasis (■), OPPH 088 (■) and saline solution (■) (the latter being already reported in a previous study [20]). Mean ± SD, $n = 6$, *significantly different according to Student's *t*-test.

According to Student's *t* test, the fluorescent area observed for Restasis was significantly different from that of OPPH 088 or saline solution. Restasis presented the highest corneal injury. Fig. 3 clearly highlights the difference between the corneal damage caused by Restasis compared to that by OPPH 088. In addition, the statistical analysis of the values obtained with OPPH 088 compared to those of the sterile saline solution showed no significant differences between the solutions.

3.2. Basal lachrymal secretion

The Schirmer test was carried out to assess the influence of the formulations on the basal tear production. Fig. 4 illustrates the experimental data. The basal mean values for all animals before drug application were between 10.6 and 12.2 mm, which are represented by the dashed line area in the figure. Data from the two rabbit groups do not significantly differ; values after administration of the formulations and baseline are either significantly different. Nevertheless, it can be noticed that rabbits treated with the prodrug solution have values above baseline or included in the baseline area, while the Restasis group presents more fluctuations below the baseline area.

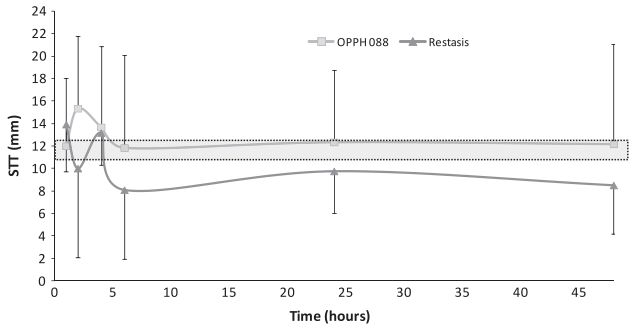

Fig. 4. Basal lachrymal production after a single instillation of Restasis and the prodrug formulation ($n = 6$) reported in STT (Schirmer tear test) in mm of wetted strip. The area with dashed line indicates the basal tear production prior to the formulation application.

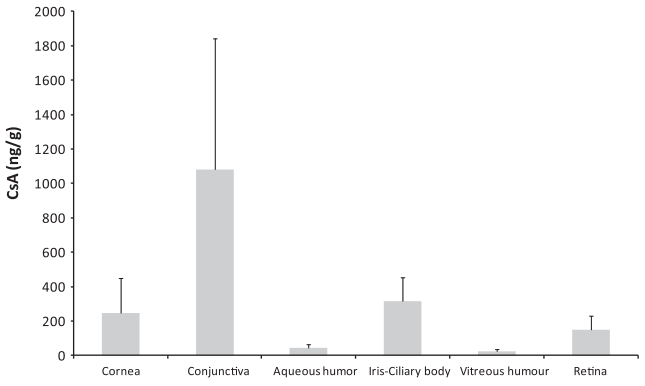

Fig. 5. CsA ocular distribution after topical application of OPPH 088 formulation bid for 5 days ($n = 6$).

3.3. Ocular distribution

The prodrug solution was administered bid during 5 days on 6 rats, and CsA levels were determined in ocular tissues and fluids. The average CsA concentrations were estimated at 247 ± 203 ng/g for the cornea, 1082 ± 761 ng/g for the conjunctiva, 45 ± 18 ng/g for the aqueous humour, 315 ± 139 ng/g for the iris-ciliary body, 24 ± 13 ng/g vitreous humour and 149 ± 83 ng/g for the retina, as illustrated in Fig. 5. The anterior and posterior structures of the eye were exposed to CsA, the highest levels being located in the anterior chamber.

3.4. In vivo precorneal kinetics

3.4.1. Kinetic profiles

Fig. 6 illustrates the CsA tear fluid kinetic profile after administration of Restasis up to 180 min. Different kinetic phases were identified. The first phase was characterised by the rapid and marked decrease of CsA levels observed during the first 20 min. The second phase was characterised by a plateau from 20 to 180 min. Analysis of the tear samples for longer time points exhibited concentrations of CsA of 5 ± 1 ng/mL and 2 ± 1 ng/mL for 24 and 48 h, respectively. The second phase was characterised by sustained drug concentrations in tears.

Fig. 7 presents the CsA tear fluid kinetic profile after administration of OPPH 088 formulation for the first 180 min. OPPH 088 appeared to have a very different general trend compared to Restasis, especially concerning the first time points and the maximum CsA concentrations.

A supplementary phase was observed in addition to the ones already identified for Restasis. In fact, the profile showed a marked

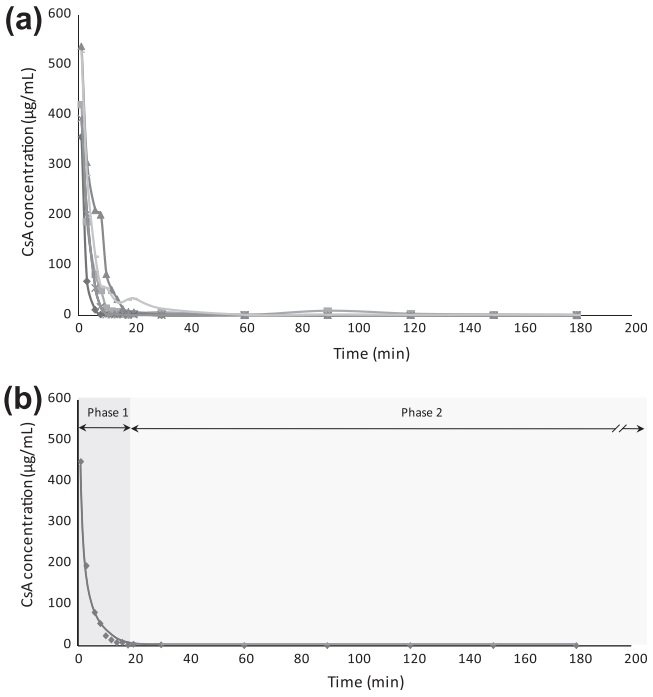

Fig. 6. CsA tear fluid kinetic profile after instillation of Restasis (a) for six rabbits and (b) based on median values ($n = 6$). The kinetic phases are represented in (b).

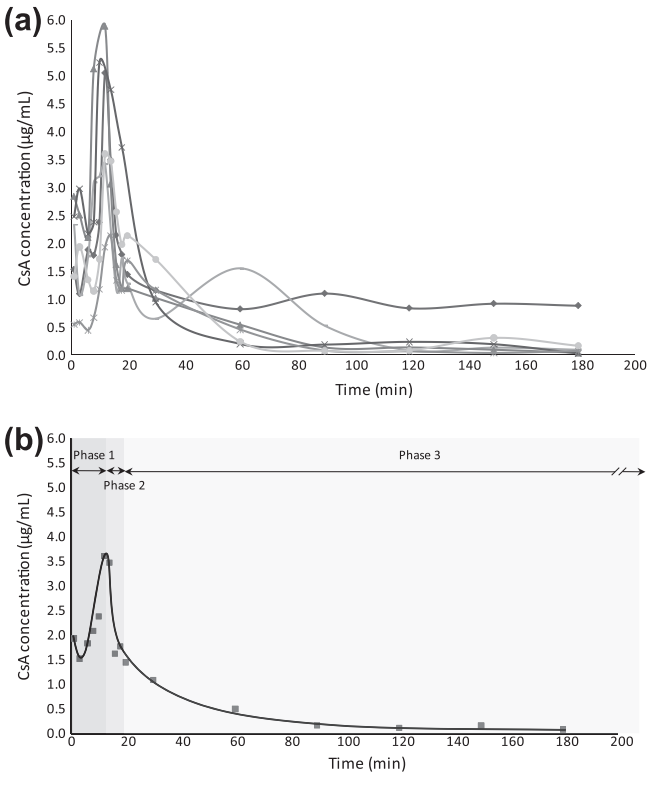

Fig. 7. CsA tear fluid kinetic profile after instillation of OPPH 088 formulation (a) for six rabbits and (b) based on median values ($n = 6$). The kinetic phases are represented in (b).

increase of CsA during the first 15 min. This corresponded to the first biotransformation step required in order to allow CsA to be released into the tear film from the prodrug formulation. This first phase was linked to the formulation characteristics and explained the specificity of the profile. Later, a rapid decrease in CsA tear

levels was observed and defined as the second phase. A plateau was visible up to 3 h. Furthermore, the analysis of the lachrymal fluid samples after 24 and 48 h showed levels of CsA of 5 ± 1 ng/mL and 2 ± 1 ng/mL, respectively. The sustained concentrations of CsA observed with Restasis were also seen for OPPH 088, constituting the third phase of its kinetic profile.

In addition, maximum CsA concentration in tears was approximately 100 times higher for Restasis than the one assessed for OPPH 088 formulation, which was expected as in the case of Restasis CsA was directly applied on the surface of the eye.

3.4.2. Statistical analysis and comparison

A non-parametrical method was selected for this study, assuming that the distribution of in vivo CsA concentrations for each time point was not necessarily Gaussian. Hence, the kinetic profiles were summarised with median values for each time point, and the Mann–Whitney statistical *U*-test was chosen to analyse the differences between the kinetics of the studied formulations. Thus, Figs. 6 and 7 show two sets of data: (a) for the six rabbits and (b) the median profile.

The major cause of disparities between the profiles was directly linked to the constitution of the formulations themselves. The observed differences during the first 15 min were linked to the nature of the formulations and other factors such as, for example, the ocular protective mechanisms or the enzymatic biotransformation specific to OPPH 088. Thus, an appropriate comparison can only be made if this variability factor is avoided. Consequently, the time points taken before 15 min were discarded from comparison, as illustrated in Fig. 8.

No statistically significant differences were found for the time points between 15 min and 48 h when applying the *U*-test to the two rabbit groups.

Therefore, the in vivo behaviours of both tested formulations were considered equivalent regarding CsA levels in tears between 15 min and 48 h.

3.5. Model development

The development of a model based on the experimental data can be of great interest for a deeper understanding of the in vivo behaviour of each formulation, particularly of their elimination.

A two-compartment model was selected based on the above presented results, and the fact that the experimental kinetic curves clearly showed a profile with, at least, two phases with different linear regression constants. The central compartment was defined as the tear fluid (in which the measurements were performed during the kinetic evaluation); the peripheral one was not precisely physically delimited and included the other structures, tissues and fluids that could interact with CsA.

The bicompartmental equation took into account two main phenomena; the distribution and elimination of CsA from the tear fluid [21].

The model used for the Restasis formulation was based on the following equation:

$$C = A_0 e^{-\alpha t} + B_0 e^{-\beta t} \quad (1)$$

where A_0, B_0, α and β were macroconstants for the hybrid disposition processes and were determined according to the method of residuals. From these macroconstants, the microconstant for the pure elimination process was calculated using the following equation:

$$K_{el} = \frac{\alpha\beta(A_0 + B_0)}{A_0\beta + B_0\alpha} \quad (2)$$

For the formulation containing OPPH 088, the additional biotransformation step had to be considered. The model equation was written as it follows:

$$C = A_0 e^{-\alpha t} + B_0 e^{-\beta t} + P_0 e^{-\gamma t} \quad (3)$$

where A_0, B_0, P_0, α, β and γ are macroconstants for the hybrid processes that were determined according to the method of residuals. The microconstant for the pure elimination process was calculated using the following relationship:

$$K_{el} = \frac{\alpha\beta\gamma(A_0 + B_0 + P_0)}{A_0\beta\gamma + B_0\alpha\gamma + P_0\alpha\beta} \quad (4)$$

The β elimination half-life was calculated as showed in Eq. (5):

$$t_{1/2\beta} = \frac{0.693}{\beta} \quad (5)$$

The coefficients of determination, r^2, for the developed models were 0.9452 for Restasis and 0.9643 for OPPH 088.

According to the above presented equations, the values of the elimination constants were 0.2193 min^{-1} for Restasis and 0.0160 min^{-1} for OPPH 088. The Restasis elimination constant was more than ten times higher than for the prodrug regarding CsA concentration in tears. The β elimination half-life values were 32 min for Restasis and 87 min for OPPH 088; approximately three times lower for the commercial emulsion than for the prodrug. These results suggest a higher CsA elimination process with Restasis than with OPPH 088 formulation.

Model characteristics are summarised in Table 1.

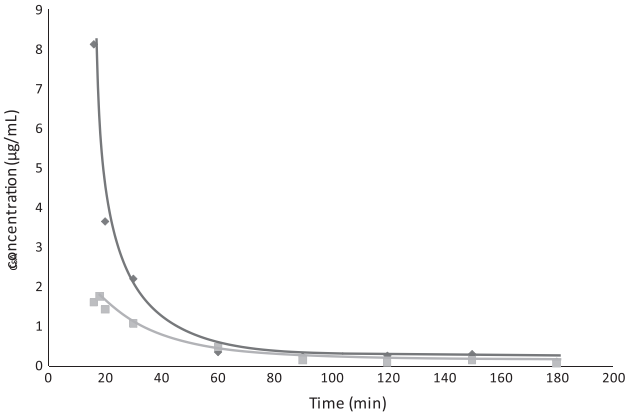

Fig. 8. Restasis (♦) and OPPH 088 (■) kinetic profiles between 15 and 180 min based on median values ($n = 6$).

Table 1
Bicompartment model characteristics for Restasis and OPPH 088. In (a) are shown the bicompartment model equations where A_0, B_0, P_0, α, β and γ are macroconstants for the hybrid processes that were determined according to the method of residuals, in (b) the elimination constant equation and values, in (c) the β elimination half-life equation and value and in (d) the coefficient of determination (r^2) values.

		Restasis	OPPH 088
(a) Bicompartment model equation		$C = A_0 e^{-\alpha t} + B_0 e^{-\beta t}$	$C = A_0 e^{-\alpha t} + B_0 e^{-\beta t} + P_0 e^{-\gamma t}$
(b) Elimination constant	Equation	$K_{el} = \frac{\alpha\beta(A_0+B_0)}{A_0\beta+B_0\alpha}$	$K_{el} = \frac{\alpha\beta\gamma(A_0+B_0+P_0)}{A_0\beta\gamma+B_0\alpha\gamma+P_0\alpha\beta}$
	Value	0.2193 min^{-1}	0.0160 min^{-1}
(c) β Elimination half-life	Equation	$t_{1/2\beta} = \frac{0.693}{\beta}$	
	Value	32 min	87 min
(d) Coefficient of determination (r^2) value		0.9452	0.9643

4. Discussion

The possible alteration of the ocular surface was the first concern when developing a novel ocular formulation. Additionally, it was a key factor that had to be evaluated prior to the kinetic and distribution studies since it could influence the experimental results. The confocal microscopy technique seemed to be an appropriate method for the objective and sensitive evaluation of the in vivo ocular irritation induced by topical ophthalmic formulations, as it has been demonstrated by previous studies [20,22]. In addition, it is a simple and non-invasive procedure. As expected, the experimental results demonstrated that the two tested preparations had a good ocular tolerance (corneal damage lower than 25%) according to the classification established by Kälin [23]. Restasis has been approved by the FDA for human use; therefore, it was not surprising that no important corneal damage was determined. This result was consistent with previous in vitro and in vivo studies where no tolerability problems were evidenced [24–27]. Nevertheless, the slightly higher fluorescence observed with Restasis compared to the other formulations may be explained by the presence of surfactants, required for the oil-in-water emulsion, that act as detergents [20]. The percentage of corneal damage assessed with the saline solution is representative of the normal physiological desquamation of the surface of the rabbit eye [28]. The assessed fluorescent area for OPPH 088 did not significantly differ from the saline preparation, clearly demonstrating the excellent ocular tolerance of the OPPH 088 formulation. Moreover, the prodrug formulation is expected to exhibit an excellent ocular tolerance in humans also, as human eyes are less sensitive to ocular irritation than rabbits [29]. On the other hand, the Schirmer test demonstrated that neither the prodrug formulation nor Restasis had any negative effect on basal tear production of healthy animals. Both corneal damage assessment and Schirmer test clearly demonstrated that the formulations had no negative effect on the ocular surface properties. In addition, the precorneal behaviour and ocular distribution studies were not expected to be influenced by any corneal irritation or any variation of the lachrymal fluid production.

Once the ocular tolerance of the formulation was found to be excellent, the question of the in vivo behaviour and efficacy of the prodrug solution could be addressed. The precorneal behaviour of both formulations as well as the ocular CsA distribution following the prodrug administration were evaluated based on the CsA quantification on ocular tissues and fluids. A specific UHPLC–MS/MS method was developed to address different analytical challenges: (i) the low volume and weight of the biological fluids and tissues (2 μL of tear fluid or different parts of the rat eye), (ii) the low CsA levels expected in the samples to be analysed, (iii) the presence of very close chemical structures in OPPH 088 samples coming from the biotransformation of the prodrug into CsA, (iv) the potential interference of the biological matrices and (v) the high number of samples to be analysed. The UHPLC system fulfilled the analytical requirements regarding the high throughput (analysis time of 3 min) as well as the high robustness (variation of the retention time <1%), allowing the use of an automated quantitative procedure. In addition, the inherent properties of the tandem mass spectrometric detection provided the required elevated sensitivity (LOQ of 2 ng/mL) and high selectivity (ensuring no interferences from close chemical structures coming from the biotransformation of OPPH 088 into CsA or components from the matrix). Moreover, the use of a last generation MS/MS instrument was highly beneficial in order to allow a sufficient number of data points across the CsA narrow peak for an accurate quantification of CsA within the short analysis time provided by the UHPLC system. Finally, the systematic addition of an internal standard, d_{12}CsA, to the samples ensured the correction of the possible variability during the ionisation process [30,31]. The optimised UHPLC–MS/MS method met the analytical needs for the in vivo investigations and displayed interesting characteristics due to the high throughput and high sensitivity.

After the topical administration of the drug, CsA was distributed from the precorneal area between the ocular structures according to its partition coefficient [26]. Thus, the quantification of CsA in the lachrymal fluid provided an overview of the precorneal ongoing processes after the administration of the drug, while the determination of CsA levels in different ocular tissues allowed the evaluation of the penetration and therapeutic effect of the drug. A precorneal in vivo kinetic was selected primarily because the mechanism of action of the CsA in the dry eye disease is linked to the precorneal area [3,26,27] and because it is a well established, non-invasive in vivo procedure [32,33]. The ocular distribution experiment was necessary in order to evaluate the therapeutic effect obtained after OPPH 088 administration since there is no established standard objective test [1,2] neither an accepted animal model for the evaluation of DED [34]. Furthermore, the rabbit and rat animal models are well characterised and commonly used for ocular investigations [17,26,34–36].

The ocular distribution evaluation showed the ability of the prodrug to achieve active CsA concentrations (i.e. between 50 and 300 ng/g of tissue [37]) in all the studied ocular tissues; cornea, conjunctiva, iris-ciliary body and retina. These results demonstrated the deep ocular penetration obtained with OPPH 088 and its capacity to lead to therapeutic effect in both the anterior and posterior segments of the eye. On the contrary, similar ocular distribution studies performed with Restasis [38] showed lower CsA levels in all ocular tissues compared to the prodrug solution; CsA concentrations after the emulsion application being orders of magnitude below the therapeutic range. The comparison of the presented results with a former study performed with Restasis in rabbits and dogs [26] showed that the prodrug solution achieved higher concentrations in the whole eye, except for the cornea were both Restasis and the prodrug formulation lead to active CsA levels. Thus, the prodrug solution clearly showed a better performance in achieving pharmacologically active CsA levels in the ocular structures compared with Restasis, highlighting its higher penetration capacity.

The target areas for the treatment of DED are located in the anterior segment of the eye and are in direct contact with the lachrymal fluid from where the CsA was redistributed depending on its affinity and bioavailability towards them. The conjunctiva is highly involved in the superficial ophthalmic inflammation reported in DED patients. The above presented ocular distribution results showed that CsA coming from the prodrug solution achieves its maximum concentration in the conjunctival tissue, being highly beneficial for the treatment of the DED. In contrast, CsA delivered from a lipophilic vehicle does not preferably penetrate into that structure [26,38,39]. Lachrymal glands are susceptible to an accumulation of CsA because they have specific transporters that retrieve selectively the peptide from the surrounding environment [26,40–42]. This action could also contribute to the therapeutic effect of the drug. It has been suggested in numerous studies that the cornea plays an important role in topical ophthalmic treatments, particularly in the case of CsA formulations where the active molecule can accumulate in the corneal epithelial cells, that can be considered a lipophilic compartment acting as a reservoir [17,26,43–46]. The transport of CsA into the cornea was mainly caused by its partitioning between the highly hydrophilic lachrymal fluid and the lipophilic corneal epithelial cells, therefore leading to an accumulation following the administration. The posterior redistribution and release of the drug into the tear film could be

explained by the presence of specific active CsA transporters that cause a drug efflux from the cornea into the lachrymal compartment [43]. Another explanation could be that the continuous clearance of CsA from the eye surface displaced the equilibrium in favour of the tear fluid. In the case of the prodrug, the CsA located in the conjunctiva could also undergo the same redistribution processes. The redistribution phenomenon was only relevant for longer time points because during the first few minutes after the administration, there was a saturation of CsA in tears that makes the passive diffusion predominant. The accumulation of CsA followed by the redistribution is consistent with the measured CsA levels in tears for both formulations until 48 h (Phase 2 for Restasis and Phase 3 for OPPH 088).

According to the postulated CsA distribution mechanism, the cornea plays a key role in the kinetics acting as a CsA reservoir. Additionally, in the case of the prodrug, the conjunctiva could also take part in the phenomenon.

The kinetics could be correlated with the mentioned physiological characteristics, and some hypotheses can be made to explain their profiles. In the case of Restasis, the first phase of the kinetic profile can be related to the predominance of a strong and rapid precorneal drug loss linked to the interaction of CsA with the ocular structures and to the stimulation of the specific protective mechanisms of the eye after the administration of the eyedrop. The second phase can be explained by the establishment of equilibrium in the CsA distribution, which is due to the reservoir effect postulated from the corneal epithelium. The same phenomena took place for the kinetics of OPPH 088; however, it was essential for the prodrug to undergo an enzymatic biotransformation prior to the appearance of CsA in the tear fluid. The first phase represented a mixture of the precorneal loss of OPPH 088 and CsA, and the gradual biotransformation of the prodrug into CsA directly in the tear fluid. The increase of CsA levels during the first minutes strongly suggested that the predominant mechanism in that phase was the biotransformation of OPPH 088. The second phase of the kinetic of OPPH 088 was considered equivalent to the first phase of Restasis, which corresponded with a predominant loss of drug from the surface of the eye. The third phase is characterised by sustained drug concentrations in tears suggesting a redistribution of the drug.

Despite the major disparities exposed between the general profiles, the statistical analysis based on the Mann–Whitney *U*-test for time points starting from the 15 min mark showed no significant difference between the kinetics of Restasis and OPPH 088. Both formulations induced equivalent CsA levels in the tear fluid between 15 min and 48 h after topical ophthalmic application.

Nevertheless, it has to be noted that the quantitative approach presented is restricted to the limitations of the technique used. A more global and qualitative view can be of interest to better understand the in vivo behaviour of the studied formulations. As the elimination of CsA is a continuous and subtle phenomenon, its further analysis was only possible through the presented model that allows to unveil interesting information (which would have been unnoticed with the quantitative approach). A two-compartment model centred on the tear fluid was appropriate since it reflects the physiological situation [35,46], and it is in accordance with the experimental results.

The obtained values for the elimination constant and the β half-life clearly pointed out a higher ocular elimination for CsA when administered with the emulsion compared to the prodrug formulation. This result is consistent with the previously presented data for Restasis (higher corneal damage and lower CsA levels in ocular tissues). The difference observed in the elimination of the studied formulations could be linked to their nature; oil-in-water emulsion for Restasis versus a totally aqueous solution for OPPH 088.

It has been demonstrated that the nature of the formulation has a key influence on CsA elimination, availability and efficacy [26,27,47]. On the one hand, the elimination could be enhanced by the stimulation of the protective mechanisms of the eye. For Restasis, some reported adverse effects such as ocular burning, discharge, foreign body sensation, redness or epiphora [48] could be linked to the stimulation of the protective mechanisms of the eye that could reinforce the elimination. Previous studies showed that the ocular application of a lipophilic vehicle is not without consequences [49–51]. In the case of OPPH 088, the aqueous vehicle contributed to a lower foreign body reaction and a moderated inducement of protection mechanisms (which was consistent with the previously presented ocular tolerance results). Thus, OPPH 088 formulation could lead to a lower patient's discomfort level. On the other hand, the miscibility of the formulations with the lachrymal fluid has also to be considered. To obtain a therapeutic effect with Restasis, CsA must leave the lipophilic fraction of the emulsion to be released into the lachrymal fluid and reach the target tissues. Nevertheless, the partition coefficient of CsA between tears and its lipophilic carrier was not favourable. CsA had a higher affinity for the oily phase of the emulsion that limited its release and availability, as former studies demonstrated [47]. In addition, the lipophilic fraction of Restasis was more compatible with the superficial lipid layer of the tear film, which facilitated its elimination. In the case of OPPH 088, the prodrug was easily distributed within the lachrymal fluid layer, where the enzymatic biotransformation occurred, resulting in the release of CsA directly into the tear fluid. The generated CsA is surrounded by the lachrymal hydrophilic environment from where it had a higher tendency to interact with ocular tissues. The CsA release and bioavailability are then reinforced. This postulated higher CsA availability for OPPH 088 formulation is in accordance with the previously described lower elimination and higher ocular tissues levels.

Despite the fact that the same active molecule was involved in both formulations, the implicated mechanisms were different; OPPH 088 aqueous formulation could lead to higher penetration, higher ocular tissue concentrations, lower elimination, higher availability and higher efficacy of CsA.

5. Conclusion

This investigation demonstrated the excellent ocular tolerance of the OPPH 088 formulation and gives considerable insight on the in vivo behaviour of the studied ophthalmic formulations. On the one hand, it demonstrated the ability of OPPH 088 formulation to achieve therapeutically active CsA levels in both the anterior and posterior segments of the eye, highlighting higher CsA tissue levels and penetration capacity compared to Restasis. On the other hand, it evidenced a lower elimination and higher availability for CsA when generated from the prodrug than after direct application with the emulsion. Therefore, it can be suggested that the use of OPPH 088 represents a safe and suitable option for the treatment of DED. In addition, OPPH 088 has some interesting advantages such as an increased patient comfort and higher compliance, which are of major importance since DED requires long periods of treatment.

Ongoing in vivo investigations will allow a deeper understanding of the behaviour of CsA-based topical ophthalmic formulations.

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