Development and Characterization of Ophthalmic Potassium Dobesilate and Resveratrol Formulations for the Treatment of Pterygium

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Development and Characterization of Ophthalmic Potassium Dobesilate and Resveratrol Formulations for the Treatment of Pterygium

Abstract
Pterygium is a fleshy, inflamed, overgrowth of the conjunctiva that can invade the visual axis and cause vision loss. Histologically, pterygia tissue shows overexpression of angiogenic factors. Potassium dobesilate (KD) and resveratrol (RES) are known to inhibit angiogenesis and thereby may be useful agents in reducing pterygia size and inflammation.

This study aimed at developing and characterizing three ophthalmic formulations each of KD, RES, or KD:RES 10:1. The formulations for the individual and dual drug combination are prepared in normal saline (NS) with a viscosity enhancer (10% polyethylene glycol 400; PEG 400) or as a sol-gel using 10% PEG 400 and 21% Pluronic® F127. All formulations contained either 25 mg of KD, 2.5 mg of RES, or a combination of KD:RES 10:1 ratio and were stored at room temperature in the dark. The two types of formulations are tested to provide flexible dosing schedules of once a day (Pluronic ® F127) or twice a day (NS+PEG 10%).

The physical and chemical stability of these formulations was assessed over a 28-day period, in triplicate, on days 1, 7, 14, 21, and 28. Physical stability was determined by visual inspection and optical density measurements at 295 nm (KD) and 306 nm (RES). Chemical stability was assessed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) using a C18 column and a DAD detector at 295 nm (KD) and 306 nm (RES). The effect of the compounds on the proliferation of human umbilical vein endothelial cells (HUVEC) was used to assess the anti-angiogenic properties of KD and RES. Cell viability of HUVEC was determined using Cell-Titer Blue® 24-hours post-treatment with KD, RES, or the combination.

Formulations 5 and 6 demonstrated physical stability for 28 days when stored at room temperature protected from light. Formulation 1 in NS underwent a color change from slight yellow to darker yellow by day 7. Optical density measurements were significantly lower at day 21 and 28 when compared to day 1. The PEG 400 and sol-gel formulations did not undergo a color or optical changes during storage. All formulations were chemically stable for 28 days as assessed by RP-HPLC using United States Pharmacopeia (USP) guidelines for ophthalmic formulations. The anti-proliferative effect of KD, RES, and KD:RES combination in HUVEC indicated that RES is a more potent anti-angiogenic agent as compared to KD. Combination index analysis of the KD:RES formulation indicates the combination is more potent inhibitor of angiogenesis as compared to the individual compounds.

Our study indicates that KD in combination with RES may be a viable alternative treatment to reduce pterygium size and inflammation. The sol-gel formulation may enhance patient compliance as compared to the solution formulations with once a day, overnight, dosing. Future work for this project lies in assessing the microbiological stability of these formulations in vitro and determining effectiveness in vivo.

Degree Type
Thesis

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DEVELOPMENT AND CHARACTERIZATION OF OPHTHALMIC POTASSIUM DOBESILATE AND RESVERATROL FORMULATIONS FOR THE TREATMENT OF PTERYGIUM

By

AMY VAN HEEL

A THESIS

Submitted to the Graduate Faculty of Pacific University Vision Science Graduate Program, in partial fulfillment of the requirements for the degree of Master of Science in Vision Science

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VISION SCIENCE GRADUATE PROGRAM

This thesis of Amy Van Heel, titled "Development and Characterization of Ophthalmic Potassium Dobesilate and Resveratrol Formulations for the Treatment of Pterygium," is approved for acceptance in partial fulfillment of the requirements of the degree of Master of Science.

5/18/2016
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DEVELOPMENT AND CHARACTERIZATION OF OPHTHALMIC POTASSIUM DOBESILATE AND RESVERATROL FORMULATIONS FOR THE TREATMENT OF PTERYGIUM

Amy Van Heel

MASTER OF SCIENCE IN VISION SCIENCE
PACIFIC UNIVERSITY, 2016

ABSTRACT

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assess the anti-angiogenic properties of KD and RES. Cell viability of HUVEC was determined using Cell-Titer Blue® 24-hours post-treatment with KD, RES, or the combination.

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Our study indicates that KD in combination with RES may be a viable alternative treatment to reduce pterygium size and inflammation. The sol-gel formulation may enhance patient compliance as compared to the solution formulations with once a day, overnight, dosing. Future work for this project lies in assessing the microbiological stability of these formulations in vitro and determining effectiveness in vivo.
ACKNOWLEDGEMENTS

I would like to thank Dr. Deepa Rao for all of her help on this project. Without her, this project would not have been able to be completed. I would also like to thank my thesis committee, Dr. Len Koh and Dr. Ryan Bulson for all of their help and advising in this study. Additionally, thanks to the PU Incentive Grant for funding of our project.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Abbreviation Description</th>
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<tbody>
<tr>
<td>KD</td>
<td>potassium dobesilate</td>
</tr>
<tr>
<td>RES</td>
<td>resveratrol</td>
</tr>
<tr>
<td>NS</td>
<td>normal saline</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reverse phase-high performance liquid chromatography</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopeia</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1 IL-1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>heparin-binding epidermal growth factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>B-FGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>IGF-BP</td>
<td>insulin-like growth factor binding proteins</td>
</tr>
<tr>
<td>MMC</td>
<td>mitomycin-C</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>DAD</td>
<td>diode array detector</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>half minimal inhibitory concentration</td>
</tr>
<tr>
<td>CI</td>
<td>combination index</td>
</tr>
<tr>
<td>fa</td>
<td>Fraction of cells affected</td>
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INTRODUCTION

Introduction

Pterygium is a fleshy, inflamed, overgrowth of the conjunctiva that can invade the visual axis and cause vision loss. Pterygium is derived from the Greek word “pterygion”, which means a small wing (1). Pterygium usually forms over the prelimal conjunctiva and extends into the corneal surface (2). Clinically it presents as a non-malignant slow growing proliferation of wing shaped fibrovascular lesion (41).

Clinical Features and Epidemiology

Typical clinical presentation of a pterygium is separated into two groups, those with a minimal proliferating pterygium or those with a rapidly growing pterygium. The latter tends to be elevated with a fibrovascular component and the former has a less fibrovascular and flatter appearance (2). If excised, the first group tends to recur less frequently, while the second group takes a more aggressive clinical course with a greater likelihood of recurrence after excision (2).

Pterygia are more common in individuals within the “pterygium zone,” defined as 40° north and south of the equator (4). Within this zone, prevalence is up to 22% in the general population (5). Outside of this geographical location, pterygium prevalence is much less, around 2% and typically found in those who work outdoors due to increased exposure to sunlight (6, 7). Differences in lifestyles between genders can be attributed to a higher prevalence in males.
compared to females (6, 7). Prevalence of pterygium in migrant workers is similar to the “pterygium zone” at 22% (42).

Chronic ocular surface irritation from sun exposure, wind, and dust are environmental factors also contributing to pterygium development (4, 5). UVA and UVB radiation (290-400 nm) (8) are the wavelengths most harmful to the front surface of the eye. At the molecular level, UV radiation creates activated free radicals. Tear film proteins, including lactoferrin, deactivate free radicals (9). However, the increased number of free radicals increases oxidative stress in pterygium tissue, and in turn, increases a number of proteins, like survivin, in comparison to a normal ocular surface (10). A variety of cytokines, growth factors, and growth factor receptors are expressed with the changes mediated by trauma caused by UV radiation (11). These factors are essential for normal cornea healing and repair but have altered expression in pterygium tissue. These factors include, the interleukin-1 (IL-1) system acts with tumor necrosis factor α (TNF α). Together these lead corneal keratocytes to take on a repair role (12, 13). Specifically, IL-6 promotes epithelial cell migration through the induction of integrin receptors (14, 15) and IL-8 displays mitogenic and angiogenetic activity (16, 17). Previous literature have involved different growth factors in pterygium tissue including epidermal growth factor (EGF), heparin-binding EGF (HB-EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and insulin-like growth factor binding proteins (IGF-BP) (11). VEGF is of particular interest because it is produced by corneal fibroblasts in response to harmful inflammatory stimuli, including UV radiation (39). When comparing normal conjunctiva to pterygium tissue, VEGF concentrations are increased as quantified by immunochemistry (18).
**Current Treatments and Therapies**

Clinical treatment of pterygia includes surgical excision typically with anti-metabolite treatment during excision, β-irradiation, or treatment with growth factor inhibitors. Simple surgical excision of removing the pterygium tissue from the anterior corneal surface was early pterygium treatment (19, 20). “Bare sclera” technique involves removing the pterygium and peri-limbal conjunctiva while suturing the remaining conjunctival rim to the sclera. After removal, it is believed the newly uncovered area would re-epithelialize with epithelial cells at the conjunctival rim (19, 21). This technique has been shown to have recurrence of pterygium up to 50% (19).

Another surgical removal technique used is a conjunctival flap or conjunctival graft. Both of these techniques aim to place normal tissue where the pterygium was. In addition to normal tissue, this can provide stem cells on the sclerocorneal limbal area (22, 23). If the pterygium removal creates too large of a conjunctival defect, a preserved amniotic membrane can be used instead a conjunctival graft (24). This commercially available tissue provides a surface for re-epithelialization (24). With this more advanced excision surgery, recurrence rates can be decreased to as low as 10% (19, 22).

Since recurrence is always a concern with surgical excision of pterygium, antimetabolites, like mitomycin-C (MMC), are typically used (19, 22). MMC is a product of *Streptomyces caesiptosus*, an antibiotic, and is capable of alkylating DNA to block transcription and translation (25, 26). During surgery, MMC (0.2-0.4 mg/mL) is typically applied on the episclera for no more than 2 minutes, in intervals (25). With this surgery adjunctive, the recurrence rate of pterygium is less than 10% (19, 22, 27). MMC is also used postoperatively in
eye drops (19, 28). Another antimetabolite, 5-fluorouracil (5-FU) can be used intra-operatively or as a post-operative subconjunctival injection (19, 28).

Antimetabolites are effective in decreasing pterygium recurrence rates (19, 22, 27), however, there are serious complications that may arise including scleral melt or delayed healing (19). These complications may damage vision or require further surgery.

Drawbacks of surgical excision of pterygia include the high incidence of recurrence and the cost associated with the procedure. Cost of surgery is typically between $1800-2000 and insurance will cover much of the surgery (29), if the growth affects vision, but is not always affordable for patients without insurance, like migrant farm workers who typically fall below the poverty level (43).

β-irradiation is another option for pterygium removal. It has been one of the first attempts to remove pterygium (30, 31). The procedure involves delivering β-irradiation by strontium-/yttrium-90 sources and this reduces cells responsible for pterygium recurrence (32). Endophthalmitis is a potential complication that can threaten vision (19).

Growth factor inhibitors (anti-VEGF monoclonal antibodies) are used intraocularly for treatment of neovascular conditions like exudative macular degeneration and proliferative diabetic retinopathy (33). Bevacizumab (Avastin, Genentech) is a recombinant monoclonal antibody and Ranibizumab (Lucentis, Genetech) is a fragment, both of which are directed at VEGF (33). Bevacizumab has been studied in pterygium treatment (34, 35) but there are significant systemic side effects, including cardiovascular toxicity (33). Looking at VEGF expression in individual pterygium tissue can be used to determine if Bevacizumab or other
anti-VEGF treatment can be used to minimize recurrence of pterygium and systemic side effects.

**Objective of Study**

Since inflammation and angiogenesis are implicated in pterygia progression, a potential low cost, nonsurgical therapeutic option could include the use of agents targeted against inflammation and angiogenesis in a topical formulation. In a recent case study published in the *British Medical Journal* (36), a 53 year-old male patient exhibiting primary pterygium was treated with potassium dobesilate (KD), an anti-inflammatory with potential anti-angiogenic effects (37). The patient received KD formulated at 25 mg/mL in artificial tears administered as two drops twice daily for two weeks. At the end of the treatment period, a clear regression in the pterygium was observed and the patient remained recurrence free at 2 months post-treatment. The authors speculated that the anti-angiogenic effects of the KD were responsible for the effectiveness of the treatment. However, the anti-angiogenic effects were never directly assessed and the follow-up time of 2 months may not have been enough to fully evaluate this treatment regimen. Additionally, other known anti-angiogenic agents like resveratrol (RES) (38) were not evaluated. Thus, objective of our work is to develop several stable ophthalmic formulations of KD and RES alone and in combination to assess the anti-angiogenic effects of these agents alone and in combination for future development as low cost, alternative treatments for pterygium.

The combination KD:RES formulation was pursued to determine if the use of two anti-angiogenic agents may have a synergistic effect in inhibiting angiogenesis. The formulations
were characterized in terms of their physical and chemical stability. To assess the degree of anti-angiogenic effects of these compounds, cell proliferation studies were conducted in Human Umbilical Vein Endothelial Cells (HUVEC). We hypothesize that one or more of the formulations containing KD, RES, or KD:RES will demonstrate physical and chemical stability and the compounds will demonstrate anti-angiogenic effects in vitro. Upon completion of this work we anticipate having one or more formulations that can be tested further in vivo for efficacy in treating pterygia in a topical manner with a larger cohort.
MATERIALS AND METHODS

Materials

KD was obtained from Pfaltz and Bauer (Waterbury, CT) and RES from TCI America (Portland, OR). Polyethylene glycol 400 (PEG 400), a 10% solution was obtained from VWR (Radnor, PA), sodium perborate from Spectrum Chemicals (New Brunswick, NJ) and normal saline from Fischer Scientific (Pittsburgh, PA). Pluronic® F127 was kindly donated by BASF (Florham Park, NJ). All other chemicals and solvents (HPLC grade) were purchased from VWR (Radnor, PA). Human Umbilical Vein Endothelial Cells (HUVEC) and Medium Kit consisting of basal medium and supplement pack were purchased from PromoCell (Heidelberg, Germany). Cell-Titer Blue® cell viability assay was purchased from Promega (Madison, WI). Other cell culture supplies, including trypsin with EDTA, were obtained from VWR (Radnor, PA).

Formulation Preparation

Ophthalmic formulations of varying viscosity were prepared with KD, RES, or KD:RES 10:1 as the active drug (Table 1). All formulations contained either 25 mg of KD, 2.5 mg of RES, or a combination of KD:RES 10:1 ratio (25 mg KD: 2.5 mg RES). The formulations for the individual and dual drug combination were prepared in normal saline (NS) with a viscosity enhancer (10% PEG 400) or as a sol-gel using 10% PEG 400 and 21%

<table>
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<tr>
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<td>KD</td>
<td>NS</td>
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<tr>
<td>Formulation 2</td>
<td>RES</td>
<td>NS</td>
</tr>
<tr>
<td>Formulation 3</td>
<td>KD + RES</td>
<td>NS</td>
</tr>
<tr>
<td>Formulation 4</td>
<td>KD</td>
<td>21% F127</td>
</tr>
<tr>
<td>Formulation 5</td>
<td>RES</td>
<td>21% F127</td>
</tr>
<tr>
<td>Formulation 6</td>
<td>KD + RES</td>
<td>21% F127</td>
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Table 1 KD and RES formulations for stability testing. All formulations contained 0.005% sodium perborate and 10% PEG.
Pluronic® F127. The sol-gel formulation was optimized to gel at 37° C, to mimic physiological temperature and ensure extended contact time on the ocular surface post-administration. All formulations were preserved using with 0.005% sodium perborate. Once prepared all formulations were stored at room temperature and protected for light. All formulations were assessed for physical and chemical stability, as well as anti-angiogenic effects in cell culture.

**Formulation Evaluation-Physical Stability**

Physical stability of KD and RES formulations was completed over a 28-day period. Assessment of physical stability was performed by visual inspection and optical density measurements on days 1, 7, 14, 21, and 28. Optical density measurements were performed at 295 nm (KD) and 306 nm (RES) using a BioTek Synergy 2 spectrophotometer. Vehicles without any drug(s) were used as controls. Data for the visual inspections is presented in tabular format and optical density measurements are presented as Mean OD ± SD of three replicates.

**Formulation Evaluation- Chemical Stability**

RP-HPLC was used to determine chemical stability over a 28-day period by UV detection at 295 nm (KD) and 305 nm (RES) with Agilent 1200 series instrument. The Agilent 1200 HPLC is equipped with a vacuum degasser, binary pump, autosampler, thermostatted column oven and a diode array detector (DAD). A Zorbax SB-C18 column (Agilent Technologies) stabilized at 40° C with a mobile phase of H₂O:ACN 70:30 stabilized with 1 % methanol and 0.1% H₃PO₄ at a flow rate of 0.3 mL/min. Samples of 3 µL were injected and KD and RES were detected at 295 nm and 305 nm respectively. Retention time for KD was 0.57 min and RES was 1.20 min. Samples
were diluted 1:100 in methanol and assessed on days 1, 7, 14, 21, and 28. The calibration curves for both drugs with the line of best-fit equation and goodness of fit \( (r^2) \) are provided in Figure i and Tables ii and iii, in Appendix A. Stability data for three replicates is reported as Mean drug concentration \( \pm \) SD.

**Formulation Evaluation- *In vitro* Anti-angiogenic Effect Assessment**

Anti-angiogenic properties of KD and RES were tested with HUVEC cell line between the passages of two and five. Cells were cultured in DMEM (Dulbecco’s Modified Eagle Medium) with supplemental growth mix (PromoCell) following the protocols provided by PromoCell. Cells were plated at a density of 10,000 cells/well in a 96-well plate and allowed to attach for 24 hours. Cells were treated for 24 hours with KD, RES, and KD:RES 10:1 ratio at RES 0.01-1,000 \( \mu\text{M} \) and KD from 1,000-10,000 \( \mu\text{M} \) and the same concentrations were used for the KD:RES 10:1. Post 24 hours, cells were assessed for viability using Cell-Titer Blue®. After a 2-hour incubation with the reagent, fluorescence was measured at 560\text{EX}/590\text{EM} on a BioTek Synergy 2 spectrophotometer. Half minimal inhibitory concentration (IC\text{50}) values were calculated using GraphPad prism (La Jolla, CA) and viability data is presented as mean IC\text{50} \( \pm \) SD for KD, RES, and the combination KD:RES.

Combination index (CI) analysis was calculated using CompuSyn software version 1.00 from ComboSyn, Inc (Paramus, NJ) by Chou and Martin (44) with the KD:RES formulation to determine the interaction of each drug. The Median-Effect Principle and Combination Index-Isobologram Theorem were used to determine whether KD and RES interaction was antagonistic or synergistic. The CI equation used was:
\[
\frac{(D_1)}{(D_0)_1} + \frac{(D_2)}{(D_0)_2} = CI
\]

where D is Drug, and the subscripts 1 and 2 indicate each individual drug. Subscript x is the percent inhibition in cell proliferation with each particular drug. CI values less than 1 indicate synergistic drug effects, while CI values greater than 1 indicate antagonism, and a CI value equal to 1 shows additivity. The CI can be calculated for different fractions of cells affected (fa).

Plotting CI vs fa shows the interaction at all affected levels (1-99%) for a given combination of drugs. Mean CI vs fa plot is shown for passage five of HUVEC cells in Figure 4B.

**Statistical Analysis**

Two-way ANOVA is utilized to determine statistical significance at a p-value of 0.05 for physical stability. Chemical stability was analyzed according to USP guidelines for ophthalmic preparations. Statistical Analysis was performed using GraphPad Prism (La Jolla, CA).
RESULTS

Formulation Preparation

Formulations 2 and 3 were unable to be tested due to RES precipitating immediately when placed in NS. RES has low water solubility (0.003mg/mL) (47) and formulations 2 and 3 use only normal saline as the vehicle, therefore, only formulations 1, 4, 5, and 6 were prepared and assessed for physical and chemical testing.

Formulation Evaluation—Physical Stability

KD in NS (Formulation 1) had a slight yellow color on day 1. During visual inspection on day 7, the yellow color changed to a deeper yellow. The color remained unchanged for the rest of the 28-day period. KD in formulations 4 and 6 did not undergo any color change during the 28-day period. Additionally, formulations containing RES, formulations 5 and 6, remained colorless for the 28-day period. Visual inspection data can be found in Table 2 below.

<table>
<thead>
<tr>
<th>Day</th>
<th>KD in NS</th>
<th>KD in 21% F127</th>
<th>RES in 21% F127</th>
<th>KD:RES in 21% F127</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Color</td>
<td>Clarity</td>
<td>Color</td>
<td>Clarity</td>
</tr>
<tr>
<td>1</td>
<td>Slightly yellow</td>
<td>Clear</td>
<td>Colorless</td>
<td>Clear</td>
</tr>
<tr>
<td>7</td>
<td>Deeper yellow</td>
<td>Clear</td>
<td>Colorless</td>
<td>Clear</td>
</tr>
<tr>
<td>14</td>
<td>Deeper yellow</td>
<td>Clear</td>
<td>Colorless</td>
<td>Clear</td>
</tr>
<tr>
<td>21</td>
<td>Deeper yellow</td>
<td>Clear</td>
<td>Colorless</td>
<td>Clear</td>
</tr>
<tr>
<td>28</td>
<td>Deeper yellow</td>
<td>Clear</td>
<td>Colorless</td>
<td>Clear</td>
</tr>
</tbody>
</table>

Table 2: Color and clarity of KD and RES formulations (n = 4) by visual inspection for physical stability measurements over a 28-day period.

Optical density measures for each formulation can be found in Figure 2. Formulations 1, 4, and 6 were assessed at 295nm to optimally detect KD. Formulations 1 and 4 demonstrated a
statistical decrease in optical density at day 21 and day 28 respectively, indicating a shorter shelf-life (Figure 2A). Formulation 6 remained stable for the duration of the study with respect to both KD and RES (Figure 2A and 2B). RES in formulation 5 also showed no statistical changes in optical density measures for the 28-day period (Figure 2B). Two-Way ANOVA tables for formulations containing KD and RES are found in Appendix A (Table i).

![Figure 2](image)

**Figure 2.** Optical density measurements at 295 nm to detect the physical stability of KD over a 28-day period (n=4) (A). RES physical stability measured by optical density at 306 nm for the 28-day period (n=4) (B).

**Formulation Evaluation- Chemical Stability**

Chemical stability for each formulation was assessed by RP-HPLC over 28 days and is presented in Figure 3. Average concentration of each drug was determined from a collated calibration curve with KD and RES dissolved in various solvents and in the presence and absence of each other. The individual conditions and the collated curves are presented in Appendix A.

USP guidelines require ophthalmic formulations to remain within 10% of their labeled potency for the duration of their shelf-life. In formulations 1, 4, and 6, KD was measured at 25mg and formulations 5 and 6 RES was measured at 2.5mg. Figure 3 shows concentration of
KD and/or RES in each formulation with USP drug concentration guidelines shown as dotted gray lines. KD concentration in formulation 1 is within the USP guidelines for the 28-day period. At day 28, the upper limit of USP guidelines is shown with formulation 1. Formulations 4 and 5 show an increase in drug concentration from day 1 than what was weighed out for each sample. Pluronic® F127 cannot be shaken due to formation of air bubbles. Increased drug concentration of day 1 samples of formulations 4 and 5 may be due to incomplete mixing and is borne by the fact that for the remainder of the study the concentrations remained consistent. For the remaining 28-day period, formulations 4 and 5 fit within the USP guidelines for labeled drug concentration. KD concentration in formulation 6 is shown in purple and RES concentration in orange in Figure 3. KD and RES concentration falls below USP guidelines at day 7. This point is an outlier due to the fact that post 7 days the concentrations remain within 10% of the labeled potency. The variability at day 7 is also higher as compared to the rest of the days indicating that experimental error and not formulation failure is the reason for the drop in potency. The remaining time course for KD and RES are within the usable range for ophthalmic preparations according to USP.
Figure 3. KD and RES concentrations in each formulation determined by RP-HPLC. USP guidelines for ophthalmic drug labeling shown by dotted gray lines. Data analyzed using GraphPad Prism.
Formulation Evaluation- *In vitro* Anti-angiogenic Effect Assessment

Anti-angiogenesis data for KD, RES, and the combination KD:RES are shown in Figure 4. IC₅₀ values, minimum concentration of a compound to inhibit growth in 50% of cells, were calculated in GraphPad Prism (La Jolla, CA) and are shown in Figure 4A. Data was collected after a 24-hour treatment period with each drug. KD (n=4) showed the highest IC₅₀ value of greater than 3500 µM. RES IC₅₀ value was much lower than KD at 81 µM (n=4). The combination in a 10:1 ratio gave an average IC₅₀ value close to 53 µM (n=3) indicating less drug is needed to inhibit the growth of 50% of HUVEC cells. CI for all fa values are shown in Figure 4B for passage five of the HUVEC cells. KD and RES show a synergist effect (CI <1) up until 80% fa when the combination shows an antagonistic effect (CI>1). This indicates that at lower doses the combination is synergistic and can exhibit stronger anti-angiogenic effects. Based on the IC₅₀ values in HUVECs future work will lie in exploring other combinations of KD:RES such as 40:1 to determine if this ratio is synergistic at all concentrations tested.

![Figure 4](image-url)

*Figure 4.* Average IC₅₀ values for KD (n=4), RES (n=4), KD:RES (n=3) calculated in GraphPad Prism (A). Combination index effects of KD:RES calculated in CompuSyn (B).
DISSCUSSION

Stable formulations in Pluronic® F127 vehicle gives a clinician a muco-adhesive option for pterygium treatment. Pluronic® F127 is a poloxamer made of triblock copolymers of polyethylene oxide-polypropylene oxide-polyethylene oxide that are thermoreversible and increases viscosity of a solution (49). In ophthalmic solutions, increased viscosity by use of carboxy methylcellulose is common, especially in artificial tears that are used to lubricate the ocular surface and increase the contact time of the artificial tear with the ocular surface (50). Increased contact with the ocular surface works to decrease the dosing schedule while maintaining a therapeutic concentration of a topical drug or artificial tear. For example, Vigamox® and Moxeza® are two pharmacologically identical antibiotics, but Vigamox® requires three times a day dosing, while Moxeza® is twice daily dosing (56, 57).

Stability of drugs can influence shelf-life and bioavailability of compounds. Our formulations showed no precipitates and formation of crystal precipitates over time is an indication of physical instability in a drug compound (51). RP-HPLC was used to detect KD and RES, as this method is highly sensitive. Each drug has different retention times and this allowed us to detect each compound when used in combination (formulation 6). Variation in chemical stability of each formulation, although usable based on USP guidelines, can be attributed to sample preparation. Each sample was individually weighed out using a calibrated Mettler Toledo AL54 decimal balance (Columbus, OH). Although the scale is calibrated, the measurement is not guaranteed. In the manufacturing setting, a stock solution for the batch would be prepared to eliminate the variability associate with individual measurements.
The combination of KD and RES showed a synergistic effect in *in vitro* anti-angiogenic effect. Both KD and RES have been used to treat proliferative conditions like vitreal hemorrhages associated with diabetic retinopathy and melanoma (52, 53). Using the combination of KD and RES as anti-angiogenic compounds can help reduce the dosing schedule with the same effects to decrease pterygia vascularization.

**Clinical Relevance of Topical Pterygium Treatment**

Looking at this study in a larger scheme, it is relevant for future pterygium treatment. As of right now pterygium treatment is limited. Surgical excision is indicated when a pterygium becomes so large it is having significant effects on the cornea, ocular irritation, or if a pterygium is cosmetically unappealing (3). Insurance companies typically consider pterygium removal an elective surgery until there are impacts on visual acuity (54) however; patients experience symptoms much sooner than this point. An ophthalmic solution with a sol-gel formulation can lead to earlier treatment for patients experiencing ocular irritation due to pterygia inflammation and growth and reduce the significant corneal effects. Additionally a topical treatment could potentially reduce the costs associated with this condition, since the mainstay surgical treatment costs $1800-2000 (29). Commonly patients with a pterygium are told to use artificial tears throughout the day to lubricate the ocular surface and to limit UV and wind exposure by wearing sunglasses (2). However, for many patients these are not enough to halt the progression of a pterygium because they do not stop the vascularization and the growth is continually fed a blood supply it needs to grow.
Many migrant and field workers surrounding the Pacific EyeClinics have higher UV exposure due to their occupation. Since UV exposure is such a large risk, a majority of patients seen in Pacific EyeClinics and on community vision screenings have pterygia. This study may lead to future in vivo trials with the population base surrounding the Pacific EyeClinics.

Our study has shown that two anti-angiogenic drugs are capable of stopping cell growth and are stable physically and chemically. This is the first step in being able to treat patients with our ophthalmic formulations before the visual consequences of pterygium growth and inflammation reach surgical levels.

**Limitations of study**

Limitations of our study include the inability to test KD and RES anti-angiogenic properties on pterygia tissue. The formulations were tested in a cell line, which most closely resembled human vessels, a key component of vascularized pterygium tissue. Conclusions can be drawn to the efficacy of KD and RES on reducing pterygia vascularization from the results of the anti-angiogenesis study in HUVECs. The case study by Cuevas et. al. (36) showed the effect of KD in a one patient study and our study complements this by the anti-angiogenic effects we saw with KD and RES in the HUVEC cell line. We have established the stability of KD and RES, however, the role of RES on pterygia tissue has not been studied. Overall, our study has shown promising results for future treatment in vivo of vascularized pterygium.
Future studies

Given the stability profile of KD and RES, the next steps include further optimizing of the formulations and testing the microbiological stability of each formulation. USP Antimicrobial Effectiveness Testing (55) is the proposed methodology for microbiological testing. *Candida albicans, Aspergillus niger, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* are the microbes to be tested by USP guidelines with the formulations by inoculating each sample separately and determining the microbial killing rate. USP category 1 guidelines for ophthalmic products would be followed to determine the microbiological stability of each formulation and each formulation will be assessed in triplicate.

Testing these formulations *in vivo* is another step to optimize KD and RES effects to decrease pterygia vascularization. The Pacific University EyeClinics has a large Hispanic patient population and the prevalence of pterygia is common in this population due to having occupations outdoors, including farming, construction, and grounds maintenance (40). As aforementioned, pterygia excision surgery is costly and not always an option for patients (29). The sol-gel formulation is ideal because it is meant for once a day dosing to reduce patient non-compliance. Ideally, a future trial using our formulations to see their effects on reducing pterygia size and vascularization is the next step with the outcomes of our study.
CONCLUSION

Overall, this study has shown KD and RES are viable options for future treatment of pterygia. These drugs are stable physically and chemically over a 28-day period. KD and RES are able to inhibit angiogenesis in HUVEC cells, while the KD:RES 10:1 combination showed a synergistic effect and a lower required dosage to inhibit cell growth to the same level as KD or RES individually. Our study has shown promising results for future topical pterygium treatment.
References

27. Young A., et. al. (2009) Prospective study on the safety and efficacy of combined conjunctival rotational autograft with intraoperative 0.02% mitomycin C in primary pterygium excision. Cornea 28: 166-169
43. Moreno, A. Migrant Health Fact Sheet. *Department of Human Services*.
APPENDIX A

A

<table>
<thead>
<tr>
<th>ANOVA table</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
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<tbody>
<tr>
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<td>0.4218</td>
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<td>0.05273</td>
<td>F (8, 40) = 15.17</td>
<td>P &lt; 0.0001</td>
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<td>F (4, 40) = 70.58</td>
<td>P &lt; 0.0001</td>
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<tr>
<td>Residual</td>
<td>0.139</td>
<td>40</td>
<td>0.003476</td>
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<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>ANOVA table</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.001306</td>
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<td>0.0003264</td>
<td>F (4, 20) = 0.7658</td>
<td>P = 0.5599</td>
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<td>Row Factor</td>
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<td>0.1199</td>
<td>F (1, 20) = 281.4</td>
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</tr>
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<td>Column Factor</td>
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<td>0.001944</td>
<td>F (4, 20) = 4.561</td>
<td>P = 0.0088</td>
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<tr>
<td>Residual</td>
<td>0.008525</td>
<td>20</td>
<td>0.0004262</td>
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<td></td>
</tr>
</tbody>
</table>

Table I. Two-Way ANOVA tables for optical density measures of physical stability for each formulation. Formulations containing KD are in A and formulations containing RES are in B. Columns in the table are SS is sums of squares, DF is degrees of freedom, MS is mean squares, and F is F statistic. Values obtained using GraphPad Prism.

Figure 1. Calibration curves for KD were prepared in methanol and 21% F127 with 10% PEG to mimic formulations (A). RES was prepared in 21% F127 with 10% PEG for each calibration curve (B).
Table ii. Individual calibration curves of KD and RES with various solvents and in the absence or presence of each other. KD absorbance was quantified at 295 nm and RES at 306 nm following separation by RP-HPLC (n = 4). Linear regression curves and goodness of fit values were obtained using GraphPad Prism.

<table>
<thead>
<tr>
<th>HPLC Calibration Conditions</th>
<th>Linear Regression Equation</th>
<th>Goodness of Fit ($r^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KD in Methanol</td>
<td>$Y = 7727x + 481.3$</td>
<td>0.9999</td>
</tr>
<tr>
<td>KD in F127</td>
<td>$Y = 7517x + 498.4$</td>
<td>1.0000</td>
</tr>
<tr>
<td>KD in F127 + RES</td>
<td>$Y = 8189x + 490.5$</td>
<td>0.9997</td>
</tr>
<tr>
<td>RES in F127</td>
<td>$Y = 89274x - 261.6$</td>
<td>0.9997</td>
</tr>
<tr>
<td>RES in F127 + KD</td>
<td>$Y = 91241x - 118.2$</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

Table iii. Collated KD and RES calibration curves utilized to analyze concentration of the compounds in various formulations. KD absorbance was quantified at 295 nm and RES at 306 nm following separation by RP-HPLC (n = 4). Linear regression curves and goodness of fit values were obtained using GraphPad Prism.

<table>
<thead>
<tr>
<th></th>
<th>Linear Regression Equation</th>
<th>Goodness of Fit ($r^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KD</td>
<td>$Y = 9829x + 490.1$</td>
<td>0.9979</td>
</tr>
<tr>
<td>RES</td>
<td>$Y = 90258x - 189.9$</td>
<td>0.9993</td>
</tr>
</tbody>
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