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European Journal of Pharmaceutics and Biopharmaceutics 59 (2005) 51–56
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Conversion of cyclosporine A prodrugs in human tears vs rabbits tears

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Received 25 May 2004; accepted in revised form 1 July 2004
Available online 1 September 2004

Abstract

The aim of this study was to evaluate the rate and mechanism of conversion of two water-soluble prodrugs of cyclosporine A (CsA) intended for topical delivery to the eye. The new molecules were designed according to the double prodrug concept: a solubilizing moiety was grafted onto CsA via an ester function, which could be hydrolysed via a two-step process (enzymatic and chemical). Prodrug solutions were prepared extemporaneously in an isotonic and neutral aqueous medium compatible with ophthalmic use. The rates of conversion into the parent molecule were determined by incubating the prodrugs in fresh rabbit or human tears or in a phosphate buffer solution (PBS) at pH 7.4. Both prodrugs were converted into CsA within the first minute in the presence of rabbit tears with rate constants of $k=5.9\times10^{-3}$ min^{-1} and $k=3.8\times10^{-3}$ min^{-1}, respectively, for UNIL088 and UNIL089, whereas chemical conversion in PBS was negligible ($k=0.5\times10^{-3}$ min^{-1} for both molecules). Incubation of UNIL088 in human tears showed a significantly high conversion rate. It is concluded that the developed double prodrugs underwent a bioconversion in physiological media and thus represent promising candidates for topical delivery of CsA to the eye.

Keywords: Cyclosporine A; Prodrug; Ex vivo model; Ocular drug delivery; Conversion; Rabbit tears; Human tears

1. Introduction

The immunosuppressive drug cyclosporine A (CsA) is nowadays commonly used in the management of several ocular conditions with an immune component such as uveitis [1], dry eye syndrome [2] and in the prevention of corneal graft rejection [3]. These pathological states can be treated by i.v. or oral administration but systemic levels of CsA induce severe side effects such as nephrotoxicity and cardiotoxicity [4]. The use of local formulations such as collyria should circumvent these side effects. However, due to its lipophilic nature, CsA cannot be formulated as an aqueous solution. Hence, numerous approaches have been investigated for topical CsA administration but, so far, only an emulsion for human use (Restasis®, Allergan Inc., Irvine, CA) has been approved by the Food and Drug Administration (FDA) [5]. Emulsions, however, present the major disadvantages of causing blurred vision and providing relatively low drug availability due to the high affinity of CsA for the oily phase of the formulation. The water-soluble prodrug approach is a very efficient way to solubilize CsA in aqueous media avoiding these drawbacks. This concept has already been applied to several molecules in ophthalmology to modify the hydrophilicity of drugs [6] (e.g. esters of steroids [7] and amino acid esters of acyclovir [8]). As CsA possesses (on position 1) a residue with a free hydroxyl group, the synthesis of ester prodrugs is a feasible option. A series of CsA ester derivatives have been synthesized [9] and among

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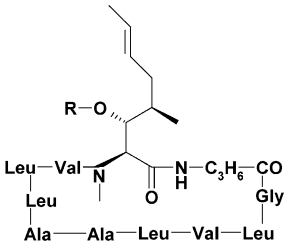
doi:10.1016/j.ejpb.2004.07.002



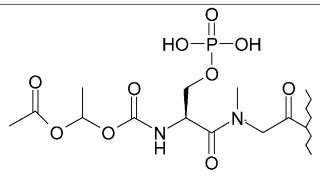
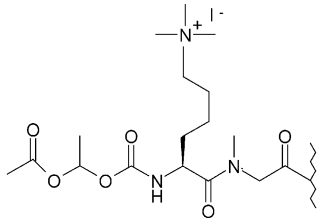
Compound	Mw	R
CsA	1202	H
UNIL088	1570	
UNIL089	1574	

Fig. 1. Chemical formula of CsA, UNIL088 and UNIL089.

them, UNIL088 and UNIL089 (Fig. 1) were selected for further investigation. The chain of the prodrugs to be removed to release the drug is the dipeptide sarcosine-serine (or lysine)-(acyloxy)alkyl-oxy-carbonyl, carrying a phosphate group (UNIL088) and an ammonium group (UNIL089) as solubilizing moieties. These two molecules have been designed according to the double prodrug theory: the prodrugs are converted into the active parent molecule via a two-step enzymatic and chemical mechanism. The rapid elimination from the precorneal area of any topically applied solution requires the prodrug derivatives to undergo quantitative conversion within the first minute after application. The conversion rate of a prodrug into the parent molecule is commonly evaluated by incubating the compound in the presence of commercial enzymes [7,10], human plasma [11] or homogenates of ocular tissues [12–14]. However, these models are not representative of the tear composition in enzymes emphasizing the need for a reliable tool able to evaluate the potential of the prodrugs to be converted into their parent molecule under clinical conditions and able to determine the nature of the underlying mechanism (enzymatic or chemical).

The aim of the present study was firstly to demonstrate that after solubilization at a concentration equivalent to 0.2% (w/v) of CsA in an aqueous medium, UNIL088 and UNIL089 could generate CsA when incubated in the presence of rabbit tears using a new and reliable ex vivo model. Secondly, the prodrugs were incubated in a phosphate buffer solution at pH 7.4 to determine whether or not the conversion was purely chemical or enzymatic. Finally, the prodrugs were incubated in human tears to test the validity of the rabbit tear model. The prodrug with the fastest kinetics of transformation would then be chosen for further investigations.

2. Materials and methods

2.1. Materials

Prodrugs (UNIL088 and UNIL089) were synthesized and characterized according to Wenger et al. [9]. Mannitol for isotonic solutions was purchased from Acros Organics (Geel, Belgium). Isotonic phosphate buffer at pH 7.4 was composed of 179 mg of monopotassium phosphate, 953 mg of disodium phosphate, 415 mg of sodium chloride and distilled water to 100 ml. Chemicals of analytical grade were obtained from Fluka (Buchs, Switzerland) and all solvents (water and acetonitrile (SDS, Peypin, France)) were of analytical grade.

2.2. Methods

2.2.1. Preparation of prodrug solutions

Prodrug solutions (UNIL088 and UNIL089) were 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 1 N NaOH. The prodrug powder is solubilized immediately at room temperature and under slight mechanical agitation. Isotonicity (Automatic osmometer type Digital/L, Knauer, Germany) and pH (Metrohm 691 pH meter, Herisau, Switzerland) were assessed prior to experiment.

2.2.2. Rabbit and human tear collection

Animal tears were collected from non-anaesthetized New Zealand albino rabbits (weighing approximately 4.0 kg) with disposable 2 μl glass micro-capillaries (Microcaps Drummond, Thomas Scientific, New Jersey). No artificial tear secretion stimulation was performed. Immediately after collection, capillaries were blown into a vial under a gentle nitrogen flow. Kinetics of conversion of the prodrugs into CsA were performed within 5 min after tear collection so that tear proteins and enzymes are still intact. Human tears were self-collected by volunteers using the same method as for rabbit tears. The experiments were approved by the local Ethics Committee.

2.2.3. Analytical method

CsA and prodrugs were analysed and quantified by high performance liquid chromatography (HPLC). This method was developed specifically to quantify in the same run hydrophobic CsA and hydrophilic prodrugs. Analytical separations were conducted 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 (ACN) as organic modifier and acidified water (0.09%, v/v TFA). An organic gradient (60–100% ACN) was carried out over 15 min using volumetric mixing by the HPLC pump (W600 controller and multisolvent delivery pump, Waters, Massachusetts). The flow rate was set at 0.8 ml/min and the column oven at 40 °C. Seventy microliters of sample were injected after treatment via an automatic injector (W717 plus Autosampler Waters, Massachusetts).

Samples underwent treatment prior to injection to eliminate tear proteins. The samples (2 μl) were placed into a vial containing 20 μl of acetonitrile to precipitate proteins. These 22 μl were then diluted with 55 μl of water, centrifuged for 5 min at 10,000 rpm (Biofuge pico, Heraeus instruments, Germany), and the supernatant (70 μl) was collected and injected into the HPLC system. The absorbance was measured at 210 nm (W2487 Dual λ Absorbance Detector, Waters, Massachusetts). The 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 by 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. Under these conditions, compounds were separated with retention times of 10, 11 and 12 min for UNIL088, UNIL089 and CsA, respectively.

2.2.4. Ex vivo kinetics

A volume of 25 μl of the prodrug solution was incubated under slight agitation with 8 μl of fresh rabbit or human tears or with phosphate buffer solution (PBS) pH 7.4 at 37.0±0.5 °C. Samples of 2 μl were collected at 1, 2, 3 and 30 min and replaced each time by 2 μl of fresh corresponding medium. Each kinetic run was conducted in triplicate. The volume of tears chosen (8 μl) corresponds approximately to the tear volume usually present in the rabbit and human eye and 25 μl of the prodrug solution represents the volume of solution commonly administered topically to avoid solution spilling. A mathematical exponential decay was fitted to the conversion curves of prodrugs in an attempt to estimate prodrug rate constants of conversion in the various media. The observed first-order constants were deduced from the slopes of the fitted curves. These constants are not absolute values but are used primarily for comparison in the present study.

All values are expressed as mean±SD. The statistical significance of differences between the means of the data was evaluated by means of the unpaired Student's *t*-test. A *p* value of less than 0.05 was considered statistically significant.

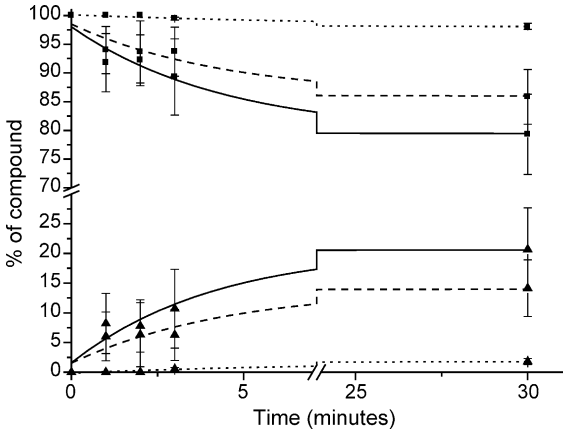


Fig. 2. Ex vivo conversion kinetics of UNIL088 (■) (0.2%, w/v in mannitol solution 5%, w/v) into CsA (▲) incubated with rabbit tears —, human tears – – – and isotonic PBS pH 7.4 ··· (mean±SD, $n = 3$).

3. Results and discussion

As expected, the chemical modifications on CsA enabled the prodrugs to be solubilized at 2.6 mg/ml in an aqueous solution compatible with ophthalmic use, providing a concentration equivalent to 0.2% (w/v) of CsA. The solubility of CsA in aqueous media is reported to range from 6.6 [15] to 27.67 μg/ml [16]. The ionisation, at neutral pH, of the phosphate (UNIL088) and ammonium (UNIL089) groups renders these molecules polar enough to solubilize the hydrophobic cyclopeptide CsA.

The conversion profiles of UNIL088 and UNIL089 in PBS, rabbit and human tears are represented in Figs. 2 and 3. Both prodrugs generated CsA at a faster rate in the presence of tears compared to PBS pH 7.4, suggesting that enzymatic processes must have been responsible for the main part of ester hydrolysis. On the other hand, both prodrugs proved to be relatively stable towards chemical conversion, i.e. in PBS pH 7.4; the slight hydrolysis of the esters in absence of enzymes being negligible compared to enzymatic hydrolysis. The conversion profile of the molecules is characterized

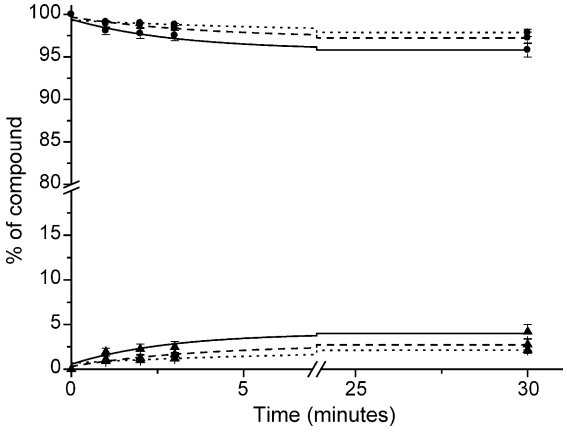


Fig. 3. Ex vivo conversion kinetics of UNIL089 (●) (at 0.2%, w/v in mannitol solution 5%, w/v) into CsA (▲) incubated with rabbit tears —, human tears – – – and isotonic PBS pH 7.4 ··· (mean±SD, $n = 3$).

Table 1
Estimated first order conversion rate constants and half-lives of UNIL088 and UNIL089 after incubation in rabbit, human tears and PBS at 37 °C ($n=3$)

	Rabbit		Human		PBS	
	UNIL088	UNIL089	UNIL088	UNIL089	UNIL088	UNIL089
$k \pm$SD (10^{-3} min^{-1})	5.9±2.3	0.9±0.2	3.8±0.8	0.6±0.2	0.5±0.1	0.5±0.1
$t_{1/2} \pm$SD (min)	128±42	738±147	186±38	1196±468	1347±333	1368±371

by biphasic curves, cleavage of the prodrugs being very rapid within the first minute and slower in the second phase. The conversion of the double prodrugs resulted in quantitative generation of CsA (1 mol of prodrug giving 1 mol of CsA). The conversion curves were relatively well described by an exponential decay model ($R^2 \sim 0.9$), suggesting a first order transformation of UNIL088 and UNIL089 into the parent drug CsA. The conversion rate constants in the various medium and the half-lives of the prodrugs (Table 1) were determined from the slopes of the fitted exponential curves. The rate constants of conversion, estimated through curve fitting, are valid in the context of experimental set-up and are used to compare the different kinetics.

From the comparison of rate constants, it appears that enzyme-catalysed breakdown of UNIL088 and UNIL089 are significantly different ($P<0.05$), UNIL088 being more sensitive to the tear fluid. No significant difference in the hydrolysis pattern of UNIL088 in rabbit ($k=5.9\times10^{-3}\pm 2.3\times10^{-3}$ min^{-1}) and human tears ($k=3.8\times10^{-3}\pm 0.8\times10^{-3}$ min^{-1}) was observed but there is a tendency of the prodrug to be more reactive with the rabbit tears. In these biological media, the calculated constants were approximately 10 times higher than the conversion constant in PBS, indicating that conversion of UNIL088 is mainly of enzymatic nature. UNIL089 appeared to be more stable in biological media with estimated rate constants of $k=0.9\times10^{-3}\pm0.2\times10^{-3}$ and $k=0.5\times10^{-3}\pm0.1\times10^{-3}$ min^{-1} in rabbit tears and PBS, respectively. The constant of conversion of this prodrug in human tears is not statistically different from that in PBS.

Focusing on kinetics at three minutes (Fig. 4), 6.0±4.0% of UNIL088 and 1.7±0.1% of UNIL089 are converted into CsA in the presence of human tears; these percentages correspond to CsA concentrations of approximately100 and 25 μg/ml, respectively, in the reacting vial. UNIL088 conversion resulted in a CsA concentration that was four times greater than that achieved with UNIL089. Under these ex vivo conditions, the prodrugs produced concentrations of CsA above the immunosuppressive blood concentration which is reported to be 0.05–0.30 μg/ml [17]. Although in vivo ocular conditions are not fully reproduced by this ex vivo test, particularly the naso-lachrymal drainage, these prodrugs have demonstrated the attainment of immunosuppressive concentrations in the precorneal medium over the first minute after contact with tears.

Bioconversion may be explained by two possible mechanisms. The first mechanism to consider is a cleavage of the molecules by esterases present in tears. However, this enzyme has, to-date, not been reported as being present in human or rabbit tears; for example Redell [18] showed that, although esterases are present in high amounts in human ocular tissues, there are no esterases in tears. Nonetheless, protein and enzyme composition in tears is not fully known and it should be noted that the work of Redell was conducted over twenty years ago. A second mechanism could proceed due to the presence of compounds in the tears exhibiting an esterase-like activity. Enzymes such as carbonic anhydrases, peptidases, oxidoreductases, N-dealkylating enzymes and carbonyl reductases, reported to possess this property [19] are not present in tears. However, numerous other enzymes have been reported [20] to be present in human tears. The similarity of the catalytic mechanism displayed by other enzymes (i.e. phosphatases) may be responsible for a significant part of the bioconversion. It is realistic to assume that one or several of these enzymes are able to hydrolyse the first ester function of the (acyloxy)alkyl-oxy-carbonyl group of the pro-moiety and contribute to the release of CsA. Other components of tears could also exert such activity on ester functions. Albumin present in both human [21] and rabbit tears [22] was reported to exert an esterase-like activity [23,24], although this activity is controversial [25]. It is known that most acidic drugs that bind to plasma proteins are bound to albumin [26]; as the phosphate group present in UNIL088 is acidic, this prodrug may easily bind to albumin. Immunoglobulins are, likewise, present in human [27] and rabbit tears [22]. Monoclonal IgGs have been shown to enhance hydrolysis of esters of the homologous hapten [28]. Hence, the prodrugs may be recognized as haptens and be hydrolysed. In addition, complement proteins particularly C3 protein [29] are present in the precorneal fluid. Thus, C3 can act as an esterase-like enzyme on acyl ester bonds [30] and it can be hypothesized that this complement protein

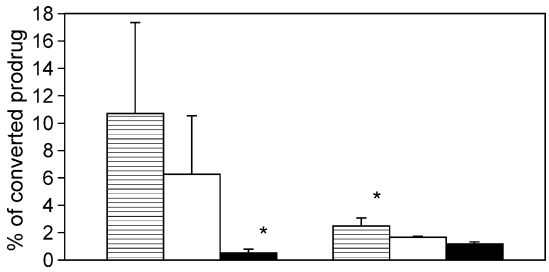


Fig. 4. Ex vivo conversion percentages at 3 min of UNIL088 and UNIL089 (at 0.2%, w/v in mannitol solution 5%, w/v) into CsA incubated with rabbit tears (▤), human tears (□) and isotonic PBS pH=7.4 (■) (mean±SD, $n=3$, Student t-test: * statistically different from other media).

plays an active role in the hydrolysis of the studied ester prodrugs. Unfortunately, the study of tear composition has progressed only at a slow pace in these past 10 years, with most of the work on enzymology of tears being published in the seventies and eighties.

A difference of conversion rates between rabbit and human tears is apparent for both molecules. Comparing the ratios of $k_{rabbit088}/k_{human088}$ and $k_{rabbit089}/k_{human089}$, similar values (1.5) are found, demonstrating that the difference of conversion rates, in the present study, between human and rabbit tears is not dependent on the molecule type but on tear composition. It is obvious that rabbit and human tears do not have exactly the same composition of enzymes and proteins. Further investigations on the tear composition are necessary to explain this observation; indeed, a detailed comparison of rabbit and human tear enzymatic composition should be conducted, as rabbit is a model commonly used in ophthalmology. Although the behaviour of the prodrugs in human tears was not exactly reproduced in the rabbit tears, the fact that rabbit tears are more reactive with the ester prodrug is an advantage for prodrug screening and selection.

In this study, the two prodrugs have different estimated hydrolysis rate constants when incubated in tears, UNIL088 being more sensitive to bioconversion than UNIL089. In contrast, the transformation rate constants of the two prodrugs in PBS are similar. The only difference in the chemical structure between the two prodrugs is the nature of the solubilizing group. In UNIL088 a phosphate group is linked to the promoiety via a CH_2, and in UNIL089 an ammonium group is linked via four CH_2 groups serving as a flexible linker (Fig. 1). In solution, the lateral chain holding the ammonium group should adopt a low energy conformation. Due to its positive charge and the high flexibility of its linkage, the ammonium ion may fold back to an oxygen atom of the promoiety. This electrostatic stabilisation could be the origin of a steric hindrance preventing contact of the (acyloxy)alkyl-oxy-carbonyl group with enzymatic tear components thus slowing biological hydrolysis. On the other hand, similar steric or electronic effects are absent in UNIL088 resulting in higher rates of hydrolysis. Furthermore, the double prodrug approach has the benefit of facilitating the enzymatic attack of the prodrug, the terminal ester function being more accessible to enzymes compared to the ester function directly grafted onto CsA. Although the rate of conversion of a prodrug in the precorneal area is usually assumed to be slow and negligible [19], it has been demonstrated in the present study that tears are able to catalyse the conversion of ester prodrugs to CsA.

This ex vivo test has proved to be a rapid and simple tool for evaluating ophthalmic prodrugs and studying their metabolism in the precorneal area. As the composition of enzymes in tears is still not clearly established, fresh tears have been used rather than a standard commercially available enzyme kit or plasma. Although fresh tears (2 μl) are added to the reacting medium after each collection of sample thus replacing the volume withdrawn, this method cannot mimic the normal tear turnover, the naso-lachrymal drainage and the induced lachrymation when medication is applied in vivo [31]. The addition of fresh tears is a way of maintaining the level of enzymes and proteins.

In conclusion, it has been demonstrated that UNIL088 and UNIL089 generated CsA in the presence of fresh human and rabbit tears faster than in a simple aqueous medium such as PBS indicating the important contribution of the biological medium in the conversion mechanism. This ex vivo test has been useful in the selection of convertible prodrugs and in demonstrating the capacity of these molecules to provide significant concentrations of CsA within a few minutes in both the human and rabbit precorneal environment as required for this application site. The results make UNIL088 a valuable candidate for the treatment of ocular surface syndromes like dry eye disease or for the prevention of corneal graft rejection. In vivo ocular tolerance, precorneal retention time of the prodrug after instillation as well as mechanisms of transformation are the subject of ongoing investigations.

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