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A novel water-soluble cyclosporine A prodrug: Ocular tolerance and in vivo kinetics

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Abstract

The purpose of this study is to demonstrate that a novel water-soluble prodrug of cyclosporine A (CsA) intended for topical ocular administration, does not induce eye irritation in a rabbit model and is able to generate therapeutic concentrations of CsA in the precorneal area immediately after administration. The eye irritancy of the prodrug and CsA control solution was assessed by the Draize test and by confocal laser ophthalmoscopy (CLSO). Residence time and tear concentrations of prodrug and CsA in the rabbit eye were assessed by HPLC. The Draize test showed an excellent tolerance for the prodrug solution while the reference CsA oil solution induced lachrymation and irritation. The CLSO-measured corneal lesions, subsequent to treatment with the prodrug and reference solutions, were 3% and 9%, respectively. The prodrug transformed rapidly, leading to relatively stable CsA concentrations in tears with a maximal concentration of 94 $\mu g\,ml^{-1}$ over the observation period. This study demonstrated that the prodrug solution was well tolerated and that clinically significant CsA tear concentrations were achieved. UNIL088 is a promising molecule in the treatment of immune-related disorders of the eye.
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Keywords: Cyclosporine A; Prodrug; Tolerance; Kinetics; Tears; Ocular delivery

1. Introduction

Cyclosporine A (CsA), an immunosuppressive drug, is commonly used in the management of ocular conditions with an immunological component, particularly for the prevention of corneal graft rejection and dry eye disease. Corneal transplantation is the only

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treatment for many blinding disorders. However, rejection is the most frequent cause of corneal graft failure (Ardjomand et al., 2003). CsA is now routinely used systemically for prophylaxis against corneal graft rejection (Poon et al., 2001). However, therapeutic blood concentrations after systemic administration are associated with severe systemic sides effects (Mihatsch et al., 1998). Local delivery of CsA can avoid such drawbacks.

Dry eye disease has a prevalence of 6% in the population aged above 40 years (Pflugfelder, 2004) and 14.6% above 65 years old (Perry and Donnenfeld, 2004). This syndrome is not a vision-threatening condition but it causes considerable discomfort and substantially reduces the sufferer's quality of life. Currently, the main treatments are palliative, as these therapies are aimed to replace or conserve the patient's tears without correcting the underlying disease process. It is now well established that CsA is effective in the treatment of this syndrome (Pflugfelder, 2004). Corneal graft and dry eye disease are both pathologies of the eye surface; hence, there is an obvious need for a topical delivery system to administer CsA to the eye. This mode of administration is convenient, non-invasive, easy enough for self-administration, offers localized effect of the medicament, and reduces systemic drug concentration. But like some other ophthalmic drugs, CsA possesses lipophilic properties forbidding formulation of an aqueous solution. Local delivery of CsA has been extensively investigated but, so far, none of these systems have been found to be fully satisfactory (Lallemand et al., 2003).

Recently, a new approach has been proposed, namely the use of water-soluble prodrugs of CsA (Hamel et al., 2004). Several prodrugs of CsA were synthesized and preformulation tests demonstrated that, besides being highly soluble in biocompatible aqueous media, some of these molecules were converted by tears and able to generate high levels of CsA within the first minute of contact with rabbit or human tears (Lallemand et al., 2005). A screening procedure has allowed the selection of prodrug UNIL088 (Fig. 1) as the most promising candidate for further in vivo study, due to its high conversion rate in tears. As a new chemical entity and due to its amphiphilic and acidic nature, UNIL088 must be submitted to ocular tolerance tests. In addition, therapeutic efficacy is dependent on drug concentration and duration of exposure at the site of action. Therefore, it is of great importance to determine the amount of CsA released and its residence time in the precorneal area.

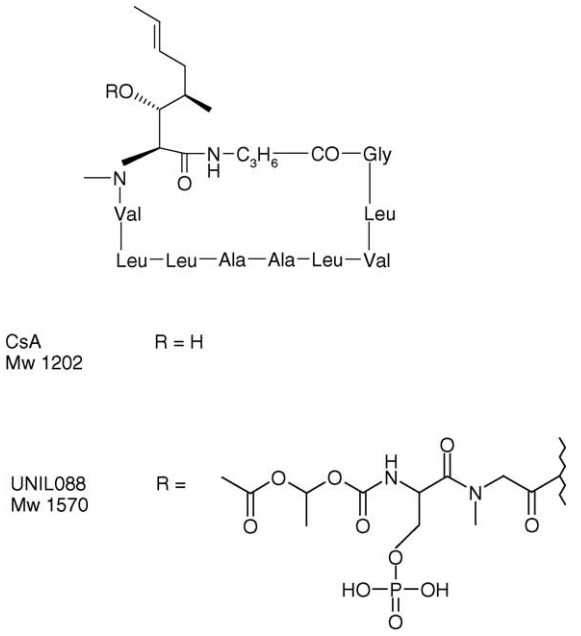

Fig. 1. Chemical structure of UNIL088 and CsA.

The aim of this work was to present selection criteria and investigation tools for the evaluation of local ocular irritation induced by topically applied UNIL088. As a control, an oil solution of CsA was used. The Draize test was performed to assess acute irritation and confocal laser scanning ophthalmoscope (CLSO) was employed to assess subchronic tissue injury after repeated topical administration. In a second step, tear concentrations of UNIL088 and CsA were determined after instillation of the prodrug and the control CsA solutions in the rabbit eye.

2. Materials and methods

2.1. Materials

UNIL088 was synthesized by the Institute of Chemical Sciences and Engineering (ISIC), Ecole Polytechnique Fédérale de Lausanne (EPFL), Switzerland as described by Wenger et al. (2002), and cyclosporine A (CsA) was kindly provided by the ISIC. Mannitol and

olive oil were purchased from Acros Organics (Belgium) and trifluoroacetic acid (TFA) was purchased from Fluka (Buchs, Switzerland). Water and acetonitrile (ACN) were of analytical grade (SDS, France).

2.2. Preparation and characterisation of formulations

An UNIL088 solution was extemporaneously prepared at a concentration equivalent to 0.2% (w/v) of CsA, in an aqueous 5% (w/v) mannitol solution adjusted to pH 7 with 1N NaOH. The prodrug was solubilized at room temperature under slight mechanical agitation. The solution was filtered through a 0.22 μm membrane (type Millex-GV 13 mm low protein retention, Millipore, Cork, Ireland) prior to instillation in the rabbit eye. Isotonicity (Automatic osmometer type Digital/L, Knauer, Germany) and pH (Metrohm 691 pH meter, Switzerland) were assessed prior to experiment. CsA was solubilized at 0.2% (w/v) in pure olive oil as reference formulation.

Refractive indexes of 5% (w/v) mannitol solution, UNIL088 in mannitol solution, olive oil, and CsA oil solution were measured at 35 °C (Refractometer, Carl Zeiss, Germany). Surface tensions of these same solutions were also measured in triplicate at 35 °C by the De Nouy ring method with a digital tensiometer K10 (Kruss GmbH, Germany).

2.3. Animals

Male albino New Zealand rabbits weighing approximately 4–5 kg and free of any ocular damage were used throughout the study. The experimental protocol was approved by the canton of Geneva's local Ethics Committee for animal experimentation.

2.4. Modified Draize test

This irritation test was conducted using a 0 (absence) to 3 (highest) clinical evaluation scale of discharge, conjunctival chemosis and conjunctival redness, as described in Table 1 (Baeyens et al., 2002). The test protocol was carried out on six rabbits after a single instillation of 50 μl of the solutions; the untreated contralateral eye was used as a control. Each animal was observed at 0.5, 1, 2, 3, 6, 9, 12, 24, 32 and 48 h after instillation. An index of overall irritation (I_{irr}) was calculated by summing up the total clinical evaluation scores over the observation time points. A score of 2 or 3 in any category or an I_{irr} greater than 4 are considered as indicators of clinically significant irritation.

Table 1
Modified Draize's grading scale for clinical evaluation of ocular irritation

Conjunctival discharge	Normal	0
	Slight discharge	1
	Severe discharge covering a small area around the cornea	2[a]
	Severe discharge covering a large area around the cornea	3
Conjunctival chemosis	Normal	0
	Slight chemosis including nictitating membrane	1
	Severe chemosis with eye partially closed	2[a]
	Severe chemosis with eye closed	3
Conjunctival redness	Blood vessels normal	0
	Some blood vessels definitely hyperaemic	1
	Diffuse colour, individual vessels not easily discernible	2[a]
	Diffuse beefy red	3

[a] A score superior or equal to 2 in any category or a total added score $I_{irr} > 4$ are considered as indicators of non-tolerance.

2.5. Confocal laser scanning ophthalmoscope test

Twenty-five microliters of the solution to be tested was instilled onto 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 instillation, rabbits were sedated with an intramuscular injection of ketamine HCl (15 mg/kg body weight) and xylazine (3 mg/kg body weight). A volume of 25 μl of a 0.5% (w/v) sodium fluorescein solution was instilled to allow the injured areas to be selectively marked. The eye was then rinsed for 1 min with 0.9% (w/v) NaCl kept at body temperature. Finally, the cornea was observed with a confocal laser scanning ophthalmoscope (CLSO® Zeiss, Germany) modified as previously described (Furrer et al., 1997). Briefly, a set of lenses was added to the original ophthalmoscope in order to examine the cornea instead of the retina. The CLSO was coupled to an image-processing system (Semper6, Synoptics, UK) to enable three-dimensional reconstruction from digitized frames and

evaluation of injured areas, which were represented by fluorescent zones. The percentage of corneal lesion is then reported on a tolerance evaluation scale. Each formulation was tested on three rabbits.

2.6. In vivo kinetics

The day before the experiment, one drop of fluorescein solution (0.5%, w/v in phosphate buffered solution pH 7) was instilled in the right eye of each rabbit to verify the normal elimination process of the nasolachrymal drainage system. Twenty-five microliters of the formulations were instilled in the right eye of three non-anaesthetized rabbits for each formulation. Tear fluid samples were collected every minute from the lower marginal strip using disposable 2 μl glass microcapillaries (Microcaps Drummond, Thomas Scientific, New Jersey). Immediately after collection, capillaries were blown under a gentle nitrogen flow into a vial containing 20 μl of ACN to precipitate proteins and to stop the enzymatic reaction catalysing the prodrug conversion.

2.7. Analytical method

CsA and the prodrug were analysed and quantified by high performance liquid chromatography (HPLC). The method was developed specifically to quantify in the same run the hydrophobic CsA and the hydrophilic prodrug. Analytical separation was achieved using a C4 column (300 Å, 5 μm, 4.6 mm i.d. × 250 mm, type 214TP54, Vydac, Hesperia, California). The mobile phase contained acetonitrile as organic modifier and acidified water (0.09%, v/v TFA). An organic gradient (60–100% ACN) over 15 min, using volumetric mixing by the HPLC pump (W600 controller and multisolvent delivery pump, Waters, Massachusetts) was used to separate the components. The flow rate was set at 0.8 ml min^{-1} and the column oven at 40 °C.

Tear samples underwent treatment to eliminate tear proteins prior to injection. The samples were then diluted with 55 μl of water, centrifuged for 5 min at 10,000 rpm (Biofuge pico, Heraeus Instruments, Germany), and the supernatant (77 μl) was collected and 70 μl of sample were injected via an automatic injector (W717 plus Autosampler Waters, Massachusetts). The absorbance was measured at 210 nm (W2487 Dual λ Absorbance Detector, Waters, Massachusetts). Millennium®32 chromatography manager software (version 3.2) was used for peak integration. The analyte peak was compared to the total peak area and was expressed as a percentage. The limit of quantification was estimated using the signal to noise ratio approach (S/N = 10) and confirmed by injections of an independent standard sample at a concentration of 2 μg ml^{-1}. Under these conditions, UNIL088 and CsA were separated with retention times of 10 and 12 min, respectively.

3. Results and discussion

3.1. Eye irritation

The solution of UNIL088 was prepared under optimal conditions for topical delivery, so that physicochemical properties of the solution (pH, osmolality, etc.), microorganisms or particles could not interfere with the irritation evaluation. The characteristics of the formulations tested are summarized in Table 2. The surface tension of UNIL88 solution (35 mN m^{-1}) is very close to the value for normal tears, reported to be 40–50 mN m^{-1} (Ibrahim et al., 1988). The surface tension of the solution of UNIL088 was lowered by 50% compared to its vehicle due to its amphiphilic nature

Table 2
Physico-chemical properties of human tears, UNIL088 solution and CsA oil solution

	Human tears[a]	UNIL088 solution	CsA in olive oil
pH	7.1–7.6	7	–
Osmolality (mOsm kg^{-1}) ($n = 3$)	310–334	295–305	–
Surface tension (mN m^{-1}) ($n = 3$)	40–50	35.3–36.1	32.1–32.3
Refractive index	1.336–1.357	1.341	1.462

[a] According to reference Ibrahim et al. (1988).

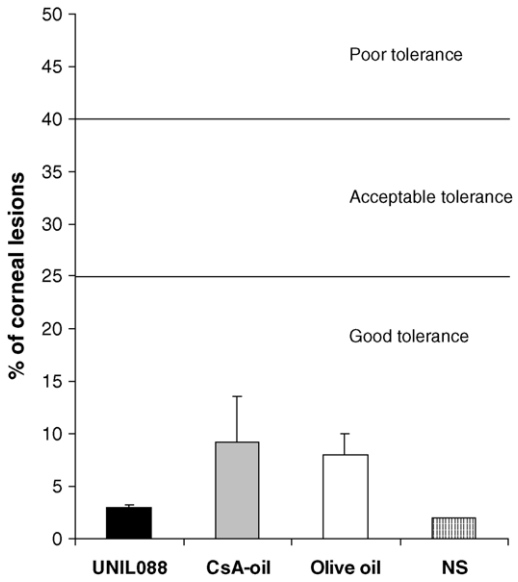

Fig. 2. Percentages of corneal lesions induced by application of UNIL088 and CsA solutions, olive oil and a normal saline solution (NS) reported using a tolerance scale for CLSO (Furrer et al., 2002a) ($n = 3$, mean ± S.D.).

(Table 2). The refractive index of the prodrug solution (1.341) is in the physiological range (Ibrahim et al., 1988). Surface tension and refractive index of the CsA oil solution (Table 2) are relatively different from physiological values indicating that ocular tolerance of such a solution may not be optimal.

The modified Draize test revealed a very slight redness of the conjunctiva and a slight reflex lachrymation but no chemosis during the first half hour after application of the UNIL088 solution. In none of the six rabbits did the I_{irr} score exceed 4 at 30 min, time of highest observed irritation. It has to be noted that all these signs decreased until complete disappearance 1 h post-administration. The administration of CsA in oil and olive oil alone induced a strong lachrymation and redness within a few minutes after application. These results are in accordance with those of Fiscella et al. (1996) who reported that patients treated by olive oil solution of CsA complained of the "oily" feeling and "eye discharge" following administration. Olive oil is not tolerated due to the disparity between the physico-chemical properties of the oil and tear fluid. The corneal lesion data are reported in Fig. 2. No significant difference is observed between CsA in oil and the oil alone, where percentages of corneal lesions calculated by CLSO were, respectively, 9.2 ± 4.4% and 8.1 ± 2.0%. CsA in oil does not appear to induce additional irritation relative to the vehicle alone. This drug has already been reported to be safe when administered locally at concentrations up to 1% in oil or emulsion formulations (Belin et al., 1990; Acheampong et al., 1999). The application of UNIL088 led to only 3.0 ± 0.3% corneal lesions whereas the instillation of a sterile saline solution results in a fluorescent surface of 2% due to normal desquamation (Furrer et al., 2002b).

Despite its amphiphilic and acidic nature, UNIL088 possesses a very low irritation potential and the use of an aqueous vehicle greatly improves the tolerance of this formulation. In addition, studies (Hutak and Jacaruso, 1996) have shown that the rabbit eye is more sensitive than the human eye, and has a longer epithelial repair time (three times longer). It is reasonable, therefore, to expect better tolerance to the prodrug solution in the human eye.

Topical administration of UNIL088 can also lead to CsA systemic toxicity if the prodrug is absorbed by the nasal mucosa and converted into the parent molecule. Since, the instillation of a 25 μl drop of UNIL088 solution contains 50 μg of CsA, complete systemic absorption of that amount of CsA would be unlikely to achieve a blood concentration above the upper limit of non-toxic concentration (300 ng ml^{-1}; Bowers and Canafax, 1984). In addition, the prodrug progressively releases CsA in the blood, exerting a controlled release-like effect that prevents CsA from achieving high blood concentrations. Also, systemic absorption of CsA after long-term topical administration of a 0.1% CsA emulsion led to blood concentrations below the quantification limit of an mass spectrometry analytical method (0.1 ng ml^{-1}) (Acheampong et al., 1999). Furthermore, preliminary results in a rat model showed that UNIL088 administered by intravenous route in the tail vein at a dose equivalent to 10 mg/kg body weight CsA was not associated with systemic toxicity (data not shown).

3.2. *In vivo kinetics*

After topical administration, UNIL088 and CsA are subjected to normal physiological processes of elimination such as naso-lachrymal drainage, tear turnover, corneal and conjunctival absorption and plasma protein binding. In addition, as a prodrug, UNIL088 undergoes

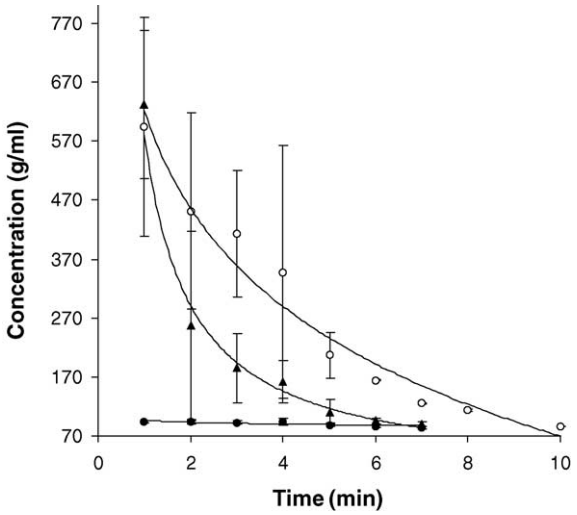

Fig. 3. Lachrymal kinetics of UNIL088 (▲), prodrug-generated CsA (●) and CsA from olive oil formulation (◯) ($n = 3$, mean ± S.D.).

a rapid first-order enzymatic hydrolysis into the parent molecule as demonstrated previously (Lallemand et al., 2005). These phenomena illustrate the major complexity of applying a conventional pharmacokinetic model to UNIL088 and CsA in the eye environment.

The tear concentration–time profiles of UNIL088 and CsA released from the prodrug and oil formulation are represented in Fig. 3. The curves describing elimination of UNIL088 and CsA in oil are biphasic, elimination being very rapid within the first 4 min and slower thereafter. The initial phase is mainly explained by the mechanical elimination of the extra volume of precorneal liquid within the first minute. Once the tear volume is back to normal, the elimination of UNIL088 and CsA in oil should follow the normal tear turn-over, reported to be 7% per minute in rabbits (Chrai et al., 1973). The observed maximum tear concentration of UNIL088 and CsA generated by the prodrug are $633 \pm 125\ \mu g\ ml^{-1}$ and $94 \pm 4\ \mu g\ ml^{-1}$ at 1 min, respectively. This concentration is thought to be clinically efficient as a concentration equivalent to $79\ \mu g\ ml^{-1}$ of CsA was measured in the tears of rabbit after topical administration of 50 μl of a 0.2% CsA emulsion (Acheampong et al., 1999) performing significant improvements in the dry eye symptoms when administered in humans (Stevenson et al., 2000). Also, a concentration of $0.10\ \mu g\ ml^{-1}$ in the vitreous has been reported to suppress T-cell activation (Kumar et al., 2001). It is assumed that similar concentrations in the cornea or in the local immune system can prevent corneal graft rejection. Concentrations of CsA generated were followed until the limit of quantification of the HPLC method was reached ($77\ \mu g\ ml^{-1}$ in tears) at 7 min. The concentration of CsA generated from UNIL088 remained relatively stable for the three rabbits, decreasing from 95 to 80 $\mu g\ ml^{-1}$ in 7 min (Fig. 3). The elimination rate is three times slower than the normal tear turn-over of 7% per min (Chrai et al., 1973). This very uncommon profile can be explained by the physico-chemical properties of CsA. Throughout the studied period, the CsA generated by UNIL088 precipitates, since its concentration is always above its solubility limit ($7.3\ \mu g\ ml^{-1}$ at 37 °C in water; Ismailos et al., 1991). Precipitates can accumulate in the conjunctival cul-de-sac supplying a reservoir of solid CsA. The precipitates may then redissolve progressively in the tears, resulting in a continuous release of CsA from the reservoir and constant levels (within the range of 80–95 $\mu g\ ml^{-1}$) of CsA in the fluid. The phenomenon can also be explained by the accumulation of CsA precipitates in the cornea as already described (Kachi et al., 2000). The corneal uptake of small particles of CsA is consistent with the uptake of ciprofloxacin precipitates in the cornea due to decreased solubility of this molecule in a physiological environment after topical administration (Sinnaeve et al., 2003). The progressive redissolution of ciprofloxacin from the cornea, was shown to exert a longer clinical effect against bacteria (Madhavan et al., 1999; Eiferman et al., 2001). Such precipitates, as well as CsA precipitates, may act as a drug depot, leading to a controlled-release of the drug in the precorneal environment.

The concentrations of CsA observed in tears were about 10 times higher than solubility in water. This enhanced solubility can be explained by the binding of CsA to lipophilic proteins of the lipid layer of the tears such as cholesterol (Bron et al., 2004). In plasma, CsA interacts primarily with lipoproteins, approximately 45 to 60% are bound to high-density lipoproteins, 30 to 35% to low-density lipoproteins, and smaller amounts to intermediate, very low density lipoproteins and chylomicrons (Strong and Ueda, 1997).

For the CsA control solution, the maximum tear concentration was $595 \pm 185\ \mu g\ ml^{-1}$ at 1 min and concentrations were detectable until 10 min. CsA in oil

remained longer than UNIL088 and generated CsA on the corneal surface mainly due to the higher viscosity of the olive oil. However, the CsA provided by the oil solution and the CsA generated from UNIL088 will not have exactly the same fate. As CsA has a very high affinity for the oily phase, there is a likelihood of clearance before CsA is able to partition from the olive oil to the tissue. Therefore, the tissue availability of CsA generated from UNIL088 might be higher than that from a CsA formulation in olive oil, despite tear concentrations being greater in the latter case.

The main metabolism pathway of CsA is via oxidation by cytochromes P450 present in microsomes (Christians and Sewing, 1993). Although cytochrome P450 levels in the eye tissues are relatively high (Kumar, 1996), to the best of our knowledge, it is not present in the tears. Therefore, CsA metabolism in the precorneal area is unlikely.

4. Conclusions

Ocular tolerance of the prodrug solution was very good in both single and chronic administration. Subsequent to topical administration of UNIL088, therapeutic concentrations of CsA were observed in the tears over 7 min. A reservoir-like effect results in relatively constant and prolonged concentrations of CsA in the tears. The CsA generated is readily available for tissue absorption as the aqueous vehicle is favourable to the tissue partitioning of the lipophilic parent molecule. Hence, UNIL088 appears to be a very promising molecule for the treatment of the dry eye syndrome and the prevention of corneal graft rejection. The clinical efficacy of the prodrug solution is the subject of ongoing investigations using a rat keratoplasty model.

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