Published in final edited form as:

Ocul Surf. 2019 April ; 17(2): 257–264. doi:10.1016/j.jtos.2019.02.006.

Manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin, a superoxide dismutase mimetic, reduces disease severity in *in vitro* and *in vivo* models for dry-eye disease

Agn Žiniauskaitė, M.S.¹, Symantas Ragauskas, Ph.D.¹, Anita K. Ghosh, B.S.^{2,3}, Rubina Thapa, M.S.¹, Anne E. Roessler, B.S.⁴, Peter Koulen, Ph.D.⁵, Giedrius Kalesnykas, Ph.D.¹, Jenni J. Hakkarainen, Ph.D.¹, and Simon Kaja, Ph.D.^{1,2,3,4}

¹Experimentica Ltd., Kuopio, Finland ²Graduate Program in Neuroscience, Health Sciences Division, Loyola University Chicago, Maywood, IL, USA ³Departments of Ophthalmology and Molecular Pharmacology & Therapeutics, Stritch School of Medicine, Loyola University Chicago, Maywood, IL, USA ⁴K&P Scientific LLC, Oak Park, IL, USA ⁵Vision Research Center, Departments of Ophthalmology and Biomedical Sciences, School of Medicine, University of Missouri – Kansas City, Kansas City, MO, USA

Abstract

Purpose: To determine the efficacy of the superoxide dismutase mimetic, manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin (Mn-TM-2-PyP), *in vitro* in human corneal epithelial (HCE-T) cells and *in vivo* in a preclinical mouse model for dry-eye disease (DED).

Methods: *In vitro*, HCE-T cultures were exposed either to *tert*-butylhydroperoxide (*t*BHP) to generate oxidative stress or to hyperosmolar conditions modeling cellular stress during DED. Cells were pre-treated with Mn-TM-2-PyP or vehicle. Mn-TM-2-PyP permeability across stratified HCE-T cells was assayed, *In vivo*, Mn-TM-2-PyP (0.1% w/v in saline) was delivered topically as eye drops in a desiccating stress / scopolamine model for DED. Preclinical efficacy was compared to untreated, vehicle- and ophthalmic cyclosporine emulsion-treated mice.

Results: Mn-TM-2-PyP protected HCE-T cells in a dose-dependent manner against *t*BHP-induced oxidative stress as determined by calculating the IC₅₀ for *t*BHP in the resazurin, MTT and lactate dehydrogenase release cell viability assays. Mn-TM-2-PyP did not protect HCE-T cells

Corresponding author: Simon Kaja, Ph.D., Loyola University Chicago, Visual Neurobiology and Signal Transduction Laboratory, 2160 South First Avenue, Center for Translational Research and Education, Maywood, IL 60153, USA, Phone: +1 (708) 216-9223, Fax: +1 (708) 216-8318, skaja@luc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure/Conflicts of Interest:

Employment: AŽ, SR, RT, GK, JJH (Experimentica Ltd.); Stock/equity ownership: SR, GK, JJH, SK (Experimentica Ltd.); SK (K&P Scientific LLC); AKG (eyeNOS Inc.); Consulting: AKG, AER (K&P Scientific LLC); SK (Experimentica Ltd.); Board membership: AKG (eyeNOS Inc.). SK also conducts academic research in areas of interest similar to the business interests of Experimentica Ltd and K&P Scientific LLC. The terms of this arrangement have been reviewed and approved by Loyola University Chicago in accordance with its conflict of interest policy.

from hyperosmolar insult. Its permeability coefficient across a barrier of HCE-T cells was $1.1 \pm 0.05 \times 10^6$ cm/s and the mass balance was $62 \pm 0.6\%$. *In vivo*, topical dosing with Mn-TM-2-PyP resulted in a statistically significant reduction of corneal fluorescein staining, similar to ophthalmic cyclosporine emulsion. Furthermore, Mn-TM-2-PyP significantly reduced leukocyte infiltration into lacrimal glands and prevented degeneration of parenchymal tissue. No protective effect against loss of conjunctival goblet cells was observed. Notably, Mn-TM-2-PyP did not produce ocular toxicity when administered topically.

Discussion: Our data suggest that Mn-TM-2-PyP, a prototypic synthetic metalloporphyrin compound with potent catalytic antioxidant activity, can improve signs of DED *in vivo* by reducing oxidative stress in corneal epithelial cells.

Keywords

antioxidant; cornea; corneal epithelial cells; dry eye disease; keratoconjunctivitis sicca; lacrimal gland; manganese porphyrin; oxidative stress

1. Introduction

Dry-eye disease (DED) represents a common ophthalmic condition worldwide with prevalence estimates ranging from 5 – 50% [1]. DED is considered a multifactorial disease characterized by tear fluid dyshomeostasis and ocular surface inflammation [2], manifesting as discomfort and visual disturbance. DED is a chronic disease that creates a significant socioeconomic burden as a result of health care costs and workplace productivity in addition to lowering quality of life of affected individuals [1].

Inflammation is critically implicated in the pathogenesis of DED and contributes to the progression of signs and symptoms associated with DED [3]. Two prescription drugs have been approved in the United States: 0.05% cyclosporine ophthalmic emulsion (formulated as Restasis®; “indicated to increase tear production in patients whose tear production is presumed to be suppressed due to ocular inflammation associated with keratoconjunctivitis sicca” [4, 5]) and 5% lifitegrast ophthalmic solution (formulated as Xiidra®; indicated “for the treatment of the signs and symptoms of dry eye disease” [6, 7]). Both drugs target the inflammatory component of the disease, but are associated with a significant incidence of adverse effects in up to 25% of patients [8, 9], highlighting the need for novel safe and well-tolerated therapeutic approaches.

Clinical and translational studies have proposed that oxidative stress in ocular tissues of the anterior segment is a major contributor to the exacerbation of DED symptoms. For example, levels of oxidative stress were significantly higher in the conjunctival epithelium of DED patients compared with controls [10], and late lipid peroxidation markers in the tear film correlated with disease severity [11]. Furthermore, inducing mitochondrial oxidative stress in mice resulted in DED-like pathology [12]. Lastly, *in vitro* studies support the notion that hyperosmolar conditions can generate oxidative stress in corneal epithelial cells, which may exacerbate ocular surface damage [13]. However, despite the apparent role of oxidative stress in DED, only few studies have tested the efficacy of antioxidants in preclinical models for non-autoimmune DED [14–17]. Herein, we tested the efficacy of manganese(III) tetrakis

(1-methyl-4-pyridyl)porphyrin (Mn-TM-2-PyP) in the desiccating stress/ scopolamine murine model for DED.

Manganese-porphyrins belong to the metalloporphyrin group and possess broad antioxidant specificity, which includes scavenging O_2^- , H_2O_2 , $ONOO^-$, $NO^\cdot$, and lipid peroxy radicals. They are well characterized and proposed to offer protection in a variety of oxidative stress injuries such as stroke, diabetes, radiation injury and ischemia [18–23]. Furthermore, a detailed non-clinical safety assessment of the structurally related manganese (III) meso- tetrakis(N-ethylpyridinium-2-yl)porphyrin in mice and monkeys provided a favorable safety profile following intravenous injection that was not associated with any specific target organ toxicity [24]. Given their ready solubility in aqueous buffers [24, 25], manganese porphyrins are well-suited for topical ocular formulations.

2. Methods

2.1. Tissue culture

Human corneal epithelial (HCE-T) cells [26] were obtained under Material Transfer Agreement from RIKEN Research Institute (Tokyo, Japan) and cultured according to the provider's instructions as described by us previously [27]. Cells were maintained in standard tissue culture flasks (Techno Plastic Products, MidSci, St. Louis, MO) in DMEM:F12 media (Thermo Fisher Scientific, Waltham, MA) supplemented with 5 μ g/ml insulin (Millipore Sigma, St. Louis, MO), 100 U/ml penicillin - 100 μ g/ml streptomycin, 10 ng/ml human recombinant epidermal growth factor, 10 ng/ml human epithelial growth factor (all from Thermo Fisher Scientific), 5% fetal bovine serum (Gemini Bio Products, West Sacramento, CA) and 0.5% dimethylsulfoxide (Millipore Sigma).

2.2. Induction of oxidative stress and hyperosmolar conditions

HCE-T were seeded in 96-well plates at 50,000 cells/cm² and grown for 72 hr. Oxidative stress was chemically induced by incubation with increasing concentrations (1 μ M - 30 mM) of tert-butylhydroperoxide (tBHP) for 4 hr. Cells were pre-treated for overnight with either Mn-TM-2-PyP (0.0001% - 0.5% for cytotoxicity analysis and 0.001% - 0.05% w/v for cytoprotection assays) or vehicle (growth media).

Hyperosmolar conditions were induced by supplementation of the growth media with NaCl (5 – 150 mM), as described previously [13]. Osmolarity of the media was confirmed using a vapor pressure osmometer (Vapro®, Wescor, Logan, UT).

2.3. Cell viability assays

The resazurin cell viability assay was performed as described by us previously for HCE-T cells [27]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and lactate dehydrogenase (LDH) release assays were conducted essentially as described by us for other ocular cells types [28, 29].

2.4. Permeability assay

Permeation studies were conducted by seeding HCE-T cells at a density of 100,000 cells on 12 mm Snapwell inserts with 0.4 µm pore size polycarbonate membrane (Costar®, Corning Inc., Corning, NY). Cells were grown for one week and subsequently stratified in an air-liquid interface for three weeks [30]. Successful stratification was assessed by staining against the tight junction markers, zona occludens 1 and quantification of the apparent permeability coefficient (P_{app}) for permeability standards 6-carboxyfluorescein and rhodamine B (data not shown). Stratified HCE-T cell layers were washed using buffer solution (BSS Plus®, Alcon, Hänenberg, Switzerland). The permeation experiments were conducted in orbital shaker at 35 °C. The solution containing 0.05 % Mn-TM-2-PyP was placed in the donor chamber and blank buffer was added in the receiver chamber. Samples were taken from the receiver side and the donor side at 10, 20, 30, 45, 60, 75, 90, 105, 120, 150 and 180 min and replaced with an equal volume of fresh buffer. Absorbance of the samples were measured at 460 nm using Cytation 3 multi-mode reader (BioTek Instruments, Winooski, VT, USA). The rate (the apparent permeability coefficient, P_{app} , cm/s) was calculated according to the following equation:

$$P_{app} = \frac{\Delta Q_r / \Delta t}{A \times C_d} \quad \text{Equation 1,}$$

where Q_r / t is the slope of the linear region of the cumulative amount of the Mn-TM-2-PyP in the receiver chamber versus time plot, and where C_d is the average concentration of the Mn-TM-2-PyP in the donor chamber during the experiment, while A is the surface area (1.12 cm²). The mass balance (%) was calculated and defined as the sum of the Mn-TM-2-PyP in the receiver chamber (the amounts of the samples withdrawn at different time points) at 180 min, and the Mn-TM-2-PyP remaining in the donor chamber at 180 min, divided by the initial donor amount. P_{app} values and mass balances are presented as mean ± standard error of mean (SEM).

2.5. Animals

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the EC Directive 86/609/EEC for animal experiments, using protocols approved and monitored by the Animal Experiment Board of Finland. C57BL/6J mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and housed at a constant temperature (22 ± 1 °C) and in a light-controlled environment (lights on from 7 am to 7 pm) with *ad libitum* access to food and water. Male mice (8 – 10 weeks of age) were used for experiments.

2.6. Induction of chronic experimental DED and drug administration

DED was induced using a combination of desiccating environment (5%-15% humidity and 15 L/min airflow; SiccaSystem™; K&P Scientific LLC, Oak Park, IL) and concurrent transdermal scopolamine administration for a period of two weeks. Mn-TM-2-PyP (0.1% dissolved in physiological saline) was administered topically three times daily as eye drops (10 µl into the conjunctival sac using a P20 micropipettor) for the entire two-week induction

period. Preclinical efficacy of Mn-TM-2-PyP was compared against saline (administered topically three times daily) and against twice daily topical administration of 0.05% cyclosporine ophthalmic emulsion (Restasis; Allergan Inc., Irvine, CA).

2.7. Quantification of tear volume

Tear volume quantification was performed using a sterile phenol red-soaked cotton thread (ZoneQuick™) that was applied in the lateral canthus for a duration of 30 s, using forceps. The wetting length of the thread was read by an examiner blinded for treatment group under a microscope and estimated using a ruler. Resolution of the measurements was 0.5 mm. Tear volume was measured in all groups, at baseline and at the end of the two-week follow-up time.

2.8. Quantification of ocular surface inflammation

To quantify ocular surface damage, we scored corneal fluorescein staining, essentially as described by us previously [31].

2.9. Tissue collection and histology

Mice were euthanized by inducing a deep plane of general anesthesia by intraperitoneal administration of a cocktail of 75 mg/kg ketamine and 1 mg/kg xylazine. Eyes and intraorbital lacrimal glands were dissected and post-fixed overnight in 4% paraformaldehyde. Paraffin sections (5 µm) of lacrimal glands and frozen sections (10 µm) of eyeballs were processed for histological analysis using hematoxylin-eosin staining for quantification of immune cell infiltration into the lacrimal gland and Periodic Acid Schiff (PAS) staining to quantify the number of goblet cells in the inferior conjunctiva, as described by us in detail previously [32].

2.10. Corneal 8-hydroxy-2'-deoxyguanosine (8-OHdG) staining

Cryosections of cornea were labeled with an anti-8-OHdG antibody (clone N45.1, 1:200 dilution, Japan Institute for the Control of Aging, NIKKEN SEIL Co., Ltd., Shizuoka, Japan). Briefly, antigen retrieval was performed using a combination of trypsin (0.1%) and denaturing of nuclear DNA by incubating sections in 2N HCl for 30 min at 37 °C followed by neutralization by immersing sections in 0.1 M borate buffer twice for 5 min each. Sections were incubated in primary antibody overnight at 4 °C. The next day, sections were washed three times for 10 min each with 10 mM phosphate-buffered saline (pH 7.4) and 8-OHdG was detected with Histostain-Plus IHC Kit (Thermo Fisher Scientific) and 3,3'-diaminobenzidine substrate (Liquid DAB Substrate Kit, Invitrogen, Thermo Fisher Scientific). Images were acquired using a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany). 8-OHdG immunoreactivity was quantified by measuring density of nuclei in the corneal epithelial layer. Density of eight randomly-selected nuclei in three regions of approx. 100 µm² was measured using Fiji ImageJ software [33]. Values were averaged to obtain the mean for that eye.

2.11. Statistics

Data are presented as mean $\pm$ standard error of mean (SEM) or as box and whisker plots showing medians, interquartile ranges, mean and min/max values with individual data points presented as symbols. Data were analyzed using either Kruskal-Wallis ANOVA (for non-parametric data) or One-Way ANOVA (for parametric data). Differences between groups were subsequently determined using either Dunn's, Dunnett's or Tukey's multiple comparisons tests as appropriate. Differences were considered statistically significant at the $P < 0.05$ level.

3. Results

3.1. Mn-TM-2-PyP protects HCE-T cells from oxidative insult in vitro

In order to determine the possible cytotoxicity of Mn-TM-2-PyP to corneal epithelial cells, we tested various concentrations of Mn-TM-2-PyP (0.0001% - 0.5% w/v in media) in the resazurin cell viability assay. Concentrations up to 0.01% did not show any significant loss of cell viability and the estimated LD₅₀ for Mn-TM-2-PyP was 0.19% (Fig. 1A). Next, we tested the ability of Mn-TM-2-PyP to protect HCE-T cells against chemically-induced oxidative stress using the resazurin assay. Fluorescence values were corrected for the fluorescence of the corresponding Mn-TM-2-PyP concentration in media. Mn-TM-2-PyP at concentrations between 0.001% and 0.05% exerted potent dose-dependent cytoprotection against α BHP insult ($n = 4$ Fig. 1B). The EC₅₀ values for α BHP were 102.6 μ M (control), 187.3 μ M (0.001%), 1.95 mM (0.005%) and 3.43 mM (0.05%), as determined by 4th parameter non-linear curve fitting of the log-dose vs. response curve (Fig. 1B). Given the absorbance of fluorescent light by Mn-TM-2-PyP, we validated our results in the MTT cell viability and proliferation assay (Fig. 1C). We observed similar cytoprotection by Mn-TM-2-PyP with EC₅₀ values for α BHP of 72.8 μ M (control) and 2.03 mM (0.005%). Similarly, Mn-TM-2-PyP completely prevented LDH release from HCE-T cells in response to α BHP treatment (Fig. 1D).

In contrast, Mn-TM-2-PyP (0.05%) did not protect HCE-T cells from hyperosmolar insult. NaCl concentrations of 5 – 150 mM (equivalent to 350 – 1,250 mOsm) resulted in a dose-dependent loss of cell viability of up to 25%, which was not affected by pre-treatment with Mn-TM-2-PyP (Fig. 1E).

3.2. Mn-TM-2-PyP shows significant uptake into corneal epithelial cells

Permeation across the stratified human corneal epithelial barrier was assessed using a snapwell system. Mn-TM-2-PyP (0.05%) showed low permeability, with a P_{app} of $1.1 \pm 0.05 \times 10^{-6}$ cm/s as determined based on the cumulative amount of drug in the receiver chamber at multiple time points and a penetration of $3.9 \pm 0.1\%$ at 180 min (Fig. 2). The calculated mass balance was $62 \pm 0.6\%$, indicative of significant cellular uptake. In addition, a long lag time (28 ± 4 min) prior to steady-state flux, i.e. linear transport phase of Mn-TM-2-PyP across corneal epithelial cells further suggests intracellular accumulation.

3.3. Topical application of Mn-TM-2-PyP reduces corneal fluorescein staining

Next we tested the efficacy of Mn-TM-2-PyP in a preclinical mouse model for DED. Tear volume in mice exposed to the desiccating environment/ scopolamine induction paradigm for 14 days showed significantly lower tear volumes compared to naive mice (3.5 ± 0.5 mm vs. 5.8 ± 0.7 mm; $n = 9$; $P < 0.05$), suggestive of successful disease induction. Dosing with saline (4.3 ± 0.5 mm), Mn-TM-2-PyP (4.1 ± 0.5 mm) or cyclosporine (4.6 ± 0.9 mm) did not result in a statistically significant increase in tear volumes at the end of the two-week study period ($n = 9 - 10$; ANOVA $P = 0.16$; Fig. 3A).

However, three times daily topical administration of 0.1% Mn-TM-2-PyP in saline statistically significantly attenuated corneal fluorescein staining (Non-parametric Kruskal Wallis ANOVA $P < 0.001$; Fig. 3B-C). Mn-TM-2-PyP reduced the median fluorescein score (median score: 2; $n = 29$ eyes) compared with untreated (median score: 3, $n = 27$ eyes, $P < 0.05$) and saline-treated (median score: 3, $n = 17$ eyes, $P < 0.01$) eyes. While cyclosporine showed a trend toward lower fluorescein score after 2 weeks of treatment, this effect did not reach statistical significance ($n = 21$, $P = 0.29$ vs. untreated and $P = 0.09$ vs. saline-treated eyes).

3.4. Topical application of Mn-TM-2-PyP reduces oxidative DNA damage

In order to determine whether topical administration of Mn-TM-2-PyP reduces oxidative DNA damage, we quantified immunoreactivity of 8-OHdG in corneal epithelial cells. Untreated and saline-treated eyes showed significant nuclear 8-OHdG immunoreactivity, suggestive of oxidative DNA damage. Mn-TM-2-PyP-treated eyes showed a noticeable reduction in 8-OHdG staining, while immunoreactivity in cyclosporine-treated eyes was similar to untreated eyes (Fig. 4A). Quantification of nuclear immunoreactivity (density), revealed a statistically significant difference in 8-OHdG staining between treatment groups (One-Way ANOVA, $P < 0.05$). Post-hoc analysis using Tukey's multiple comparisons test revealed a statistically significant reduction in 8-OHdG immunoreactivity in Mn-TM-2-PyP-treated eyes ($n = 3$) compared with untreated ($n = 4$, $P < 0.05$; Fig. 4B) and saline-treated ($n = 4$, $P < 0.05$; Fig. 4B) eyes. 8-OHdG staining in cyclosporine-treated eyes was lower, but that difference did not reach statistical significance ($n = 4$, $P = 0.54$ vs. untreated eyes and $P = 0.45$ vs. saline-treated eyes; Fig. 4B).

3.5. Topical application of Mn-TM-2-PyP attenuates lacrimal gland pathology

DED is characterized by infiltration of immune cells into the lacrimal gland. To determine whether topical administration Mn-TM-2-PyP can attenuate lacrimal gland pathology, we scored immune cell infiltration as described by us previously [32]. Lacrimal glands showed a large number of foci of mononuclear cells, and in some cases, degeneration of parenchymal tissue resulting in a median score of 3 in saline-treated animals ($n = 8$; Fig. 5A, B). In contrast, Mn-TM-2-PyP reduced lacrimal gland pathology significantly by one score ($n = 15$; Kruskal-Wallis test with Dunn's multiple comparisons test, $P < 0.05$ compared with saline). The improvement by Mn-TM-2-PyP was slightly greater in magnitude as observed in cyclosporine-treated eyes ($n = 8$; $P = 0.08$ compared with saline, $P = 0.99$ compared with Mn-TM-2-PyP).

3.6. Topical Mn-TM-2-PyP treatment does not protect against loss of conjunctival goblet cells

Mucin-producing conjunctival goblet cells were identified using PAS staining on frozen sections of the inferior conjunctiva. Goblet cells and total conjunctival length were quantified. The number of goblet cells was 30.7 per mm conjunctiva in saline-treated eyes ($n = 5$), representing an approximate 50% reduction compared to naive animals (data not shown). There was no statistically significant difference in the number of goblet cells in Mn-TM-2-PyP-treated eyes (29.8 per mm conjunctiva, $n = 13$) and cyclosporine-treated eyes ($n = 8$; One-Way ANOVA $P = 0.99$; Fig. 5C, D).

4. Discussion

In the present study, we tested the efficacy of the prototypic manganese porphyrin antioxidant, Mn-TM-2-PyP in corneal epithelial cells *in vitro* and in a desiccating stress/scopolamine mouse model for DED.

Mn-TM-2-PyP exerted strong protection against oxidative stress in human corneal epithelial cells. The observed low permeability across corneal cells is likely a result of cellular uptake and/or binding to the epithelial barrier. Our data thus support the notion that Mn-TM-2-PyP can act as a potent cytoprotectant for the corneal epithelium. Furthermore, our data indicate that three times daily topical administration provides a significant therapeutic benefit against ocular surface alterations and lacrimal gland pathology *in vivo* in a DED model over untreated or vehicle-treated eyes. Notably, Mn-TM-2-PyP showed similar or mildly superior efficacy as 0.5% ophthalmic cyclosporine emulsion (Restasis), an FDA-approved topical immunomodulator indicated to increase tear production in patients with keratoconjunctivitis sicca.

In the present study, we used HCE-T cells to predict the toxicity to and the permeability of Mn-TM-2-PyP across the corneal epithelium. HCE-T cells have been widely used as *in vitro* model for the corneal epithelium as they form a stratified epithelium with barrier properties and a characteristic morphology (for review, see [34]). However, it must be noted that an altered genomic content suggestive of heterogeneous cellular origin has been described for HCE-T cells [35], which needs to be considered when interpreting our *in vitro* findings. Our mouse model for DED is based on a well-established paradigm that employs low-humidity air flow and concurrent scopolamine administration to induce DED in wild-type mice [36]. The model and its quantitative readouts used to determine DED severity were published by us previously [32] and are of similar magnitude to those reported by us and others using a desiccating stress/scopolamine model to induce DED in mice [32, 36].

The central role of oxidative stress in ocular surface disease, which has been demonstrated in preclinical and clinical studies, is the basis for the scientific premise of utilizing antioxidants as therapeutics in DED. In aqueous-deficient DED, lacrimal gland dysfunction alters the composition of the tear film causing hyperosmolarity [3]. Hyperosmolar conditions on the corneal epithelium facilitate the generation of oxidative stress by disturbing the cellular balance of generation and elimination of Reactive Oxygen Species, as previously demonstrated in human corneal epithelial cells [13]. Elevated levels of oxidative stress are

considered an important contributor to ocular surface inflammation through activation of nuclear factor- κ B (NF- κ B) by increased Reactive Oxygen Species [37]. NF- κ B controls the expression of components of the antioxidant system and is also a mediator of pro-inflammatory signaling through toll-like receptors, such as TLR4, which by itself has been implicated in corneal surface inflammation [38]. Elevated levels of oxidative stress can thus cause apoptosis and damage to the corneal epithelium and facilitate pro-inflammatory signaling [37]. We show the presence of oxidative stress-induced DNA damage in the desiccating stress/ scopolamine model for DED (Fig. 4A, B), which renders support to studying the preclinical efficacy of topical antioxidants for the treatment of DED in this model. Topical treatment with Mn-TM-2-PyP resulted in a statistically significant reduction of corneal oxidative stress-induced DNA damage (Fig. 4A, B). This finding supports our *in vitro* data from permeability studies that suggest significant cellular uptake of Mn-TM-2-PyP into corneal epithelial cells (Fig. 2). Notably, the effect of manganese porphyrins in preventing NF- κ B up-regulation in response to oxidative stress has been well documented [39]. Furthermore, topically applied manganese porphyrins showed potent anti-inflammatory effects in a mouse model of allergic dermatitis [25].

Topical antioxidant treatment using Mn-TM-2-PyP resulted in an attenuated lacrimal gland pathology with a significantly lower number of foci of infiltrating immune cells (Fig. 5). It is well known that mitochondrial oxidative stress in the lacrimal gland can induce lacrimal dysfunction. Specifically, deletion of the *mev-1* gene that encodes the succinate dehydrogenase cytochrome b protein resulting in a defect in complex II of the mitochondrial electron transport chain resulted in increased generation of reactive oxygen species in the lacrimal gland and an ocular phenotype reminiscent of dry-eye disease, including reduced tear volumes, increased corneal fluorescein staining, and infiltration of immune cells into the lacrimal gland [12, 40].

Ongoing studies are investigating the extent by which topical administration of Mn-TM-2-PyP can alter the expression of genes and proteins associated with elevated tissue levels of oxidative stress in DED. Data from these studies will provide additional mechanistic insight needed for the translation of antioxidant treatments of DED to the clinic.

In this study, we also compared the efficacy of Mn-TM-2-PyP against 0.05% ophthalmic cyclosporine emulsion (Restasis), which is FDA-approved and the current clinical standard of care for patients with moderate to severe DED [4, 8]. When comparing three times daily topical administration of Mn-TM-2-PyP to twice daily administration of cyclosporine, Mn-TM-2-PyP showed similar or better efficacy in reducing corneal surface inflammation (Fig. 3B, C) and partially preventing the infiltration of immune cells into the lacrimal gland (Fig. 5A, B). Notably, Mn-TM-2-PyP reduced oxidative DNA damage in corneal epithelial cells, while cyclosporine did not show any effect (Fig. 4A, B). Neither drug was able to increase the number of conjunctival goblet cells (Fig. 5C, D), in agreement with previous reports [32].

Manganese porphyrins have an established preclinical safety profile [24] and are currently used in early stage clinical studies administered subcutaneously as adjunctive therapy concurrent with radiation therapy (Clinical trials #NCT03386500 and # NCT02655601).

Together with their solubility and stability in aqueous buffers and formulations, manganese porphyrins, including Mn-TM-2-PyP, have the potential for a rapid translation to the clinic. Precedent for the use of topical antioxidants was recently shown by encouraging results of the mitochondrially-targeted antioxidant, SkQ1, in a Phase 2 Safety and Efficacy Trial [41].

5. Conclusions

Topical administration of the superoxide dismutase mimetic, Mn-TM-2-PyP, showed efficacy against corneal surface inflammation, infiltration of immune cells into the lacrimal gland and degeneration of parenchymal tissue in a preclinical model for DED. Efficacy was similar or better than ophthalmic cyclosporine emulsion, which is the current clinical standard of care. Given their chemical and safety profile, manganese porphyrins show great potential for translation to the clinic.

Acknowledgements:

This study was conducted with support from a Fight for Sight Grant-in-Aid (SK), the Illinois Society for the Prevention of Blindness (SK, AKG), the Dr. John P. and Therese E. Mulcahy Endowed Professorship in Ophthalmology (SK), and the Richard A. Perritt M.D. Charitable Foundation. Additional support by the Felix and Carmen Sabates Missouri Endowed Chair in Vision Research, a Challenge Grant from Research to Prevent Blindness and the National Institutes of Health (EY015672; PK) is gratefully acknowledged. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Additional support by Experimentica Ltd. and K&P Scientific LLC is gratefully acknowledged. The authors would like to thank Anne Mari Haapaniemi and Anni Tenhunen for excellent technical support.

Abbreviations:

ANOVA	analysis of variance
DED	dry eye disease
HCE-T	human corneal epithelial cells
KCS	Keratoconjunctivitis sicca
LDH	lactate dehydrogenase
Mn-TM-2-PyP	manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide
P_{app}	apparent permeability coefficient
S.D.	standard deviation
S.E.M.	standard error of the mean
tBHP	<i>tert</i> -butylhydroperoxide

References

1. Stapleton F, Alves M, Bunya VY, Jalbert I, Lekhanont K, Malet F, et al. TFOS DEWS II Epidemiology Report. *Ocul Surf* 2017;15:334–365 [PubMed: 28736337]

2. Craig JP, Nichols KK, Akpek EK, Caffery B, Dua HS, Joo CK, et al. TFOS DEWS II Definition and Classification Report. *Ocul Surf* 2017;15:276–283 [PubMed: 28736335]
3. Baudouin C, Messmer EM, Aragona P, Geerling G, Akova YA, Benitez-Del-Castillo J, et al. Revisiting the vicious circle of dry eye disease: a focus on the pathophysiology of meibomian gland dysfunction. *Br J Ophthalmol* 2016;100:300–6 [PubMed: 26781133]
4. Ames P and Galor A. Cyclosporine ophthalmic emulsions for the treatment of dry eye: a review of the clinical evidence. *Clin Investig (Lond)* 2015;5:267–285
5. Department of Health and Human Services, Food and Drug Administration. Restasis (cyclosporine ophthalmic emulsion) 0.05%, Application Number 21-023, Approval Letter, Rockville, MD 2000
6. Lifitegrast (Xiidra) for Dry Eye Disease. *JAMA* 2017;317:1473–1474 [PubMed: 28399249]
7. Department of Health and Human Services, Food and Drug Administration. Xiidra (lifitegrast ophthalmic solution) 5%, Application Number 208073, Approval Letter, Silver Spring, MD 2000
8. Jones L, Downie LE, Korb D, Benitez-Del-Castillo JM, Dana R, Deng SX, et al. TFOS DEWS II Management and Therapy Report. *Ocul Surf* 2017;15:575–628 [PubMed: 28736343]
9. Donnenfeld ED, Karpecki PM, Majmudar PA, Nichols KK, Raychaudhuri A, Roy M, et al. Safety of Lifitegrast Ophthalmic Solution 5.0% in Patients With Dry Eye Disease: A 1-Year, Multicenter, Randomized, Placebo-Controlled Study. *Cornea* 2016;35:741–8 [PubMed: 27055211]
10. Macri A, Scanarotti C, Bassi AM, Giuffrida S, Sangalli G, Traverso CE, et al. Evaluation of oxidative stress levels in the conjunctival epithelium of patients with or without dry eye, and dry eye patients treated with preservative-free hyaluronic acid 0.15 % and vitamin B12 eye drops. *Graefes Arch Clin Exp Ophthalmol* 2015;253:425–30 [PubMed: 25398660]
11. Choi W, Lian C, Ying L, Kim GE, You IC, Park SH, et al. Expression of Lipid Peroxidation Markers in the Tear Film and Ocular Surface of Patients with Non-Sjogren Syndrome: Potential Biomarkers for Dry Eye Disease. *Curr Eye Res* 2016;41:1143–9 [PubMed: 26731289]
12. Uchino Y, Kawakita T, Miyazawa M, Ishii T, Onouchi H, Yasuda K, et al. Oxidative stress induced inflammation initiates functional decline of tear production. *PLoS One* 2012;7:e45805 [PubMed: 23071526]
13. Deng R, Hua X, Li J, Chi W, Zhang Z, Lu F, et al. Oxidative stress markers induced by hyperosmolarity in primary human corneal epithelial cells. *PLoS One* 2015;10:e0126561 [PubMed: 26024535]
14. Choi W, Lee JB, Cui L, Li Y, Li Z, Choi JS, et al. Therapeutic Efficacy of Topically Applied Antioxidant Medicinal Plant Extracts in a Mouse Model of Experimental Dry Eye. *Oxid Med Cell Longev* 2016;2016:4727415 [PubMed: 27313829]
15. Lee HS, Choi JH, Cui L, Li Y, Yang JM, Yun JJ, et al. Anti-Inflammatory and Antioxidative Effects of *Camellia japonica* on Human Corneal Epithelial Cells and Experimental Dry Eye: In Vivo and In Vitro Study. *Invest Ophthalmol Vis Sci* 2017;58:1196–1207 [PubMed: 28245300]
16. Zheng Q, Ren Y, Reinach PS, She Y, Xiao B, Hua S, et al. Reactive oxygen species activated NLRP3 inflammasomes prime environment-induced murine dry eye. *Exp Eye Res* 2014;125:1–8 [PubMed: 24836981]
17. Oh HN, Kim CE, Lee JH, and Yang JW. Effects of Quercetin in a Mouse Model of Experimental Dry Eye. *Cornea* 2015;34:1130–6 [PubMed: 26203745]
18. Mackensen GB, Patel M, Sheng H, Calvi CL, Batinic-Haberle I, Day BJ, et al. Neuroprotection from delayed postischemic administration of a metalloporphyrin catalytic antioxidant. *J Neurosci* 2001;21:4582–92 [PubMed: 11425886]
19. Piganelli JD, Flores SC, Cruz C, Koepf J, Batinic-Haberle I, Crapo J, et al. A metalloporphyrin-based superoxide dismutase mimic inhibits adoptive transfer of autoimmune diabetes by a diabetogenic T-cell clone. *Diabetes* 2002;51:347–55 [PubMed: 11812741]
20. Vujaskovic Z, Batinic-Haberle I, Rabbani ZN, Feng QF, Kang SK, Spasojevic I, et al. A small molecular weight catalytic metalloporphyrin antioxidant with superoxide dismutase (SOD) mimetic properties protects lungs from radiation-induced injury. *Free Radic Biol Med* 2002;33:857–63 [PubMed: 12208373]
21. Bloodworth A, O'donnell VB, Batinic-Haberle I, Chumley PH, Hurt JB, Day BJ, et al. Manganese-porphyrin reactions with lipids and lipoproteins. *Free Radic Biol Med* 2000;28:1017–29 [PubMed: 10832063]

22. Ferrer-Sueta G, Batinic-Haberle I, Spasojevic I, Fridovich I, and Radi R. Catalytic scavenging of peroxynitrite by isomeric Mn(III) N-methylpyridylporphyrins in the presence of reductants. *Chem Res Toxicol* 1999;12:442–9 [PubMed: 10328755]
23. Day BJ, Batinic-Haberle I, and Crapo JD. Metalloporphyrins are potent inhibitors of lipid peroxidation. *Free Radic Biol Med* 1999;26:730–6 [PubMed: 10218663]
24. Gad SC, Sullivan DW Jr., Crapo JD, and Spainhour CB. A nonclinical safety assessment of MnTE-2-PyP, a manganese porphyrin. *Int J Toxicol* 2013;32:274–87 [PubMed: 23704100]
25. Stover K, Fukuyama T, Young AT, Daniele MA, Oberley R, Crapo JD, et al. Topically applied manganese-porphyrins BMX-001 and BMX-010 display a significant anti-inflammatory response in a mouse model of allergic dermatitis. *Arch Dermatol Res* 2016;308:711–721 [PubMed: 27709295]
26. Araki-Sasaki K, Ohashi Y, Sasabe T, Hayashi K, Watanabe H, Tano Y, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci* 1995;36:614–21 [PubMed: 7534282]
27. Hakkarainen JJ, Reinisalo M, Ragauskas S, Seppanen A, Kaja S, and Kalesnykas G. Acute cytotoxic effects of marketed ophthalmic formulations on human corneal epithelial cells. *Int J Pharm* 2016;511:73–78 [PubMed: 27374205]
28. Kaja S, Payne AJ, Naumchuk Y, Levy D, Zaidi DH, Altman AM, et al. Plate reader-based cell viability assays for glioprotection using primary rat optic nerve head astrocytes. *Exp Eye Res* 2015;138:159–66 [PubMed: 26048476]
29. Maciulaitiene R, Pakuliene G, Kaja S, Pauza DH, Kalesnykas G, and Januleviciene I. Glioprotection of Retinal Astrocytes After Intravitreal Administration of Memantine in the Mouse Optic Nerve Crush Model. *Med Sci Monit* 2017;23:1173–1179 [PubMed: 28265105]
30. Toropainen E, Ranta VP, Talvitie A, Suhonen P, and Urtti A. Culture model of human corneal epithelium for prediction of ocular drug absorption. *Invest Ophthalmol Vis Sci* 2001;42:2942–8 [PubMed: 11687540]
31. Lin Z, Liu X, Zhou T, Wang Y, Bai L, He H, et al. A mouse dry eye model induced by topical administration of benzalkonium chloride. *Mol Vis* 2011;17:257–64 [PubMed: 21283525]
32. Ziniauskaite A, Ragauskas S, Hakkarainen JJ, Rich CC, Baumgartner R, Kalesnykas G, et al. Efficacy of Trabodensin in a Mouse Keratoconjunctivitis Sicca (KCS) Model for Dry-Eye Syndrome. *Invest Ophthalmol Vis Sci* 2018;59:3088–3093 [PubMed: 30025146]
33. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012;9:676–82 [PubMed: 22743772]
34. Ronkko S, Vellonen KS, Jarvinen K, Toropainen E, and Urtti A. Human corneal cell culture models for drug toxicity studies. *Drug Deliv Transl Res* 2016;6:660–675 [PubMed: 27613190]
35. Yamasaki K, Kawasaki S, Young RD, Fukuoka H, Tanioka H, Nakatsukasa M, et al. Genomic aberrations and cellular heterogeneity in SV40-immortalized human corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2009;50:604–13 [PubMed: 18824731]
36. Dursun D, Wang M, Monroy D, Li DQ, Lokeshwar BL, Stern ME, et al. A mouse model of keratoconjunctivitis sicca. *Invest Ophthalmol Vis Sci* 2002;43:632–8 [PubMed: 11867577]
37. Lan W, Petznick A, Heryati S, Rifada M, and Tong L. Nuclear Factor-kappaB: central regulator in ocular surface inflammation and diseases. *Ocul Surf* 2012;10:137–48 [PubMed: 22814642]
38. Lee HS, Hattori T, Park EY, Stevenson W, Chauhan SK, and Dana R. Expression of toll-like receptor 4 contributes to corneal inflammation in experimental dry eye disease. *Invest Ophthalmol Vis Sci* 2012;53:5632–40 [PubMed: 22789921]
39. Batinic-Haberle I, Spasojevic I, Tse HM, Tovmasyan A, Rajic Z, St Clair DK, et al. Design of Mn porphyrins for treating oxidative stress injuries and their redox-based regulation of cellular transcriptional activities. *Amino Acids* 2012;42:95–113 [PubMed: 20473774]
40. Uchino Y, Kawakita T, Ishii T, Ishii N, and Tsubota K. A new mouse model of dry eye disease: oxidative stress affects functional decline in the lacrimal gland. *Cornea* 2012;31 Suppl 1:S63–7 [PubMed: 23038038]
41. Petrov A, Perekhvatova N, Skulachev M, Stein L, and Ousler G. SkQ1 Ophthalmic Solution for Dry Eye Treatment: Results of a Phase 2 Safety and Efficacy Clinical Study in the Environment

and During Challenge in the Controlled Adverse Environment Model. *Adv Ther* 2016;33:96–115
[PubMed: 26733410]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1: Mn-TM-2-PyP exerts potent cytoprotective effects against oxidative stress, but not hyperosmolar insult.

A. Cytotoxicity of HCE-T cells was assessed using the resazurin cell viability assay. Non-linear curve fitting using a four parameter (log)inhibitor vs. response curve yielded an estimated LD₅₀ for Mn-TM-2-PyP of 0.19% w/v. Concentrations of up to 0.01% did not show any significant cytotoxicity after exposure for 16 – 20 hr. **B.** One hour pre-treatment with Mn-TM-2-PyP exerted potent dose-dependent cytoprotection of HCE-T cells against tBHP-induced oxidative stress. Individual data points were fitted using non-linear 4th parameter algorithm. Individual data points represent mean ± S.E.M. from 5 different experiments. **C.** We confirmed the results from the resazurin cell viability assay using an MTT cell viability and proliferation assay. Estimated EC₅₀ values for tBHP were similar to those derived from the resazurin assay shown in B. Data are shown as mean ± S.E.M. from four separate experiments with eight technical replicates each. **D.** In a matched experiment, media supernatants from the same wells as from the MTT assay were used to quantify LDH release. Mn-TM-2-PyP completely prevented tBHP-induced LDH release from HCE-T cells. Data are shown as mean ± S.E.M. from four separate experiments with eight technical replicates each and fitted using a four-parameter algorithm. **E.** In contrast, Mn-TM-2-PyP did not exert any cytoprotective effects against hyperosmolarity-induced loss of cell viability (n = 4 separate experiments). Data were normalized against the 0 μM tBHP condition and are presented as individual data points representing the mean ± S.E.M. and were fitted using a sixth order polynomial equation.

Figure 2: Mn-TM-2-PyP shows significant uptake into corneal epithelial cell.

Permeation across the corneal epithelial barrier was assessed using a snapwell system. Samples were taken at indicated time points for up to three hours and the P_{app} for Mn-TM-2-PyP was calculated. The dotted line indicates the steady state flux, i.e. the linear transport phase of Mn-TM-2-PyP across corneal epithelial cells, and was determined by linear curve fitting, indicating a lag-time of 28 ± 4 min. The calculated mass balance was $62 \pm 1\%$, indicative of significant cellular uptake.

Figure 3: Topical administration of Mn-TM-2-PyP does not increase tear volume but reduces corneal fluorescein staining.

A. Tear volumes were significantly reduced in untreated mice exposed to the DED induction paradigm for two weeks compared with naive mice, indicative of successful induction of DED. No statistically significant differences between treatment arms were detected. **B.** Mn-TM-PyP was administered three times daily in a desiccating environment/ scopolamine mouse model for DED. Mice treated with Mn-TM-2-PyP showed significantly lower corneal fluorescein staining compared with untreated and saline-treated animals. The effect of Mn-TM-2-PyP on corneal fluorescein staining was similar or better than that of the FDA-approved reference compound, 0.5% ophthalmic cyclosporine emulsion (Restasis). Data are presented as box and whisker plots, indicating individual data points (circles), median (line), mean (+), interquartile ranges (box) and the min/max values (whiskers). * $P < 0.05$ compared with untreated; ## $P < 0.01$ compared with saline-treated. **C.** Representative images of corneal fluorescein staining are shown for each treatment group. Scale bar: 2 mm.

Figure 4: Topical application of Mn-TM-2-PyP reduces oxidative DNA damage.

A. Corneal sections were labeled for 8-OHdG as marker for oxidative stress-induced DNA damage. Strong immunoreactivity in untreated eyes is suggestive of significant DNA damage in animals exposed to the desiccating stress/ scopolamine model for DED. Nuclear 8-OHdG immunoreactivity was reduced in Mn-TM-2-PyP-treated eyes compared with both untreated and saline-treated eyes (One-Way ANOVA, $P < 0.05$; Tukey's multiple comparisons test, $P < 0.05$ compared with both untreated and saline-treated eyes). In comparison, topical cyclosporine treatment had no statistically significant effect on 8-OHdG staining ($P = 0.59$ compared with untreated and $P = 0.45$ compared with saline-treated). Data are presented as mean $\pm$ standard error of the mean, individual data points are depicted as circles. * $P < 0.05$ compared with untreated, # $P < 0.05$ compared with saline-treated. **B.** Representative grayscale images are shown. Note the lighter nuclear staining in Mn-TM-2-PyP-treated eyes, suggestive of reduced oxidative stress-induced DNA damage. Scale bar: 10 μ m.

Figure 5: Mn-TM-2-PyP reduced immune cell infiltration in the lacrimal gland.

A. Induction of DED resulted in significant infiltration of immune cells into the lacrimal gland in saline-treated animals. Mn-TM-2-PyP significantly reduced lacrimal gland pathology (Kruskal-Wallis test with Tukey's multiple comparisons test, $P < 0.05$). Cyclosporine-treated eyes showed a reduction in the number of infiltrates, however, the difference did not reach statistical significance ($P = 0.08$). Data are presented as box and whisker plots, indicating individual data points (circles), median (line), mean (+), interquartile ranges (box) and the min/max values (whiskers). * $P < 0.05$. **B.** Representative examples of H&E-stained lacrimal gland showing reduced number of infiltrates in both the Mn-TM-2-PyP- and cyclosporine-treated groups and showed a potent prevention of degeneration of parenchymal tissue. Scale bar: 200 μm . **C.** Mn-TM-2-PyP did not prevent loss of mucin-producing conjunctival goblet cells. The number of goblet cells was similar between saline-, Mn-TM-2-PyP- and cyclosporine-treated groups (One-Way ANOVA, $P = 0.99$). Data are presented as box and whisker plots, indicating individual data points (circles), median (line), mean (+), interquartile ranges (box) and the min/max values (whiskers). **D.** Representative examples of PAS staining on conjunctival cryosections. Scale bar: 50 μm .