A Structural and Functional Comparison Between Two Recombinant Human Lubricin Proteins: Recombinant Human Proteoglycan-4 (rhPRG4) vs ECF843

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Abstract (498 out of 500 words)

Proteoglycan 4 (PRG4, lubricin) is a mucin-like glycoprotein present on the ocular surface that has both boundary lubricating and anti-inflammatory properties. Full-length recombinant human PRG4 (rhPRG4) has been shown to be clinically effective in improving signs and symptoms of dry eye disease (DED). In vitro, rhPRG4 has been shown to reduce inflammation-induced cytokine production and NFκB activity in corneal epithelial cells, as well as to bind to and inhibit MMP-9 activity. A different form of recombinant human lubricin (ECF843), produced from the same cell line as rhPRG4 but manufactured using a different process, was recently assessed in a DED clinical trial. However, ECF843 did not significantly improve signs or symptoms of DED compared to vehicle. Initial published characterization of ECF843 showed it had a smaller hydrodynamic diameter and was less negatively charged than native PRG4. Further examination of the structural and functional properties of ECF843 and rhPRG4 could contribute to the understanding of what led to their disparate clinical efficacy. Therefore, the objective of this study was to characterize and compare rhPRG4 and ECF843 in vitro, both biophysically and functionally.

Hydrodynamic diameter and charge were measured by dynamic light scattering (DLS) and zeta potential, respectively. Size and molecular weight was determined for individual species by size exclusion chromatography (SEC) with in-line DLS and multi-angle light scattering (MALS). Bond structure was measured by Raman spectroscopy, and sedimentation properties were measured by analytical ultracentrifugation (AUC). Functionally, MMP-9 inhibition was measured using a commercial MMP-9 activity kit, coefficient of friction was measured using an established boundary lubrication test at a latex-glass interface, and collagen 1-binding ability was measured by quart crystal microbalance with dissipation (QCMD). Additionally, the ability of rhPRG4 and ECF843 to inhibit urate acid crystal formation and cell adhesion was assessed.

ECF843 had a significantly smaller hydrodynamic diameter and was less negatively charged, as assessed by DLS and zeta potential. Size was further explored with SEC-DLS-MALS, which indicated that while rhPRG4 had 3 main peaks, corresponding to monomer, dimer, and multimer as expected, ECF843 had 2 peaks that were similar in size and molecular weight compared to rhPRG4's monomer peak and a third peak that was significantly smaller in both size and molar mass than the corresponding peak of rhPRG4. Raman spectroscopy demonstrated that ECF843 had significantly more disulfide bonds, which are functionally determinant structures, relative to the carbon-carbon backbone compared to rhPRG4, and AUC indicated that ECF843 was more compact than rhPRG4. Functionally, ECF843 was significantly less effective at inhibiting MMP-9 activity and functioning as a boundary lubricant compared to rhPRG4, as well as being slower to bind to collagen 1. Additionally, ECF843 was significantly less effective at inhibiting urate acid crystal formation and at preventing cell adhesion.

Collectively, these data demonstrate ECF843 and rhPRG4 are significantly different in both structure and function. Given that a protein's structure sets the foundation for its interactions with other molecules and tissues in vivo, which ultimately determine its function, these differences most likely contributed to the disparate DED clinical trial results.

1.Introduction

Proteoglycan 4 (PRG4, also known as lubricin) is a mucin-like glycoprotein that is most well-known for its boundary lubricating ability on articular cartilage (Jay et al., 1998; Schmidt et al., 2007; Zappone et al., 2007). PRG4 has also been found on the surface of the eye (Schmidt et al., 2013), as well as in tears (Das et al., 2021), and lack of PRG4 expression in unchallenged mice results in increased ocular surface staining (Schmidt et al., 2013). In addition to its properties as a boundary lubricant (Ludwig et al., 2015) and cell anti-adhesive (Rhee et al., 2005), PRG4 has been shown to exhibit a variety of anti-inflammatory properties. A full length recombinant human PRG4 (rhPRG4), previously reported to have a higher order structure (existing as a monomer and disulfide bonded dimer) and glycosylation consistent with native PRG4 (Samsom et al., 2014), has been shown to bind to toll-like receptors in modified HEK cells (Alquraini et al., 2015), to reduce nuclear factor kappa B (NFκB) activity and pro-inflammatory cytokine expression in synoviocytes, and to inhibit fibroblast-like synoviocyte proliferation (Al-Sharif et al., 2015). Finally, rhPRG4 has previously been shown to inhibit formation and effects of monosodium urate (MSU) crystals (Elsaid et al., 2022; ElSayed et al., 2021), which are known to play a key role in the development of gout (Dalbeth & Haskard, 2005; ElSayed et al., 2021), which is another property of rhPRG4 that can be used to demonstrate functionality.

In the context of ocular diseases, endogenous ocular surface PRG4 has been shown to be diminished in a pre-clinical model of dry eye disease (DED) (Menon et al., 2021) as well as an in vitro co-culture model of evaporative DED (Seo et al., 2019). Tear levels of PRG4 have been reported to be decreased in Sjögren's Syndrome-associated DED (Das et al., 2021). Further, rhPRG4 has also been shown to reduce pro-inflammatory cytokine and chemokine secretion by human corneal epithelial cells, to bind to matrix metalloproteinase 9 (MMP-9), to inhibit MMP-9

activity (Menon et al., 2021), and to reduce inflammatory NFκB activity in these cells by altering expression of human leukocyte antigen-F adjacent transcript 10 (FAT10), a ubiquitin-like modifier that targets proteins for degradation (Menon et al., 2022). Given that PRG4 is expressed endogenously on the ocular surface and lack of endogenous expression results in increased basement membrane staining, along with PRG4 having both boundary lubricating and anti-inflammatory properties, these data suggest PRG4 plays an important role in maintaining the homeostasis of the ocular surface.

Clinically, rhPRG4 has shown encouraging performance in significantly reducing signs and symptoms of patients with moderate DED (Lambiase et al., 2017). In a small clinical trial (NCT02507934), rhPRG4 at 150 μg/ml (0.015%) was significantly more effective than 0.18% sodium hyaluronate (HA) eye drops at reducing subjective symptoms, including Visual Analogue Scale and Symptom Assessment in Dry Eye (SANDE) frequency (60.5 ±17.2 to 23.4 ± 18.6). Objective signs, including tear film break-up time (TFBUT) and corneal fluorescein staining (3.2 ± 0.9 to 1.8 ± 0.9 (right eye) & 3.2 ± 1.0 to 1.6 ± 0.8 (left eye)) were also reduced. In total, both the pre-clinical and early clinical data suggest PRG4 plays an important role in maintaining the homeostasis of the ocular surface, and that exogenous application should restore balance to the epithelium.

A recent clinical trial (NCT04391894) was conducted to assess the efficacy of a novel recombinant lubricin, ECF843, which was produced using the same cell line as rhPRG4, but publicly available data indicate it was purified and manufactured using an altogether different process (WO 2021/171165 A1). In this study, in contrast to that with rhPRG4, there were no statistically significant improvements in any of the outcomes measured comparing ECF843 to vehicle, which included change in SANDE scores (Δ = -1.8 ± 1.91 from a baseline of 65 ± 18.6)

and change in corneal fluorescein staining (Δ = -1.0 ± 0.31 compared to baseline of 6.2 ± 2.5) (Periman et al., 2022). The potential cause for the discrepancy in the clinical efficacy between ECF843 and rhPRG4 (i.e., ECF843 lacking efficacy) remains unknown and is the fundamental motivation for this study.

ECF843 has previously been characterized and studied to show its in vitro equilibrium wetting and adsorption properties (Rabiah et al., 2020), where it was reported to have a z-average hydrodynamic diameter of 60 nm. This is significantly smaller than the previously measured hydrodynamic diameter for native PRG4, 162 nm (Zappone et al., 2008). Rabiah et al. also measured the zeta potential to be approximately neutral (1.4 ± 2.1 mV), but native PRG4 has been reported to have a negative zeta potential (-25 to -40 mV) (Greene et al., 2016). However, the concentration of ions in buffers can significantly influence surface charge and zeta potential. Since the work conducted by Rabiah et al. was performed in phosphate buffered saline (PBS) with an electrolyte concentration of 150 mM, and the work conducted by Greene et al. was performed in an electrolyte concentration of 15 mM, it remains to be seen how surface charge compares between these molecules if they were in a similar buffer. Given the importance of protein structure on function, these apparent differences in structure and charge between ECF843 and native PRG4 would likely also contribute to significant differences in functionality between ECF843 and rhPRG4. However, these two recombinant human lubricin proteins have not been directly compared under similar conditions in a single study, and there are currently no peer-reviewed publications examining the hydrodynamic diameter or zeta potential of rhPRG4.

Therefore, motivated by the conflicting clinical efficiency in improving signs and symptoms of DED as well as the preliminary biophysical differences observed in different studies, the objective of this study was to characterize the structural and functional properties in vitro of

two different versions of recombinant human lubricin, rhPRG4 and ECF843~~, that demonstrated conflicting clinical efficiency in improving signs and symptoms of DED~~.

2. **Materials & Methods**

2.1. Materials – Recombinant Human Lubricin

rhPRG4 was created by purifying media from a Chinese hamster ovary (CHO) cell line transfected with the PRG4 gene (Samsom et al., 2014) and provided by Lubris BioPharma LLC (Naples, FL). ECF843, as described by (Rabiah et al., 2020), was produced using the same cell line but purified using a different process than that of rhPRG4. rhPRG4 stock ~~was~~ had a concentration of 1.33 mg/mL in PBS (10 mM sodium phosphate, 150 mM sodium chloride) + 0.01% Tween-20 (PBST), and ECF843 stock ~~was~~ had a concentration of 2 mg/mL in PBS (10 mM sodium phosphate, 140 mM sodium chloride) + 0.02 % Tween-20. Lubricin samples were >99% pure and had similar osmolarities.

2.2 Biophysical Characterization

2.2.1 Dynamic light scattering (DLS) & zeta potential

To assess the size and charge of rhPRG4 and ECF843, DLS and zeta potential measurements were made using a Zetasizer Nano-ZS90 (Malvern Panalytical). For DLS measurements, rhPRG4 and ECF843 were diluted to 1 mg/mL in PBST. 1 mL of each solution was loaded into a clear plastic cuvette (Fisher Scientific, Grand Island, NY) before being measured. Each sample was measured up to 50 times within each run, and runs were repeated three times on three separate days. Z-average hydrodynamic radii were determined by cumulant analysis, and intensity profiles were determined by distribution analysis.

For zeta potential measurements, since the concentration of ions in buffers can significantly influence surface charge and zeta potential, ECF843 and rhPRG4 were first diluted to 1 mg/mL in PBST before being buffer exchanged to MilliQ water using a 30 kDa filter (EMD Millipore) to a final concentration of 1 mg/mL. 800 µL of each sample was loaded into a zeta potential testing

cell (Malvern Panalytical) before being measured. Each sample was measured up to 20 times within each run, and runs were repeated three times on three separate days. The testing cell was cleaned with deionized (DI) water and 70% ethanol four times in between sample changes.

2.2.2 Size exclusion chromatography (SEC) with in-line multi-angle light scattering (MALS) & DLS

To assess the size of rhPRG4 and ECF843, SEC with inline MALS and DLS was conducted using a high pressure liquid chromatography (HPLC) equipped with in-line ultraviolet (UV) (1260 Infinity II HPLC with G1310B isocratic pump, G1329B autosampler, G1316A column compartment and G1365C multiple wavelength detector, Agilent Technologies), MALS (DAWN HELEOS-II multi angle light scattering detector, Wyatt Technology Corp) and DLS (Optilab T-rEX refractive index detectors, Wyatt Technology Corp). rhPRG4 and ECF843 were individually separated with a SEC column (Acquity UPLC Protein BEH SEC, 450A, 2.5 µm, 4.6 x 300mm; Waters) equilibrated in 20 mM sodium phosphate, 300 mM sodium chloride, pH 7.0. After column preconditioning, samples were injected at 10 µg and eluted with a flow rate of 0.25 mL/min (run time 20 min). Samples were run in technical triplicate. UV, MALS, DLS and refractive index data were collected and analyzed using ASTRA software (Wyatt Technology), and a dn/dc value of 0.160 mL/g was used for PRG4 (Steele et al., 2013).

2.2.3 Raman Spectroscopy

Raman spectroscopy is a technique that measures the composition of a material by exciting its surface with a laser and measuring changes in the scattered wavelength due to vibrations in its molecules (Petry et al., 2003). This can be used to identify specific bond structures in a molecule, acting like a molecular fingerprint. ~~Raman spectroscopy~~ This technique was performed using a WiTec Alpha 300 Raman Spectrometer (Oxford Instruments, Abingdon, UK). 2 µL drops of each

sample were placed on glass slides wrapped in aluminum foil. Using a WiTec Raman microscope with a 785 nm laser, 6 spectra were collected per sample using a 50x objective and an integration time of 30 x 1-second per spectra. The WiTec Project 5 software was used to deconvolute the ν_1 disulfide bonds (550 cm^{-1}) and ν_1 carbon-carbon backbone (987 cm^{-1}) peaks using a Lorentzian fit. The relative level of disulfide bonds was calculated from the ratio of the 550 cm^{-1} disulfide bond and the 987 cm^{-1} carbon-carbon bond peak areas. Values were averaged over all 6 spectra for each sample.

2.4 Analytical Ultracentrifugation (AUC)

AUC is a biophysical technique that investigates various macromolecular characteristics, including size, shape, stoichiometry, and binding properties, by monitoring a sample's sedimentation profile while it is ultracentrifuged at speeds as high as 60000 rpm (Edwards et al., 2020). ECF843 and rhPRG4 samples were diluted to 0.45 mg/mL in PBS + 0.02% Tween-20 and PBST, respectively, with these two buffers being used as controls. Sedimentation velocity analysis was conducted at 20 °C and 32,000 RPM using absorbance optics with a Beckman-Coulter Optima AUC analytical ultracentrifuge. Double sector cells equipped with quartz windows were used. The rotor was equilibrated under vacuum at 20 °C, and after an equilibration period of ~45 minutes, the rotor was accelerated to 32,000 RPM at this temperature. Absorbance scans at 230 nm were acquired at 20 seconds intervals for ~12 hours. Continuous sedimentation coefficient distributions for each sample were calculated using the program Sedfit (v16.36).

2.3 Functional Characterization - DED-associated

2.3.1 MMP-9 Inhibition

MMP-9 inhibitor testing kits (Abcam, Cambridge, MA) were used following manufacturer instructions. Briefly, MMP-9 enzyme, N-Isobutyl-N-(4-methoxyphenylsulfonyl) glycyl

hydroxamic acid (NNGH) inhibitor, rhPRG4, and ECF843 were warmed to 37°C for 15 minutes, and the enzyme was incubated with NNGH at 150 μg/mL, as well as rhPRG4 and ECF843 at 50 μg/mL and 150 μg/mL, for 1 hour. ECF843 and rhPRG4 were first diluted to 1 mg/mL in PBST. Additionally, PBST (no lubricin) was used as a negative control at the same volumes used for ECF843 and rhPRG4 samples at the tested concentrations, and the assay buffer used for all samples was 50mM HEPES, 10mM CaCl2, and 0.05% Brij-35, pH 7.5. Then, fluorogenic substrate was added, and fluorescence was measured using an excitation wavelength of 328 nm and an emission wavelength of 420 nm. All measurements were performed on a SpectraMax i3x microplate reader (N=9 per sample). The average slope was calculated within the linear portion of the curve, and all values were normalized to the average slope value of the MMP-9 control samples.

2.3.2. Boundary Lubrication

Dynamic coefficient of friction was measured between condom latex and polished glass (Davis et al., 1978) on a Bose ElectroForce 3230 (Bose, Prairie Eden, MN), as described previously (Larson et al., 2017), but without a pre-conditioning loading period as would be used for cartilage bearings. Briefly, 200 μL of rhPRG4 or ECF843 was placed onto a glass slide before an attachment covered in latex was lowered and oscillated. The entraining velocity was 0.3 mm/sec at the frictional radius and the load was 0.35 x 10^6 N/m^2. (Jay, 1992; Jay & Cha, 1999). The coefficient of friction at equilibrium was measured for each sample (N=3), and PBST was used as a control before each test. Both the glass slide and the top fixture covered in latex were thoroughly rinsed with DI water for 2 min in between each test.

2.3.3 Quartz-Crystal Microbalance with Dissipation (QCMD)

Binding kinetics to collagen was assessed by QCMD, which has been used to study PRG4 previously (Greene et al., 2015). Collagen 1 was coated onto a gold sensor before rhPRG4 or

ECF843 was flowed across the surface. Both solutions were used at 150 µg/mL with a flow rate of 400 µL/min for ~2.5 min after the onset of the frequency drop before flow was halted. Rate constant was calculated using a standard first-order linear time-invariant model starting from the drop in frequency.

2.4 Functional Characterization – non DED-associated

2.4.1 Urate Acid Crystal Formation

MSU crystal formation was performed as described previously (Elsaid et al., 2022). 50 mL of 0.1M tetraborate buffer at pH 8.5 was incubated in water bath at 37°C for 1 hour. 75 mg of monosodium urate monohydrate (MSU, Sigma Aldrich) was added to the borate buffer solution, mixed, and incubated again in a water bath at 37°C for 1 hour. 1 mL centrifuge tubes were filled with rhPRG4, ECF843, or bovine submaxillary mucins (BSM, Sigma Aldrich) at 9 gradated concentrations (0, 6.25, 12.5, 25, 50, 100, 150, 200, and 250 µg/mL) prepared in MSU-containing tetraborate buffer. These solutions were incubated at 35°C for 96 hours. At 24-hour intervals, each 1 mL tube was mixed by vortexing for 15s before 20-25 µL of sample was collected (N=6 per sample per timepoint). The number of MSU crystals formed in each sample was counted with a hemocytometer under 20X magnification on an Olympus BX51 microscope.

2.4.2 Cell Growth and Adhesion

To assess the effect of rhPRG4 or ECF843 on cell adhesion (Rhee et al., 2005), A-375 cells (ATCC) were cultured in Synoviocyte Medium (ScienCell, 4701) with 10% fetal bovine serum (FBS, Neuromics, FBS001-HI) and 5% penicillin/streptomycin (Gibco, 15070-063). 96-well plates were coated with rhPRG4 or ECF843 by diluting stock solutions to ~~at~~ 4 different concentrations (6.25, 12.5, 25, and 50 µg/mL) ~~by~~ and adding 100 µL of each ~~diluted~~ sample to a well and incubating at 37°C for 1 hour. 20,000 cells were plated per well, and the plate was

incubated for 24 hours at 37°C in a 5% CO_2 incubator. Cell viability was measured as a surrogate for cell adhesion using a CellTiter-Glo® 2.0 Cell Viability Assay Kit (Promega, G9242), following manufacturer guidelines. Briefly, the plate was washed 3 times with 250 µL DPBS before 50 µL of RT CellTiter-Glo was added to each well. The plate was shaken at 300 rpm for 5 min and then incubated for 10 min at RT without shaking. Luminescence was measured using a Spectramax M2e plate reader 3 times for 3 wells per condition.

2.5. Statistical Analysis

Data are expressed as the mean ± SEM. Differences in size and charge between rhPRG4 and ECF843 were assessed by Student's t-test with Welch's correction. Differences in size and mass of each SEC peak were assessed by Student's t-test with Welch's correction. Differences in Raman analysis ratios were assessed by Student's t-test with Welch's correction. Effects of rhPRG4 and ECF843 on MMP-9 activity were assessed by ANOVA at each concentration. Differences in uric acid crystal formation were assessed by Student's t-tests at each concentration. Differences in cell adhesion properties were assessed by Student's t-tests at each concentration. Differences in coefficient of friction were assessed by one-way ANOVA. All statistical analyses were performed using GraphPad PRISM software (v9.5.0).

3. Results

3.1 ECF843 is smaller, less negatively charged, and lower in molecular weight than rhPRG4

From the batch DLS analysis, ECF843 has a significantly smaller z-average hydrodynamic diameter (D_h) (**Fig. 1A-B**, 58.8 ± 2.3 nm vs 173.5 ± 15.7 nm, $p < 0.0001$) and a less negative ~~more positive~~ zeta potential (**Fig. 1C,** -11.6 ± 1.1 mV vs -17.7 ± 2.0 mV, $p < 0.05$) than rhPRG4. Examining specific peaks through SEC/MALS/DLS analysis, both ECF843 and rhPRG4 eluted as 3 main peaks at ~7.1, ~8.5, and ~9.6 min identified as peaks 1, 2, and 3, respectively (**Fig. 1D-E)**. For rhPRG4, peak 1 had a D_h of 137.0 ± 2.6 nm, peak 2 had a D_h of 69.4 ± 2.6 nm, and peak 3 had a D_h of 42.4 ± 2.4 nm (**Table 1**). ECF843 had a significantly smaller D_h for Peak 1 (**Table 1**, $p < 0.01$) and Peak 2 (**Table 1**, $p < 0.05$) peaks, but there was no significant difference in D_h for Peak 3 (**Table 1**). In addition to this, SEC-MALS analysis shows that although there was no significant difference in molecular weight for Peak 3 (**Table 1**), the molecular weights for both the Peak 1 (**Table 1**, $p < 0.0001$) and the Peak 2 (**Table 1**, $p<0.001$) of ECF843 were significantly smaller than the molecular weights of the rhPRG4 peaks.

3.2 ECF843 and rhPRG4 have different bond structures and sedimentation properties

Examination of bond structural differences through Raman spectroscopy revealed there was a significant difference in the 550/987 peak area ratio between ECF843 and rhPRG4 (**Fig, 2A-B**, 1.18 ± 0.3 vs 0.36 ± 0.07, $p < 0.05$). This indicates the amount of disulfide bonding relative to the carbon-carbon backbone was significantly greater in ECF843 compared to rhPRG4.

Examination of sedimentation properties revealed both ECF843 and rhPRG4 had a major species with an S-value of ~4-4.5S, which, for rhPRG4, constituted the majority of the measured signal (**Fig. 2C**). However, ECF843 showed a second, more dominant and more compact species at ~6S (**Fig. 2C**).

3.3 ECF843 was less effective at inhibiting MMP-9 activity than rhPRG4

ECF843 was significantly less effective at inhibiting MMP-9 compared to rhPRG4 at both measured concentrations. At 50 μg/mL, ECF843 was only able to inhibit MMP-9 activity to 50.2 ± 10.8% of control, while rhPRG4 was able to inhibit MMP-9 activity to 19.7 ± 2.1% of control (**Fig. 3A**, $p < 0.001$). At 150 μg/mL, ECF843 inhibited MMP-9 activity to 24.0 ± 3.2% of control, while rhPRG4 inhibited MMP-9 activity to 8.5 ± 0.5% of control (**Fig. 3A**, $p < 0.001$). While PBST alone did show some artefactual MMP9 inhibitory ability even though it contained no lubricin, it is of note that ECF843 was not significantly different from PBST at 50 μg/mL (**Fig. 3A**, 50.2 ± 10.8% vs. 63.9 ± 8.9%, $p > 0.05$) and 150 μg/mL (**Fig. 3A**, 24.0 ± 3.2 vs. 28.9 ± 5.4%, $p > 0.05$). After normalizing to PBST at each concentration, there was a significant difference between rhPRG4 and ECF843 at both 50 μg/mL (**Fig. 3B**, $p < 0.01$) and 150 μg/mL (**Fig. 3B**, $p < 0.001$).

3.4 ECF843 was less effective at acting as a boundary lubricant than rhPRG4

ECF843 had a significantly higher coefficient of friction than rhPRG4 (0.089 ± 0.009 vs 0.048 ± 0.012, $p < 0.05$) at a glass-latex interface (**Fig. 3C**). rhPRG4 had a significantly lower coefficient of friction than PBST (0.096 ± 0.005), which was used as a negative control, while there was no significant difference in coefficient of friction between ECF843 and PBST.

3.5 ECF843 was slower to bind to collagen 1 than rhPRG4

Examination of binding to Collagen 1 by QCMD revealed ECF843 was ~5X slower in binding to the Collagen 1 surface (**Fig. 3D**); rhPRG4 had a time constant of 8s, while ECF843 had a time constant of 37s.

3.6 ECF843 was less effective at inhibiting MSU crystal formation than rhPRG4

ECF843 was less effective than rhPRG4 at inhibiting MSU crystal formation, and the differences between them increased with time. After one day, there were significantly more MSU crystals formed in ECF843 than rhPRG4 at all measured concentrations except 150 μg/mL (**Fig. 4A**, $p < 0.05$ – $p < 0.0001$). After two days, there were significantly more MSU crystals formed in ECF843 than rhPRG4 at all measured concentrations except 250 μg/mL (**Fig. 4B**, $p < 0.05$ – < 0.0001). After three days, there were significantly more MSU crystals formed in ECF843 than rhPRG4 at all measured concentrations (**Fig. 4C**, $p < 0.05$ – < 0.0001). After four days, there were significantly more MSU crystals formed in ECF843 than rhPRG4 at 25, 100, and 200 μg/mL concentrations (**Fig. 4D**, $p < 0.05$ – $p < 0.001$).

3.7 ECF843 was less effective at preventing cell adhesion than rhPRG4

There was no significant difference between rhPRG4 and ECF843 at preventing cell adhesion at 6.25 μg/mL (**Fig. 4E**). There were significantly more A375 cells bound to the wells coated with ECF843 than those coated with rhPRG4 at 12.5 μg/mL (**Fig. 4E**, 1082 ± 70 cells vs 871 ± 5 cells, $p < 0.01$), 25 μg/mL (**Fig. 4E**, 1168 ± 12 cells vs 868 ± 16 cells, $p < 0.001$), and 50 μg/mL (**Fig. 4E**, 1111 ± 62 cells vs 818 ± 19 cells, $p < 0.001$).

4. **Discussion**

PRG4 is a mucin-like glycoprotein with well-established boundary lubricating properties (Coles et al., 2010; Schmidt et al., 2007) that naturally forms dimers and multimers through disulfide bonding (Schmidt et al., 2008) and is negatively charged (Greene et al., 2016). Our findings in this study show that two different recombinant human lubricin proteins (rhPRG4 and ECF843), produced from the same cell line yet purified with a different process, have very different biophysical and functional properties.

Our findings are consistent with, and significantly extend, that of a previous study examining ECF843, as well as the published patent on ECF843. Specifically, the z-average hydrodynamic diameter for ECF843 measured here agrees with a previous study (Rabiah et al., 2020), and the two peaks measured for ECF843 by AUC match those reported in a published patent describing the purification of ECF843 (WO 2021/171165 A1). In addition to this, we identified other significant structural differences between rhPRG4 and ECF843, including in molecular weight and size as well as disulfide bond structure. From the SEC-MALS-DLS analysis, it is clear that ECF843 and rhPRG4 had a similar size and molecular weight for their third peak. However, the second peak for ECF843 was significantly smaller than rhPRG4's second peak in both weight and size and was not statistically different from ECF843's third peak in size or molecular weight. These differences continue in peak 1, where ECF843 was once again significantly smaller and had a lower molecular weight. The molecular weights for rhPRG4 are consistent with a monomer (Peak 3), dimer (Peak 2), and a higher-order species (Peak 1). For ECF843, however, the second peak was not significantly different from the monomer peak, suggesting that ECF843 is potentially deficient in its ability to form dimers. From Raman analysis, there were significant differences in bond structure between ECF843 and rhPRG4, specifically in the abundance of disulfide bonds

relative to the carbon backbone. This could explain the differences in dimer and multimer formation observed in the SEC-MALS-DLS data, since disulfide bonds are a key aspect of dimer formation for PRG4 and are functionally determinant structures. Examining the zeta potential results, we report -11 mV for ECF843, which is different than the 1 mV reported previously (Rabiah et al., 2020). This is likely due to differences in the buffer used, which has been established to have large effects on zeta potential results (Burns & Zydney, 2000; Inam et al., 2022; Rabiah et al., 2020); Rabiah et al. conducted their experiments with samples diluted in PBS, while the experiments conducted here used samples which were buffer exchanged and diluted in DI water. Irrespective, the results indicate that ECF843 is much less negatively charged than rhPRG4, which is more consistent with the zeta potential reported for native PRG4 of -25 to -40 mV (Greene et al., 2016). Collectively, these data demonstrate ECF843 is significantly different that rhPRG4 in terms of size, molecular weight, bonding structure, and charge.

Given these differences in size, charge, and structure between rhPRG4 and ECF843, and the importance of protein structure on its function, it is not surprising that there are significant differences in their functionality as well. rhPRG4 has previously been shown to be an effective inhibitor of MMP-9 (Menon et al., 2021), which is of special interest due to the important role this enzyme plays in DED. MMP-9 levels increase with DED severity in human tears, as well as in mice with experimental DED (Luo et al., 2004; Pinto-Fraga et al., 2018). MMP-9 is also used as a biomarker for DED (Kaufman, 2013). ECF843 is significantly less effective at inhibiting MMP-9 activity than rhPRG4 and is indeed not significantly different from the PBST control (no lubricin) at both measured concentrations. While there was inhibition in the samples due to PBST alone, potentially due to sodium phosphate precipitating calcium present in the reaction buffer, rhPRG4 demonstrated specific MMP-9 inhibition activity while ECF843 was no different than buffer –

suggesting ECF843 lacked MMP-9 inhibitory activity all together. In addition to this lack of MMP-9 inhibitory ability, ECF843 was significantly less effective as a boundary lubricant compared to rhPRG4. Since the boundary lubrication ability of PRG4 is very well established (Jay et al., 1998; Schmidt et al., 2007; Zappone et al., 2008), and this ability could impact the molecule's efficacy as a DED therapeutic, this difference is of particular importance. ECF843 was also significantly slower to bind to collagen 1 as compared to rhPRG4. Since lubricin needs to be surface bound to be active as a boundary lubricant and collagen 1 makes up a majority of the collagen located in the corneal stroma (Meek, 2009), the alterations in ECF843's structure may have directly and adversely impacted both the pharmacokinetics and biophysical mechanism of action of this molecule.

Additional support for the claim that changes in ECF843's structure also affected its function was evident as ECF843 was deficient at inhibiting MSU crystal formation, which play a key role in the development of gout (Elsaid et al., 2022), and was less effective at preventing cell adhesion when compared to rhPRG4. Both solutions had similar osmolarities, so it is unlikely that this could have contributed to the difference seen here. In addition to this, it is important to note that the concentrations tested in the adhesion assay were lower than those used clinically. Concentrations that are needed to provide biomechanical functionality are higher than those needed to show anti-adhesion activity in a cell-based anti-adhesion assay. The latter asymptotes to maximal activity at a lower concentration based on prior work. Overall, ECF843 performed significantly worse than rhPRG4 in many aspects, some of which, such as MMP-9 inhibition, boundary lubrication, and collagen 1 binding, likely influenced its performance as a DED therapeutic.

Since drainage rates at the ocular surface are high, drugs used topically here need to be able to bind and achieve their effects quickly before they are cleared by the eye's defense mechanisms (Dosmar et al., 2022). Due to the significantly slower kinetics of binding for ECF843, it is possible that at the concentrations used in the trial, there was insufficient binding at the cornea for ECF843 to act as quickly as rhPRG4 would be able to. Since more than 80% of topically applied ocular drugs are cleared, usually by blinking (Dosmar et al., 2022), the actual concentration seen at the corneal surface would be much lower. It is worth noting that the difference in properties between ECF843 and rhPRG4 might be less pronounced in equilibrium state analyses than in kinetic assays. Indeed, in our own preliminary study examining ECF843 at 150 μg/mL, the same concentration as the clinical trial, was able to inhibit inflammatory cytokine production in human corneal epithelial cells to some extent for RANTES when stimulated by TNFα (Schmidt, 2021). However, it did not downregulate production of ENA-78 or IP-10, which were previously shown to be downregulated by rhPRG4 addition at the same time point (Menon et al., 2021). Similarly, the studies conducted by Rabiah et al. show that ECF843 is very effective at maintaining wet surfaces, but these measurements were taken at equilibrium, often after more than 3 hours, and were not directly compared to rhPRG4. Our studies here show a significant difference between ECF843 and rhPRG4 in mechanical properties, both in boundary lubrication ability and in anti-adhesive ability. If ECF843 takes longer to interact with the ocular surface than rhPRG4, it is likely that the drug would be quickly cleared from the eye before being able to bind and exert its function.

In drug development, the manufacturing process is integral to the drug product. The intellectual property (WO 2021/171165 A1) for ECF843 cites a process that exposes the harvested lubricin to high pH and cationic ammonium (from ammonium sulfate) for an extended period of time, perhaps resulting in disulfide bond reshuffling. Such a mechanism could lead to the altered

charge and size of ECF843, and if true, would explain the stark differences between rhPRG4 and ECF843 highlighted here and in their respective clinical trials. Moreover, the difference in MMP-9 inhibition and lubrication could have played a key role in the substantive differences in the clinical outcome of the DED clinical trials for rhPRG4 and ECF843. Indeed, rhPRG4 improved signs and symptoms significantly better than an HA solution, with a change in (SANDE) frequency from 60.5 ±17.2 to 23.4 ± 18.6 ($\Delta \sim -37$ from baseline), while ECF843 was indistinguishable from vehicle with very low signal (change SANDE scores of $\Delta = -1.8 \pm 1.91$ from a baseline of 65 ± 18.6). Manufacturing of biologics can be very challenging, and PRG4 is itself a complex molecule with critical, functionally determinant post-translational modifications and higher order structure. Overall, given ECF843's significant biophysical differences and in vitro functional deficiencies, the failed clinical trial of ECF843 does not diminish the therapeutic potential and previously demonstrated success of rhPRG4 (with size, structure and charge similar to native PRG4) to treat DED.

Declaration of Competing Interests

TAS, BDS, and GDJ have authored patents on rhPRG4 and hold equity in Lubris BioPharma LLC, USA. TAS is also a paid consultant for Lubris BioPharma LLC, FL. APT and GWG have also authored patents on rhPRG4. All other authors have nothing to disclose.

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