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A Highly Soluble Matrix Metalloproteinase-9 Inhibitor for Potential Treatment of Dry Eye Syndrome

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Abstract: Dry eye syndrome (DES) or *keratoconjunctivitis sicca* is an eye disease caused by the chronic lack of lubrication and moisture of the eye. The pathogenesis of DES involves the over-expression and over-activity of corneal Matrix Metalloproteinase 9 (MMP-9). We propose herein a new, non-symptomatic approach for the treatment of DES based on the inhibition of MMP-9 by a new highly soluble molecule, designed as PES_103 that has been shown to inhibit MMP-9 both *in vitro* and *in vivo*. The efficacy of PES_103 *in vivo* and the potential benefits of this treatment in restoring tear production were studied in this work using an animal model of reduced lacrimation. PES_103 did not show any significant corneal toxicity.

Matrix metalloproteinases (MMPs) are a family of zinc containing hydrolases with a broad proteolytic specificity and large structural similarity [1]. These enzymes are involved in the degradation of several extracellular proteins including extracellular matrix components (ECM) and play a crucial role in tissue remodelling and in the regulation of different cellular activities [2]. An aberrant MMPs activity often promotes an excessive degradation of the ECM and could be responsible for the genesis of diseases such as inflammation, angiogenesis and cancer [3]. Recently, relevant ocular disorders such as proliferative diabetic retinopathy and dry eye syndrome (DES), or *keratoconjunctivis sicca*, have been associated with a significant increase in the concentration and activity of MMPs in the tear fluid [4–6]. In particular, DES has been associated with the over-expression and increased activity of corneal Matrix Metalloproteinase-9 (MMP-9 or Gelatinase B) [7], which has been shown to be both a marker and a promoter of eye dryness [8]. According to the work of Pflugfelder *et al.* [9], MMP-9 knockout mice do not develop DES, whereas the topical administration of active MMP-9 to such mice significantly increased corneal epithelial permeability.

The 2007 Report of the Dry Eye WorkShop (DEWS) defined dry eye as an eye disease that results mostly in symptoms of discomfort, visual disturbance and tear film instability, with potential damage to the ocular surface that affects about 20% of the American population and 75% of the over-65-year population [10,11]. DES can be caused by a decrease in tear production or an increase in tear film evaporation, hence being represented by reduced lacrimation models. Persistent dryness, burning and a sandy-gritty eye irritation are the most common symptoms associated with DES and, in general, get worse as the day goes on [12]. Generally, both eyes are affected by DES and the physical damage occurs mainly at the corneal level. The aetiology of this syndrome is manifold, from the normal ageing process, to the effects of different drugs [13], to climatic factors together with daily routines and working-related features, such as standing in front of a computer screen, the use of air conditioning or of contact lenses [14,15]. DES is even a symptom of many systemic diseases. In particular, people affected by Sjögren syndrome often experience DES as a common complication [16,17]. Diagnostic tests comprising a grading of the disease are generally clinical [18,19]. Despite the high number of factors that can produce eye dryness, only symptomatic therapies are currently commercially available. The most effective treatment is based on the corneal lubrication through the administration, many times a day, of artificial tears or eye drops containing anti-inflammatory drugs or lubricating eye drops, which provide moisture on the corneal surface and a relief from eye dryness. However, all these symptomatic treatments require frequent eye drop administrations, which decrease the patient's compliance and might lead to a social problem, especially in case of chronic therapies [20]. Therefore, new therapeutic strategies, which could overcome the need of repeated administrations, are strongly encouraged.

In this paper, we propose a new, non-symptomatic approach for the treatment of DES that relies on the inhibition of MMP-9 by a novel water-soluble small molecule, namely PES_103 [1,21]. Here, we report the encouraging results obtained *in vitro* and *in vivo* by testing the efficacy of PES_103 for the treatment of DES, as well as the lack of corneal cytotoxicity, on an animal model of reduced lacrimation.

Materials and Methods

PES_103 (Acetamide, 2-[(2,3-dihydroxypropyl)[(4-methoxyphenyl)sulfonyl]amino]-N-hydroxy-) (fig. 1) is a synthetic MMPs inhibitor belonging to the hydroxamic aryl-sulphonamide scaffold, with a K_i

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value of 14 nM towards MMP-9 recombinant catalytic domain. Water solubility of PES_103 is higher than 40 mg/ml. Water solutions at 0.01%, 0.1% and 1% of PES_103 at neutral pH were prepared by dissolving the inhibitor in K_2HPO_4 buffer (20 mM).

Pes_103 enzymatic inhibition assay. All measurements were performed in a 50 mM HEPES-buffered solution, with 10 mM CaCl2, 0.05% Brij- 35 and 0.1 mM ZnCl2 (pH 7.0), using 1 nM enzyme and 1 μM fluorescent peptide (OmniMMP fluorogenic substrate; Enzo Life Science, Farmingdale, NY, USA) at 298 K.

Cell culture and MMPs zymography. All samples were incubated for 24 hr at 40°C before being tested by means of gelatine zymography. Active MMP-2 and MMP-9 in conditioned media were separated by electrophoresis on 8% gelatine-zymography gel (Invitrogen, Carlsbad, CA, USA) and then visualized as clear bands against a dark background with Coomassie Blue staining according to Dabizzi *et al.* [22].

Toxicological tests. In vivo tests were performed to preliminarily evaluate the toxicity of PES_103. The solutions of PES_103 were prepared using sterilized equipment; each sample was further sterilized passing through a 0.22 μm Millipore filter before being applied to mice. Three different concentrations (0.01, 0.1 and 1%) were chosen to evaluate the safety range of PES_103.

In vivo toxicity. Experiments on mice were performed at the Laboratory of Genetic Engineering for the Production of Animal Models (LIGeMA) at the Animal House of the University of Florence, Italy. All animals received care in accordance with the provisions of the Declaration of Helsinki and with the Italian law on experimentation with laboratory animals.

C57BL/6 and BALB/c 7-week-old mice, all female, were divided into four groups, each one containing the same number of C57BL/6 and BALB/c mice. The control group received no treatment; the other groups received 2 μl of PES_103 0.01%, 0.1% and 1% solutions, respectively. All solutions were topically administered at the ocular level. All mice were treated in both eyes four times a day for five consecutive days with PES_103 solutions, at the different concentrations described previously. After 5 days of treatment, toxicity was checked in the animals. A water solution of Fluorescein 2% (Sigma-Aldrich, St. Louis, MO, USA) was applied in both eyes of each mouse; a Beta 200 UV lamp (HEINE, Herrsching, Germany) was used to highlight the possible corneal ulceration damage. After a water cleavage, 5 μl of a water solution of Lissamine green (0.325 mM) were applied on both eyes of each mouse to check for keratinization process.

Histology examination. All mice were killed immediately after the last treatment, and their eyeballs were removed and fixed in 10% formalin. After dehydration, the corneal specimens were embedded in paraffin, cross-sectioned and stained with hematoxylin-eosin or Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay. The morphology of the cornea and the number of the apoptotic cells were assessed under a microscope by two independent, masked observers.

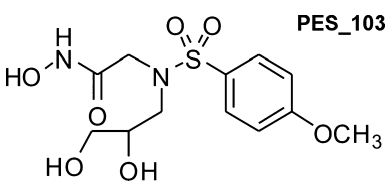

Fig. 1. Chemistry. Chemical structure of the synthetic MMPs inhibitor designed as PES_103.

Measurement of aqueous tear production. Tear production was measured with cotton threads (Zone-quick; Oasis, Glendora, CA, USA). The threads were held with forceps and applied to the ocular surface in the lateral canthus for 30 sec. The length of the wet cotton thread was measured using a millimeter scale.

Tear fluids collection and gelatin zymography. Tear fluids were collected using sterile mounted swabs (Sugi®, Kettenbach GmbH, Eschenburg, Germany) gently placed on the conjunctival area. The swabs were maintained in place for 40 sec. during tear collection. The tear fluids were then extracted by centrifugation and analysed by gelatine zymography. MMP-9 enzyme activity was assayed by SDS-PAGE zymography, using gelatin as substrate. Two different kinds of samples were assayed: tear fluid and the HT1080 cell culture supernatant. The tear fluid was collected directly from the mice eyes using sterile swaps and immediately frozen at −80°C. An extraction protocol was applied to recover the tears by wetting each swap with a standard amount of physiological solution and then spinning down by centrifugation. HT1080 human fibrosarcoma cells over-expressing MMP-2 and MMP-9 were cultured in DMEM medium supplemented with 10% FBS and 2 mM glutamine, in an atmosphere conditioned by CO_2 5% at 37°C. Cells at a density of 1×10^5/well were seeded into a 96-well plate and incubated in presence of five different concentrations of PES_103 (1, 3, 25, 50 and 75 mM) for 24 hr. Conditioned media were then collected, held on ice, centrifuged at 110× *g* for 5 min. at 4°C to pellet any non-adherent cells, and stored at −20°C until analysis. The culture supernatants were stored at −20° C without any further treatment. Samples were directly subjected to electrophoresis through an 8% polyacrylamide gel co-polymerized with 0.7 mg/ml gelatine. After electrophoresis, the gel was washed three times (15 min. each) with a 0.5% Triton X-100 TBS (Tris-buffered saline) solution at room temperature under constant shaking. Triton was then removed washing the gel three times with TBS in the same condition as previously. Finally, enzymatic activation was induced by incubating the gel in 50 mM Tris–HCl-buffered solution at pH 7.6, with 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij at 37°C under constant shaking overnight. The gel was stained with Coomassie Blue R-250 and then de-stained with milliQ water.

Induction of eye dryness by cholinergic receptor blockade. Experimental reduced lacrimation (ERL) was induced on C57BL/6 mice, all female and 7 weeks old. Commercially available transdermal scopolamine patches (TRANSCOP®), 2.5 cm^2 and 1.5 mg, were applied on the mice tail. Each patch was divided into two identical pieces and wrapped around the shaved mid-tail. To avoid patch removal by mice, a plastic tube was applied over the tail. The production of tears was measured four times a day for four consecutive days after the application of the patch, on both eyes of a group of 6 C57BL/6 mice. Six supplementary mice were used as control for the detection of the physiological mean tears volume. Patches were removed immediately after the last measurement.

Statistical analysis. Tears level is presented as the Standard Error of the Mean (S.E.M.) of at least four separate experiments. Student's *t*-test was used for statistical comparison between groups. Values of p lower than 0.05 ($p \leq 0.05$) were considered as statistically significant and were highlighted in figures. All other experiments and measurements were performed at least in triplicate and were of statistical meaning. Error bars and p values are reported in text and figures.

Results

Fluorimetric inhibitory assay on catalytic domains of MMP-2 and MMP-9.

We first tested the inhibitory activity of PES_103 on MMPs. This inhibitor belongs to the family of the aryl sulphonamide-containing hydroxamic acids and it was specifically designed and developed as a water-soluble molecule with nanomolar affinity for several MMPs [21].

The inhibition constants (K_i) of PES_103 towards the catalytic domains of MMP-2 and MMP-9 were determined by evaluating its ability to prevent the hydrolysis of the fluorescent-quenched peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2. The fluorescence ($excitation_{max}$ 328 nm; $emission_{max}$ 393 nm) was measured for 5 min. after the addition of the substrate [23]. Fitting of rates as a function of inhibitor concentration provided for PES_103 a K_i value of 14 nM towards MMP-9 and of 5 nM towards MMP-2.

Inhibitory assays on the supernatant of HT1080 cells.

To evaluate PES_103 inhibitory activity towards MMP-2 and MMP-9 in their native form, we added increasing concentrations of PES_103 to the medium conditioned by HT1080 cells, which is enriched of both MMP-2 and MMP-9 [22] and evaluated the MMPs inhibition by means of gelatine zymography [24]. The gelatin zymogram of HT1080 culture supernatant is presented in fig. 2. The supernatant of untreated HT1080 cells clearly showed three bands of molecular weights of 92, 82 and 72 kDa (fig. 2A) that correspond to the pro-MMP-9, the active MMP-9, the pro-MMP-2 and the active MMP-2, respectively, while the fused band at 72 kDa included both the pro- and the active MMP-2 [25]. When the supernatant of HT180 cells was incubated with increasing concentrations of PES_103, the intensity of the spots corresponding to the pro- and active MMP decreased in a dose-dependent manner (fig. 2B), thus confirming the inhibitory activity of PES_103 also against native MMP-2 and MMP-9.

Monitoring MMP-2 and MMP-9 levels in the tear fluid after DES induction.

The cholinergic receptors blockade is a reliable animal model of reduced lacrimation (ERL) for studying DES and, hence, for investigating the efficacy of MMPs inhibitors against ocular dryness [26–28]. ERL was induced in C57BL/6 mice by transdermal scopolamine patch (SCP). Following what was described by Dursun *et al.* [28], we expected scopolamine to produce a significant decrease in tear production followed by eye dryness. Moreover, as it has been reported that, in such conditions, MMP-2 and MMP-9 levels increase significantly [29], the ERL animal model was selected as the most suitable for evaluating the efficacy of a MMP inhibitor. Firstly, we tested the capability of SCP to induce eye dryness, as well as to promote the over-expression of active MMP-9. In detail, eye dryness was verified by measuring tear production with phenol red–impregnated cotton threads, while active MMP-9 production was checked by gelatine zymography on the collected tear samples. Our results show that the ocular dryness occurred after 24 hr from the application of transdermal SCP (fig. 3). The tear volume remained at low levels for 54 hr,

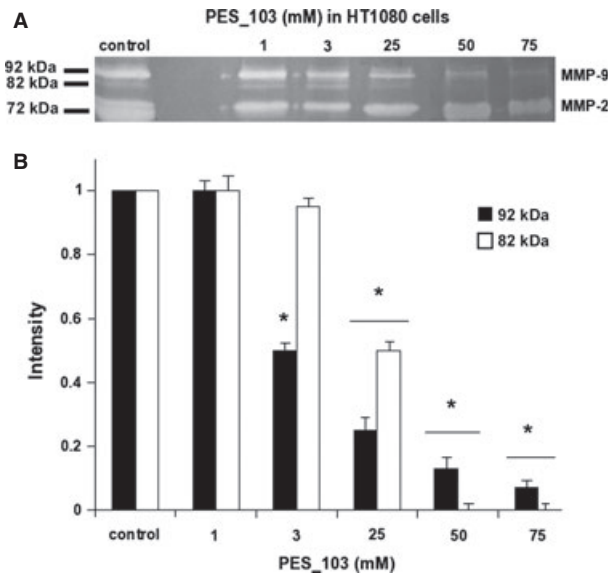

Fig. 2. Inhibition of MMP-2 and MMP-9 by PES_103. PES_103 inhibits both pro- and active MMP-9. Gelatin zymogram, performed on equal volumes of medium conditioned by HT1080 cells in presence of increasing concentrations of PES_103 shows the dose-dependent decrease in MMP-9 levels. The concentration of PES_103 added in the cell culture is reported (A). The dose-dependent decrease in MMP-9 (B). Experiments were performed in triplicate. *MMP-2 and MMP-9: Significantly different compared with control ($p < 0.05$).

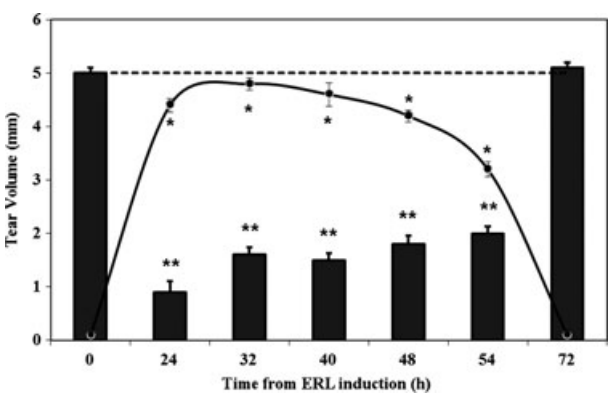

Fig. 3. Direct relationship between MMP-9 over-expression and eye dryness. Tear reduction and a significant MMP-9 over-expression ($p < 0.05$) occurred 24 hr after scopolamine patch (SCP) administration. The low levels of tear production and the high levels of MMP-9 are observable until 48 hr after the SCP treatment ($p < 0.05$) and then restored to the physiological levels. Tear volume measured in control mice is shown as a dotted line, while that measured in experimental reduced lacrimation mice is shown as black histograms. The black line shows the level of active MMP-9 detected in tear fluids. Experiments were repeated four times. *MMP-9: significantly different compared with control ($p < 0.05$). **Tear production: significantly different compared with control ($p < 0.05$).

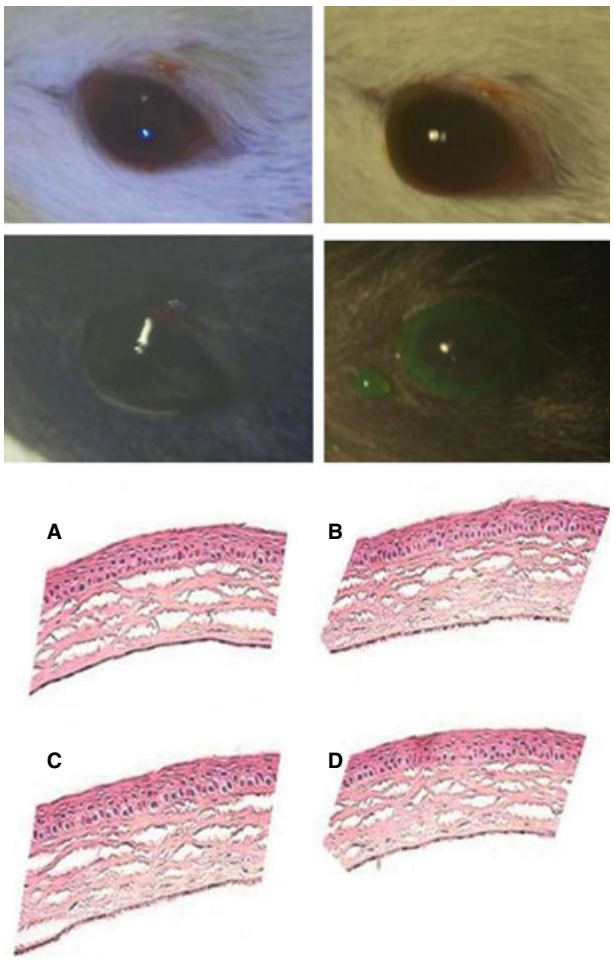

Fig. 4. Corneal toxicity and histological analysis. PES_103 solution at 1% concentration showed no significant ocular toxicity after application in both eyes, four times a day for five consecutive days. Top panel left column: eye of a BALB/c (top) and a C57BL/6 (bottom) mouse viewed and photographed under cobalt blue light 10 min. after the application of 1 µl sodium fluorescein 1% solution to the ocular surface. Right column: eye of a BALB/c (top) and a C57BL/6 (bottom) mouse after the application of 1 µl of Lissamine green standard solution. Bottom panel: histological samples of cornea after treatment with (A) saline solution; PES_103 0.01%; (B) PES_103 0.1%; and (C) PES_103 1%. Neither ulceration nor keratinization processes were demonstrated at the tested concentrations. No Lissamine staining in conjunctiva was observed.

confirming the effective occurrence of a reduced lacrimation condition. After this time, the tear volume quickly recovered to the physiological level as observed in control mice (dotted line in fig. 3). The results of zymography are reported in fig. 3 as a continuous line. The zymogram showed that significantly high levels of active MMPs in the tear fluid could be detected starting from 16 hr after SCP administration. After 24 hr, a significant MMP-9 over-expression could be observed, whereas MMP-2 levels were undetectable. The MMP-9 expression remained considerably high at least until 48 hr after SCP administration.

In summary, both reduced lacrimation and MMP-9 over-expression occurred approximately 24 hr after the treatment of mice by SCP patches. After 48 hr, both MMP-9 levels and tear production returned to their physiological levels, as observed in the control mice group. This suggests a direct relationship between MMP-9 over-expression and eye dryness.

We then decided to test the *in vivo* efficacy of PES_103, using the above-described ERL model. In particular, since 24 hr was the time required to induce eye dryness and to allow the detection of MMP-9 at the corneal level by gelatine zymography, both administration of PES_103 and sample collection for tear volume measurements were started after 24 hr of the SCP administration.

In vivo toxicity.

Toxicity of three different PES_103 concentrations (0.01, 0.1 and 1%) on the mouse ocular surface was evaluated by means of two different *in vivo* methods: (i) application of Fluorescein eye-drop and analysis of the ocular surface under UV lamp light; (ii) application of Lissamine green eye-drop and further corneal analysis. Neither ulceration nor keratinization processes were demonstrated by both methods (fig. 4). Moreover, no staining was observed in the conjunctiva of treated mice, after instillation of Lissamine green (fig. 4, right-hand panels).

Microscopy analysis performed on thin slices of eye balls after staining with hematoxylin and eosin revealed that all the tested concentrations did not induce alterations of the ocular surface morphology. The corneal epithelium turned out to be well stratified and differentiated, with four- to five-cell layers. Moreover, the number of apoptotic cells measured by TUNEL assay was almost identical in all the groups (table 1). Toxicity experiments provided the same response for both C57BL/6 and BALB/c mice. However, as C57BL/6 mice have been reported to develop a more severe keratoconjunctivitis than BALB/c mice after treatment with SCP [30], we selected C57BL/6 mice for evaluating the efficacy of PES_103 on the ERL model. In conclusion, the *in vivo* tests revealed the absence of ocular toxicity at the tested concentrations.

Evaluation of PES_103 efficacy in vivo on the ERL model.

In a preliminary dose-finding study, where the efficacy of PES_103 water solutions at 0.01, 0.1 and 1% concentration was compared on the selected animal model (fig. 5), the 0.1% concentration was found as the dosage producing the highest tear production. Hence, 0.1% PES_103 was selected for further experiments.

To evaluate PES_103 efficacy, we induced ERL by applying SCP patches, as described previously. 16 C-57BL/6 mice were equally divided into five groups i) control; ii) ERL; iii) ERL plus control vehicle; iv) ERL plus PES_103 0.1%; v) ERL plus commercially available artificial tears (Carbopol974 – Trade Name SiccaFluid®) [31,32]. The eyes of each group were selected so as to avoid both eyes of a mouse being assigned to the same treatment group, with the only exception of the control group. Immediately before ERL induction, tear production was measured with phenol red-impregnated cotton threads [33]. According to the MMP-9 expression profile

Table 1.

PES_103 toxicity. The histological analysis performed using TUNEL assay shows no significant difference in the number of apoptotic cells between the control and the experimental groups. The mean number of apoptotic cells between experimental and control groups is approximately the same in all groups, and no statistical difference was found amongst them. These data confirm the absence of damage on the corneal surface after PES_103 administration.

	Control	0.01%	*p*-value	0.1%	*p*-value	1%	*p*-value
BALB/c	15 ± 2	14 ± 3	$p = 0.87$	15 ± 3	$p = 0.58$	18 ± 3	$p = 0.98$
C57BL/6	16 ± 3	15 ± 4	$p = 0.96$	16 ± 5	$p = 0.79$	16 ± 4	$p = 0.64$

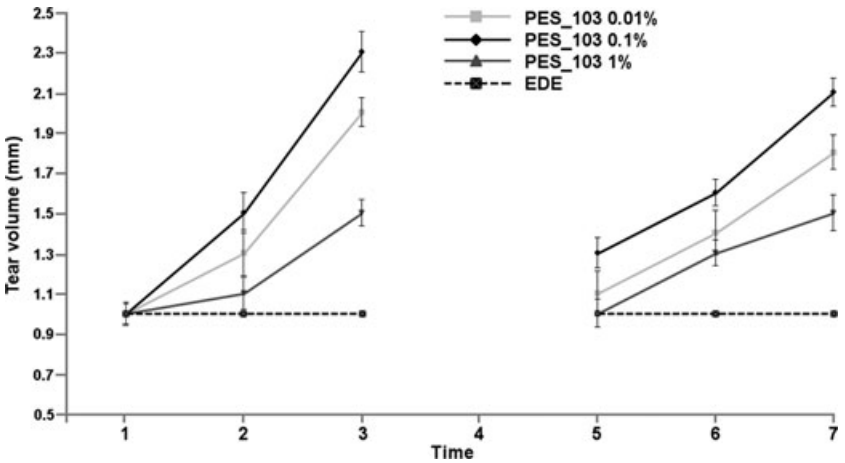

Fig. 5. Evaluation of PES_103 efficacy *in vivo*. Dose-finding experiments showed a statistically significant increase ($p < 0.05$) of the tear volume after treatment of experimental reduced lacrimation mice with each PES_103 dosage, namely 0.01, 0.1 and 1%. We observed the highest tear production using the 0.1% concentration of PES_103. Experiments were performed in triplicate.

reported in fig. 3, the vehicle, PES_103 and artificial tears were administered to groups iii), iv) and v), respectively, 24 hr after ERL induction. Administration of PES_103, artificial tears and the control vehicle was carried out twice a day, in a morning and an afternoon session. Each administration was preceded by the measurement of tear fluids (time T0), followed by two subsequent measurements performed after 30 (T30) and 60 min. (T60), respectively. The same schedule was applied to groups i) and ii), which did not receive any topic administration of tested compounds. The results obtained in three independent experiments are reported in fig. 6, where the measured tear volumes are shown at the different times of measurement in the morning and afternoon session, respectively.

PES_103 0.1% water solution determined the highest production of tears in the animal model, restoring up to 65% of the physiological tear production, while treatment with the vehicle-control produced no significant effects on tear production. A relationship between PES_103 potency and active MMP-9 corneal levels can be observed, suggesting that the direct inhibition of MMP-9 might occur because of the local administration of PES_103. On the contrary, this relationship could not be observed for artificial tears, whose mechanism of action is not specific and, to the best of our knowledge, not related to MMP-9 inhibition or modulation. Therefore, the tear production observed after corneal administration of PES_103 could be ascribable to the direct inhibition of MMP-9.

Discussion

The over-expression of MMP-2 and MMP-9 in tear fluids has been observed in DES [4–6]. To investigate the possible

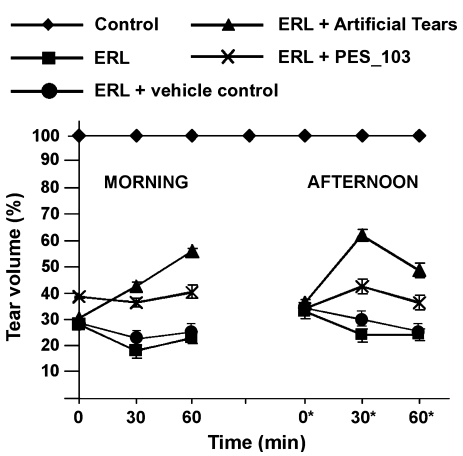

Fig. 6. Comparison between PES_103 and artificial tears. Plot of the tear volume (percentage with respect to control mice) over the time of the two measurements session. Experiments were repeated four times.

beneficial role of MMPs inhibition on ocular dryness, we designed an ERL animal model, consisting of C57BL/6 mice treated with transdermal scopolamine. In the animals, the ocular dryness occurred about 24 hr after SCP administration and the tear volume remained at low levels for 54 hr, confirming the effective occurrence of eye dryness. Significantly high levels of active MMPs in the tear fluid were detected starting from 16 hr, with a significant MMP-9 over-expression 24 hr after SCP administration. The over-expression was maintained until 48 hr after SCP treatment. On the contrary, MMP-2 was not detected in the tear fluids. This was a clear demonstration that a direct relationship exists between MMP-9 over-expression and eye dryness in the ERL model.

The same model was used to test the efficacy of PES_103 administration for the non-symptomatic treatment of DES. We first ruled out that different PES_103 concentrations (0.01, 0.1 and 1%) had any ocular toxic effects *in vivo,* as shown by Fluorescein and Lissamine green eye-drop application as well as by histological analysis. Then, we compared the effects of PES-103 and artificial tears *in vivo.* For this purpose, ERL was induced in C57BL/6 mice, PES_103 and artificial tears were topically applied 24 hr after the ERL induction, when eye dryness was present as well as MMP-9 was over-expressed at the corneal level. Administration of PES_103 or artificial tears into eyes was performed twice a day, in a morning and an afternoon session. In each session, eyes treated with PES_103 0.1% water solution always produced a tear volume 65% higher than the control group and statistically higher than all other ERL groups. Notably, tear production was higher in the PES_103-treated group compared with that treated with artificial tears.

In conclusion, PES_103 could represent a tool for studying *in vitro* and *in vivo* the effects of MMP-9 inhibition at the corneal levels, as well as a non-toxic valuable lead compound for the development of novel drug candidates for the non-symptomatic therapy of dry eye.

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