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14. ABSTRACT
Retinal detachment (RD) is a prevalent cause of blindness that is common after ocular injury to military personnel. Permanent vision loss occurs due to death of photoreceptors and formation of excessive scar tissue, known as proliferative vitreoretinopathy (PVR). There are no effective pharmaceuticals to prevent these problems. The inflammatory protein, macrophage migration inhibitory factor (MIF), is produced at high levels in RD and PVR, as well as in excitotoxic (NMDA-mediated) damage, which is important in blast injury. We tested the ability of different clinically-relevant MIF inhibitors to block photoreceptor death after NMDA damage in a chick excitotoxic retinal damage model. These inhibitors, ibudilast, AV1013, and CPSI-1306, are well tolerated in the eye, and treatment with the maximum dose of each drug does not show retinal toxicity. Ibudilast pretreatment significantly reduced the number of TUNEL positive cells in the retina after NMDA damage. CPSI-1306 also reduced TUNEL. AV1013 had no effect. Ibudilast and AV1013 also blocked epithelial mesenchymal transition in the *in vitro* PVR model.

15. SUBJECT TERMS
Retinal detachment (RD), proliferative vitreoretinopathy (PVR), N-methyl-D-aspartate (NMDA), macrophage migration inhibitory factor (MIF), MIF inhibitor, chick, rabbit, retinal pigment epithelium (RPE), ARPE-19, ibudilast, AV411, CPSI-1306, AV1013, scratch assay, MTT assay, contraction assay, epithelial mesenchymal transition (EMT)

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Our project pertains to the FY17 VRP Technology/Therapeutic Development Award Focus Areas: “Preclinical animal studies to evaluate safety and/or efficacy of treatments or technologies returning form and function after traumatic injury to: (1) orbit and ocular tissues (optic nerve, retina, and uvea), (2) eyelid, and/or (3) adnexal structures” and “Preclinical animal studies to evaluate safety and/or efficacy of treatments or technologies to reduce/control scarring and/or pathological healing response(s) after military-relevant ocular/visual system injury.” Since MIF promotes photoreceptor apoptosis and retinal gliosis and it antagonizes the anti-inflammatory effects of glucocorticoids, which are the standard pharmacologic treatment used in traumatized eyes, our hypothesis is that clinically relevant MIF inhibitors that target the inflammatory response will yield benefit in retinal neuroprotection and scar prevention in traumatic retinal damage. Our hypothesis will be tested with the following specific aims. Aim 1: Test the hypothesis that clinically-relevant MIF inhibitors block neuronal apoptosis in our in vivo RD and NMDA damage models. We will evaluate neuroprotection using electrophysiology, spectral domain OCT (SD-OCT), fundus imaging, and histology. Any potential toxicity will be evaluated. Aim 2: Test the hypothesis that clinically-relevant MIF inhibitors block gliosis and pathologic wound healing in traumatic RD. Studies with cell lines (retinal pigment epithelium and Müller glia) and our in vivo RD, RD-PVR, and NMDA models will be performed. Retinal fibrosis will be evaluated with SD-OCT and histology. Aim 3: Evaluate the effects on intraocular pressure (IOP) and the ocular pharmacokinetics of clinically relevant MIF inhibitors. Rabbits will be used to determine the effects of the drugs on IOP and the pharmacokinetics of ocular delivery of MIF inhibitors.

2. **KEYWORDS:**

Retinal detachment (RD), proliferative vitreoretinopathy (PVR), N-methyl-D-aspartate (NMDA), macrophage migration inhibitory factor (MIF), MIF inhibitor, chick, rabbit, retinal pigment epithelium (RPE), ARPE-19, ibudilast, AV411, CPSI-1306, AV1013, gel contraction assay, TUNEL assay, epithelial mesenchymal transition (EMT)

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Task 1. Evaluate the clinically relevant MIF inhibitors, CPSI-1306, ibudilast/AV1013 in our in vivo retinal damage models (RD and NMDA) for neuroprotection of photoreceptors.
This task will utilize electrophysiology, SD-OCT, fundus imaging, and TUNEL/IHC to assess the ability of MIF inhibitors to protect the retinal neurons in RD- and NMDA-induced retinal damage in chicks and find the appropriate dosage. This will include dose escalation studies in untreated animals with a single intravitreal injection in chicks to find a toxic and maximal therapeutic dose (1a). Dose ranging studies will be conducted in retinal damage models to find the optimal dose for neuroprotection (1b). Finally, drug timing studies will be performed to test the impact of timing on the neuroprotective benefit (1c).
Timeframe: Months 1-18

Subtask 1a. Perform dose escalation studies in untreated animals to find toxic and maximal therapeutic doses. This task will involve weighing, ocular examination with Nussenblat scale grading, IOP measurements, ERG, SD-OCT, and fundus imaging. This will be carried out on untreated chick eyes on a limited number of chicks (n=4).
Timeframe: Months 1-6

Y2Q1 Progress: AV1013 toxicity testing was performed at 10-5 mg/mL to match the chosen ibudilast dose. Since AV1013 is an analog of ibudilast, we were not expecting to see any adverse effects from AV1013 since ibudilast toxicity testing at 4.5mg/mL did not cause cell death significantly different from the control. We also updated our clinical exam procedure by incorporating a new tonometer. Variability in IOP measurement has been drastically reduced with the new tonometer, and this will lead to more reliable data for the remainder of the project.

Y2Q2 Progress: After reviewing cell culture experiments evaluating the MIF inhibitors, AV1013 was shown to be more potent than originally expected. In light of this, toxicity testing for AV1013 was performed at 4.5mg/mL to match the maximal dose and toxicity testing that was performed with ibudilast.

Y2Q3 Progress: No progress was made as the lab was shut down due to the university's COVID-19 restrictions.

Y2Q4 Progress: Remaining data analysis for AV1013 toxicity testing at 4.5mg/mL was completed. Results show maximal dose of AV1013 does not cause significant cell death. All toxicity testing and data acquisition is now complete.

Remaining Tasks: None.

<u>Completion:</u> 27 chicks (27/27) + 18 ERG Tests (18/18) + 19 OCT Tests (19/19) + 22 normative ERG dataset chick measurements (22/22) + 12 normative OCT dataset chick measurements (12/12) + 13 TUNEL stains/analysis (13/13) + 12 DAPI INL thickness measurements (12/12) + Training & Protocol Generation (ERG,
OCT, Injection, Intubation, Slit Lamp, Fundus Imaging) (14) + Equipment (Tonopen, OCT Datastation, Fundus Camera, Injector, Isoflurane, Brooders x4, Intubation Tubes) (10) = 147 pts: 147/147 = 100%

Subtask 1b. Perform dose ranging studies for MIF inhibitors in retinal damage models. This task will be performed on a limited number of chicks (n=4) that have undergone retinal damage (RD or NMDA). They will be treated to find the optimal dose for neuroprotection with the MIF inhibitors. TUNEL analysis will be carried out on day 3 for RDs and day 1 for NMDA damaged retina. Following this study a larger study (n=10) will be carried out on day 14 (RD) or day 7 (NMDA) chicks with IHC, SD-OCT, fundus imaging, and ERG.
Timeframe: Months 1-18

Y2Q1 Progress: Ibudilast dose testing was done at 10^{-8} and 10^{-10} mg/mL in an attempt to find the minimum effective dose and see a full dose response curve. Ibudilast at 10^{-5} mg/mL remained the optimal dose in the NMDA model at this time. Preliminary CPSI-1306 dose testing showed that 10^{-8} mg/mL was the most effective dose from the doses tested (1.0, 0.001, and 10^{-8} mg/mL). Preliminary testing with AV1013 + NDMA showed reduction in TUNEL compared to NMDA + vehicle controls. Upon reviewing the data collected to this point, there was a noticeable decrease in the amount TUNEL positive cells in the most recent experiments. We ordered a new vial of NMDA, since the most probable explanation was that our NMDA stock had lost potency over time.

Y2Q2 Progress: Additional dose testing was performed with new NMDA drug stock for ibudilast at 10^{-3} and 10^{-5} mg/mL to reconfirm the neuroprotective effect of the two most promising doses. CPSI-1306 at 1.0, 10^{-3}, and 10^{-8} mg/mL, and AV1013 at 10^{-5} and 10^{-8} mg/mL were also tested again with the new NMDA stock to match the ibudilast doses, and a lower dose of 10^{-8} mg/mL was tested to account for the increased potency of AV1013.

Y2Q3 Progress: We continued with day 1 dose testing with the new NMDA drug stock for ibudilast, AV1013, and CPSI-1306 at 1.0 mg/mL to add additional animals for statistical power and attempt to find a more significantly neuroprotective dose for each drug. We continued to have high levels of variability in TUNEL positive cells between batches of chicks. The experiment was concurrently repeated with a day 13 endpoint with SD-OCT to examine longer term effects of drug treatment after NMDA damage. SD-OCT data showed no significant difference in retinal layer thickness between ibudilast 10^{-5} mg/mL treated and NMDA + vehicle eyes.

Y2Q4 Progress: We began troubleshooting again with our NMDA stock after seeing high levels of variability in retinal cell death. We repeated day 1 dose testing with high dose 4.0 mg/mL ibudilast. We also conducted an experiment to evaluate multiple stocks and a dose response of NMDA. We refined drug preparation protocols to limit variability in NMDA concentration. We also incorporated an internal control into our analysis, evaluating the fellow eye vs the treatment eye in a paired analysis. These data suggest that ibudilast at 10^{-3} mg/mL and

CPSI-1306 at 1.0 mg/mL were the most promising inhibitors and doses. Data is pending for the last experiment.

Remaining Tasks: Complete day 3 RD TUNEL experiments on three doses of ibudilast as determined from the NMDA studies. Complete expanded study (n=10) on optimal dose for RD and NMDA, D3 and D1 respectively. Complete extended timecourse study on optimal dose for RD and NMDA, D14 and D10 respectively. Complete all experiments for CPSI-1306 and AV1013. ERG and OCT tests should be performed on a subset of 3 chicks per group in future experiments (4 groups for RD, 3 groups for NMDA) at multiple timepoints. Troubleshooting with NMDA will continue as dose testing in the RD model begins.

Completion: ~265 chicks (164/268) + 42 ERG Tests (0/42) + 48 OCT Tests (12/48) + 217 TUNEL stains/analysis (132/217) + 70 DAPI INL thickness measurements (9/70) + 48 NMDA Normative chicks (48/48) + Training & Protocol Generation (ERG, OCT, Injection, Intubation, Slit Lamp, Fundus Imaging) (14) + Equipment (Tonopen, OCT Datastation, Fundus Camera, Injector, Isoflurane, Brooders x4, Intubation Tubes) (10) = 717: 389/717 = 54.3%

Subtask 1c. Drug timing studies in retinal damage models to test the impact of timing on the neuroprotective benefit of MIF inhibitors.
RD and NMDA damage models will be treated with optimized MIF inhibitor dose 2-6 hours after damage initiation. TUNEL staining and retinal thickness measurements will be taken to evaluate the impact of MIF inhibitors on retinal neuroprotection following injury.
Timeframe: Months 4-18

Y2Q1 Progress: No significant progress. Preliminary experiments had been planned but not yet started.

Y2Q2 Progress: Preliminary drug timing studies were completed with ibudilast at 10^{-5}, 10^{-3}, and 10^{-1} mg/mL, the most promising doses of ibudilast, with treatment two hours post-NMDA injection to examine the effect of an additional drug treatment after damage was induced. A second preliminary experiment with AV1013 at 10^{-5} mg/mL was also performed to match the ibudilast dose and examine if phosphodiesterase activity had an effect on neuroprotection after post-damage treatment.

Y2Q3 Progress: No progress was made as the lab was shut down due to the university's COVID-19 restrictions.

Y2Q4 Progress: A day 1 dose testing pilot study was performed with an additional ibudilast injection at four hours pre-sacrifice to better examine the effects of post-damage treatment at a longer timepoint. Data is pending.

Remaining Tasks: We need to finish staining, imaging, and analysis of all experiments done in this subtask to date. Further experiments will add additional animals to the previous experiments to increase statistical power using both models. Additional post-damage timepoints will be explored in the future if necessary.

Completion: ~90 chicks (36/90) + 70 TUNEL stains/analysis (12/70) + 20 DAPI INL thickness measurements (0/20) + Training & Protocol Generation (Injection, Slit Lamp, Fundus Imaging) (3) + Equipment (Tonopen, Injector, Isoflo, Brooders x4) (7) = 94: 58/190 = 30.5%

Task 2. Evaluate the ability of clinically relevant MIF inhibitors, CPSI-1306 and ibudilast/AV1013 to block gliosis and pathological wound healing and to protect against PVR in traumatic RD. *In vitro* studies with ARPE19 (retinal pigment epithelium) and MIO-M1 (Müller glia) cell lines and *in vivo* RD & NMDA models will be used to assess MIF inhibitor's anti-gliotic and anti-fibrotic activity.
Timeframe: Months 3-36

Subtask 2a. Test the hypothesis that MIF inhibitors block gliosis and pathological wound healing responses *in vitro*. Human RPE (ARPE-19) and Müller glia (MIO-M1) cell lines will be incubated with TGFβ to mimic PVR conditions. The effect of clinically relevant MIF inhibitors will be tested through scratch assays to assess migration, MTT assays to assess proliferation, contraction assays, and epithelial mesenchymal transition

(EMT) assays to assess which aspects of pathological wound healing may be impacted by the MIF inhibitor treatment. Additionally, the Ibudilast/AV1013 combination will allow for elucidation to the role phosphodiesterase in wound healing.
Timeframe: Months 6-30

Y2Q1 Progress: EMT assays were performed with 0.01 and 0.1mg/mL ibudilast or 0.01 and 0.1mg/mL AV1013 with TFGβ. Staining for mesenchymal cell markers smooth muscle actin and showed ibudilast and AV1013 both blocked EMT in ARPE-19 cells.

Y2Q2 Progress: Conditions of the EMT assay were optimized for ARPE-19 cells to better express epithelial markers. Staining for ZO-1 showed that AV1013 blocked TGFβ induced-EMT in ARPE-19 cells in a dose dependent manner. TUNEL staining was also performed to study the effect of varying drug concentrations on cell apoptosis.

Y2Q3 Progress: No progress was made as the lab was shut down due to the university's COVID-19 restrictions.

Y2Q4 Progress: Contraction assays were performed with MIF inhibitors in the *in vitro* PVR model.

Remaining Tasks: Continuation of experiments to finish final tests with remaining MIF inhibitors.

<u>Completion:</u> Cell line Verification (2/2) + 1 cell line * With & Without TGFβ (2) * 4 tests * 3 drugs (not including vehicle) = 26: 24/26 = 92.3%

Subtask 2b. Test the hypothesis that MIF inhibitors block gliosis and pathological wound healing responses *in vivo*. Doses of MIF inhibitors shown to block TUNEL staining will be used to evaluate the effect on retinal gliosis in our RD and NMDA damage models. Retinas will be evaluated with immunostaining for GFAP to detect gliosis, aquaporin 4 to detect Müller glia and astrocyte changes, CD45 to detect microglia accumulation, and PCNA to evaluate cell proliferation of Müller progenitor cells at day 14 (RD) and day 7/10 (NMDA). Studies will be carried out in an *in vivo* rabbit PVR model to determine if intravitreal drug administration blocks PVR formation. Retinas will be graded with the Fastenburg grading scale.
Timeframe: Months 12-36

Y2Q1 Progress: Antibody optimization began with titrating the GFAP antibody. After unsuccessful stains in NMDA treated chick eyes with an older aliquot of antibody, a new GFAP antibody was procured and successfully titrated in chick eyes. The optimized concentration for this antibody is 1:1000.

Y2Q2 Progress: No significant progress.

Y2Q3 Progress: No progress was made as the lab was shut down due to the university's COVID-19 restrictions.

Y2Q4 Progress: Optimization and troubleshooting for CD45 has begun. An antibody recommended by Dr. Fischer's lab was purchased, but preliminary stains to titrate the antibody have been unsuccessful. Troubleshooting is underway and we have consulted with Dr. Fischer on alterations to our immunostaining protocol.

Remaining Tasks: Continued optimization, troubleshooting, and validation for CD45 and GFAP need to be completed. Work with PCNA and aquaporin 4 antibodies needs to begin. Experimental groups have not yet been started.

<u>Completion:</u> ~70 chicks (0/70) + 350 stains (GFAP, AQP4, CD45, PCNA, Sox2/9) (0/350) + ~70 Rabbits (0/70) + 50 ONL Thickness measurements (0/50) + 64 ERG tests (0/64) + 68 OCT measurements (0/68) + Training & Protocol Generation (Chick & Rabbit Injection, Chick & Rabbit Slit Lamp, Chick & Rabbit Fundus Imaging) (6/12) + Equipment (Tonopen, Fundus Camera, Injector, Isoflo, Brooders x4) (8/8) = 692: 14/692 = 2.0%

Task 3. Determine the ocular pharmacokinetics of clinically relevant MIF inhibitors and their effects on IOP in rabbits. In vivo studies in untreated chicks will be performed in parallel to Aim 1 to evaluate retinal drug levels after a single injection. The MIF inhibitor that shows the greatest promise in Aims 1/2 will be further evaluated in rabbits for pharmacokinetic properties. An assay will be developed by the Pharmacoanalytical Core Facility at OSU to analyze the plasma, vitreous, and retinal drug levels. ERG and SD-OCT will be used to assess potential retinal toxicity. Systemic organ toxicity will be evaluated through necropsy by the Veterinary Core facility.
Timeframe: Months 1-36

Task 3a. Pharmacokinetic study – A screening pilot study using chick, performed in parallel to Aim 1, will be performed to evaluate drug levels in the retina. One untreated chick will be treated with one (or two) intravitreal drug injections at multiple doses and retinal drug levels will be evaluated after 1 day using the assay developed by the Pharmacoanalytical Core facility.
Timeframe: Months 1-6

Y2Q1 Progress: No significant progress

Y2Q2 Progress: No significant progress

Y2Q3 Progress: No significant progress.

Y2Q4 Progress: No significant progress.

Remaining Tasks: Experimental groups have not yet been started.

<u>Completion</u>: ~18 chicks (18) + Assay Generation (4) + Equipment (Tonopen, Injector, Isoflurane, Brooders x4) (7) = 29: 7/29 = 24.1%

Task 3b. Pharmacokinetic study – An extended pilot study using chick, performed in parallel to Aim 1, will be performed to evaluate retinal drug levels after one (or two) intravitreal treatments of MIF inhibitors in untreated animals using an extended pilot study.
Untreated chicks (n=3) will be treated with one (or two) drug injections at 1-2 doses. Retinal and vitreous drug levels will be evaluated at day 1 and day 3.
Timeframe: Months 3-12

Y2Q1 Progress: No significant progress

Y2Q2 Progress: No significant progress

Y2Q3 Progress: No significant progress.

Y2Q4 Progress: No significant progress.

Remaining Tasks: Experimental groups have not yet been started.

<u>Completion:</u> ~36 chicks (36) + Assay Generation (4) + Equipment (Tonopen, Injector, Isoflurane, Brooders x4) (7) = 47: 7/47 = 14.9%

Task 3c. Rabbit pharmacokinetic study – The MIF inhibitor drug showing the most promise in Aims 1/2 will be further evaluated in rabbits for pharmacokinetic properties.
Untreated rabbit eyes (n=3) will be intravitreally injected with the MIF inhibitor dosing scheme established in Aim 1. Fellow eyes will remain untreated. Clinical examination and IOP measurements will be performed prior to harvest for drug level evaluation. The plasma, vitreous, and retinal drug levels will be analyzed at different timepoints with assays developed by the Pharmacoanalytical Core facility at OSU. ERG, SD-OCT, and fundus imaging will be used to assess potential retinal toxicity. Systemic organ toxicity will be evaluated by necropsy at the 168 hour timepoint by the Veterinary Core facility.

Timeframe: Months 24-36

Y2Q1 Progress: No significant progress

Y2Q2 Progress: No significant progress

Y2Q3 Progress: No significant progress.

Y2Q4 Progress: No significant progress.

Remaining Tasks: Experimental groups have not yet been started.

Completion: ~45 Rabbits (45) + ~15 ERG Tests (15) + ~15 OCT Tests (15) + Training & Protocol Generation (Rabbit ERG, Rabbit OCT, Rabbit Injection, Rabbit Slit Lamp, Rabbit Fundus Imaging) (10) + Equipment (Tonopen, OCT Datastation, Fundus Camera, Injector, Isoflo) (5) = 90: 5/90 = 5.6%

What was accomplished under these goals?

A. **Subtask 1A**
   a. **AV1013 toxicity testing:** After continuing cell studies with MIF inhibitors, it appeared that AV1013 was much more potent than initially thought. Up to this point, toxicity testing for AV1013 was only completed at 10^{-5} mg/mL to match the chosen ibudilast dose. AV1013 is more soluble in water than ibudilast, but 4.5 mg/mL was chosen as the maximal dose to match ibudilast, and maximum soluble dose was not found as it was not needed. At 4.5 mg/mL, AV1013 showed an increase in TUNEL compared to vehicle control (18.8 ± 17.6 and 2.4 ±3.4, respectively, **Figure 1**), however this difference was not significant (n=3/group, p=0.42). TUNEL analysis was done in ImageJ and the p value was done with Student's two tailed t test in Excel. These results suggest that AV1013 itself is not toxic to the retina, and is unlikely to cause any adverse effects in the eye. All data for subtask 1A is now complete.

B. **Subtask 1B**
   a. **Continued dose testing for ibudilast, CPSI-1306, and AV1013:**
   Ibudilast low dose testing was done at 10^{-8} and 10^{-10} mg/mL in an attempt to find the minimum effective dose and see a full dose response curve. Ibudilast at 10^{-5} mg/mL appeared to be the optimal dose in the NMDA model in early experiments. Additional dose testing was performed with new NMDA drug stock for ibudilast at 10^{-3} and 10^{-5} mg/mL to reconfirm the neuroprotective effect of the two most promising doses. SD-OCT data showed no significant difference in retinal layer thickness between ibudilast 10^{-5} mg/mL treated and NMDA + vehicle eyes at D13. Further dose testing was conducted at day 1 with the new NMDA drug stock for ibudilast at 1.0 mg/mL. Due to variability in TUNEL response, we began experiments to evaluate different concentrations and different stocks of NMDA as part of our quality control processes (data pending). We refined drug preparation protocols to limit variability in NMDA concentration. We also repeated day 1 dose testing with high dose 4.0 mg/mL ibudilast to determine if a higher dose would be more effective; this did not reduce cell death. We also incorporated an internal control into our analysis, evaluating the fellow eye vs the treatment eye in a paired analysis (**Fig 2**). These data suggest that ibudilast at 10^{-3} mg/mL and CPSI-1306 at 1.0 mg/mL were the most promising inhibitors and doses (**Fig 3, 4**). Data is pending for expanded testing at these doses. Dose testing for AV1013 does not show any neuroprotective effect (**Fig 5**).

C. **Subtask 1C**
   a. **Drug timing studies:** To begin drug timing studies, a third dose of ibudilast and AV1013 at 10^{-5} was injected two hours post NMDA injection to evaluate the effect of post-damage treatment on retinal cell survival. AV1013 did not provide neuroprotective benefit. Ibudilast did reduce TUNEL response, although not significantly (**Fig 6**). The experiment was repeated with ibudilast at 10^{-3} and 10^{-1} mg/mL at two hours post damage and with 1.0 mg/mL four hours pre-sacrifice to examine the effects of longer timepoints and higher dosing. Data is pending.

D. Subtask 2A

a. **Gel contraction assays:** An important aspect of wound healing related to PVR is cell contraction. We performed ARPE-19 gel contraction assays with three-dimensional collagen gels as an *in vitro* PVR model. Using a Cell Biolabs CytoSelect 24-well Cell Contraction Assay kit and following the manufacturer's protocol, we measured the contraction over a period of 24 hours. The contraction ratio was calculated by measuring the area of the gel at 24 hours and dividing it by the initial area at time 0. The ratios are shown as percent of control. MIF inhibitors were tested at two different concentrations for their ability to inhibit ARPE-19 gel contraction. Inhibition of contraction indicates potential to inhibit a mechanism of retinal scarring that occurs in the cicatricial condition of PVR (**Fig 7, 8**).

b. **EMT assays:** EMT assays were performed with 0.01 and 0.1mg/mL ibudilast or 0.01 and 0.1mg/mL AV1013 with TFGβ treated ARPE-19 cells and controls. Staining for mesenchymal cell markers showed ibudilast and AV1013 both blocked EMT. After 5 days of serum starvation, ARPE-19 cells were either left untreated (starved) or treated with 10 ng/mL TGFβ with or without the indicated concentrations of AV1013 or ibudilast for 48 hours. Cells were fixed in 4% paraformaldehyde and stained for the mesenchymal markers vimentin and SMA and epithelial cell marker zonula occludens (ZO-1) to determine level of EMT inhibition by MIF (**Fig 9, 10**).

c. **Proliferation assays.** MTT assays with CPSI-1306, ibudilast, and AV1013 were performed to evaluate cell proliferation of TGFb treated ARPE-19 cells (**Fig 11**). CPSI-1306 did not alter proliferation but proliferation was blocked significantly by AV1013 and to a lesser degree by ibudilast (**Fig 11**). TUNEL staining was also performed to rule out cell death caused by the MIF inhibitors. H_2O_2 at 1mM was used as a positive control for cell death; MIF inhibition did not increase cell death (**Fig 12**).

E. Subtask 2B

a. **Antibody optimization:** GFAP antibody titration was successful in both chick and mouse tissue. GFAP is an intermediate filament that is upregulated in gliotic conditions, so we expect to see increased fluorescence in NMDA treated chicks compared to vehicle treated. While titration was successful, more optimization may be needed to evaluate differences in fluorescence between treatment conditions when experiments for this subtask begin. In preliminary stains, 1:1000 was identified as the working concentration for this antibody (**Figure 13**). Western blotting with retinal lysates will be used to validate the target protein of this antibody. CD45 optimization and troubleshooting has begun. Preliminary titration staining has been unsuccessful. After consulting with Dr. Fischer's lab, we have made changes to our immunostaining protocol for this stain, and higher concentrations of antibody (1:200 and 1:100) will be titrated.

F. OCT

a. **Two week timecourse**: Completed a 13 day timecourse to examine long-term effects of ibudilast treatment with a dose of 10^{-5} mg/mL. Intravitreally injected 20µL of ibudilast +NMDA in the left eye and vehicle control (sterile water) in the right eye (n=6). Performed OCT and clinical exams with fundoscopy at day 1 and day 13. Histology data is pending. OCT results show treatment with ibudilast did not significantly prevent loss of retinal thickness after NMDA damage at 13 days (**Figure 14**).

b. **New normative database**: A new normative database has been acquired with a total of 20 chicks (10 undamaged, 10 NMDA damaged at 1, 2, 3, 4, and 5 weeks post hatch). While data has been captured, data processing is still ongoing.

G. Celeris ERG

a. **Established new ERG machine protocol:** With the assistance of Dr. Julie Racine, we developed a protocol for testing chicks on the new Celeris ERG system. Using a Weck-Cel sponge, the bottom eyelid is moved out of the way to position each electrode on the cornea along the eye's axis of vision. Once both electrodes are placed, the impedance of the connection is checked and adjustments to the electrode placement are made if necessary. In clinical settings, if impedence is less than 10 kilo-ohms, it is considered a good connection. We are able to reliably achieve impedances between 2 and 3 kilo-ohms. Once a good connection is established, we begin the protocol which consists of 20 steps. The steps are as follows: 1) flash ERG at 5 cd.s/m^2, 2) flash ERG at 0.05 cd.s/m^2, 3) flash ERG at 0.1 cd.s/m^2, 4) flash ERG at 0.2 cd.s/m^2, 5) flash ERG at 0.3 cd.s/m^2, 6) flash ERG at 0.4 cd.s/m^2, 7) flash ERG at 0.6 cd.s/m^2, 8) flash ERG at 1 cd.s/m^2, 9) flash ERG at 2 cd.s/m^2, 10) flash ERG at 3 cd.s/m^2, 11) flash ERG at 5 cd.s/m^2, 12) flash ERG at 8 cd.s/m^2, 13) flash ERG at 10 cd.s/m^2, 14) flash ERG at 13 cd.s/m^2, 15) flash ERG at 25 cd.s/m^2, 16) flicker ERG at 15Hz at 5 cd.s/m^2, 17) flicker ERG at 20Hz at 5 cd.s/m^2, 18) flicker ERG at 25Hz at 5 cd.s/m^2, 19) ON/OFF ERG at 5 cd.s/m^2, and 20) ON/OFF ERG at 10 cd.s/m^2 (**Figure 15**). These data are exported directly into excel for later processing.

b. New normative database: A new normative database utilizing the Celeris ERG machine has been acquired with a total of 20 chicks (10 undamaged, 10 NMDA damaged at 1, 2, 3, 4, and 5 weeks post hatch). While data has been captured, data processing is still ongoing.

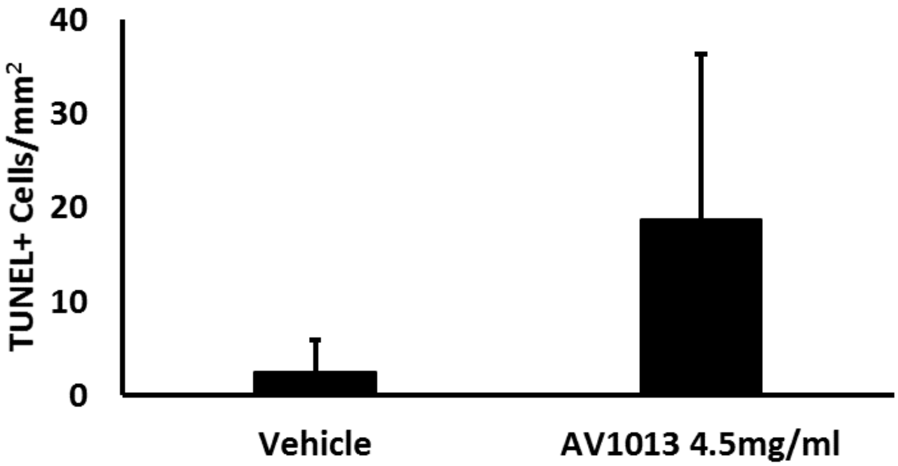

Figure 1. Maximal dose of AV1013 does not cause retinal toxicity. AV1013 at 4.5 mg/mL (18.8 ± 17.6) did not cause significant increase in TUNEL response in retinal cells when compared to vehicle control (2.4 ±3.4, n=3/group, p=0.42). This suggests AV1013 is not toxic to retinal cells and will not cause adverse effects.

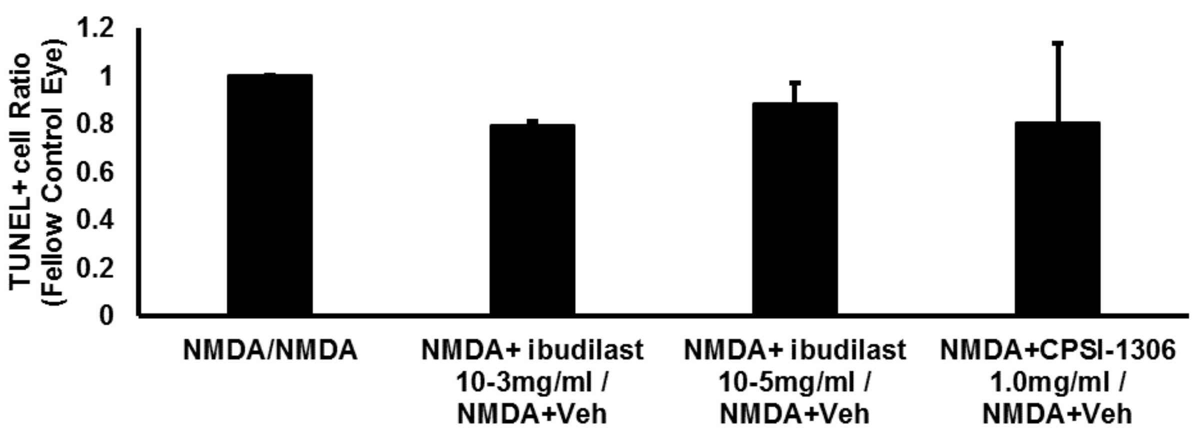

Figure 2. Paired eye cell death analysis. MIF inhibitor treatment eye was divided by the NMDA damaged fellow eye, and the ratio is presented here. NMDA (1.0±0.0), ibudilast 10^{-3} mg/ml (0.79±0.03), ibudilast 10^{-5} mg/ml (0.88±0.15), and CPSI-1306 1.0 mg/ml (0.81±0.66) exhibited the greatest reduction in cell death compared to their fellow eye controls. Error bars shown as standard error of the mean.

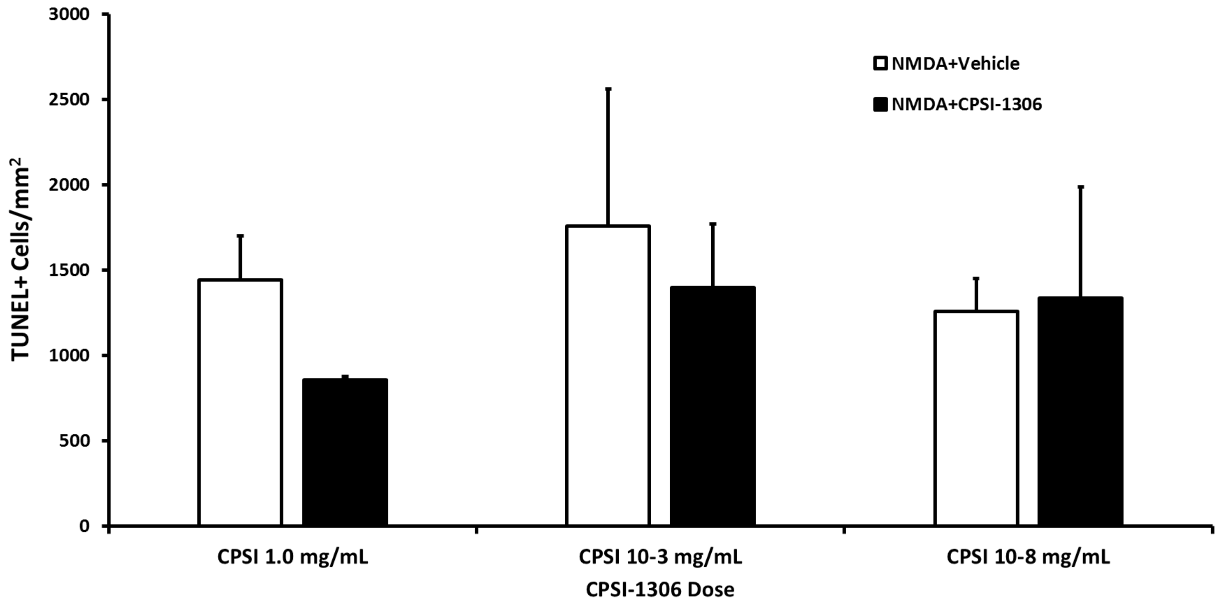

Figure 3. CPSI-1306 reduces TUNEL in a dose dependent manner. CPSI-1306 at 1.0 mg/mL had the most effect of the doses tested. Compared to control (1442.8±260.8), treatment with 1.0 mg/mL reduced TUNEL but did not reach significance (855.9±23.1, $p=0.18$). Treatment with 10-3 mg/mL trended to reduce cell death compared to control (1396.6±376.5 vs 1760.8±799.1, respectively, $p=0.64$). CPSI-1306 at 10-8 mg/mL did not reduce cell death after NMDA treatment (1338.0±650.8 vs 1257.5±195.0, $p=0.91$). Higher doses are needed to significantly reduce TUNEL response. Larger subject numbers could cause significance to be achievable.

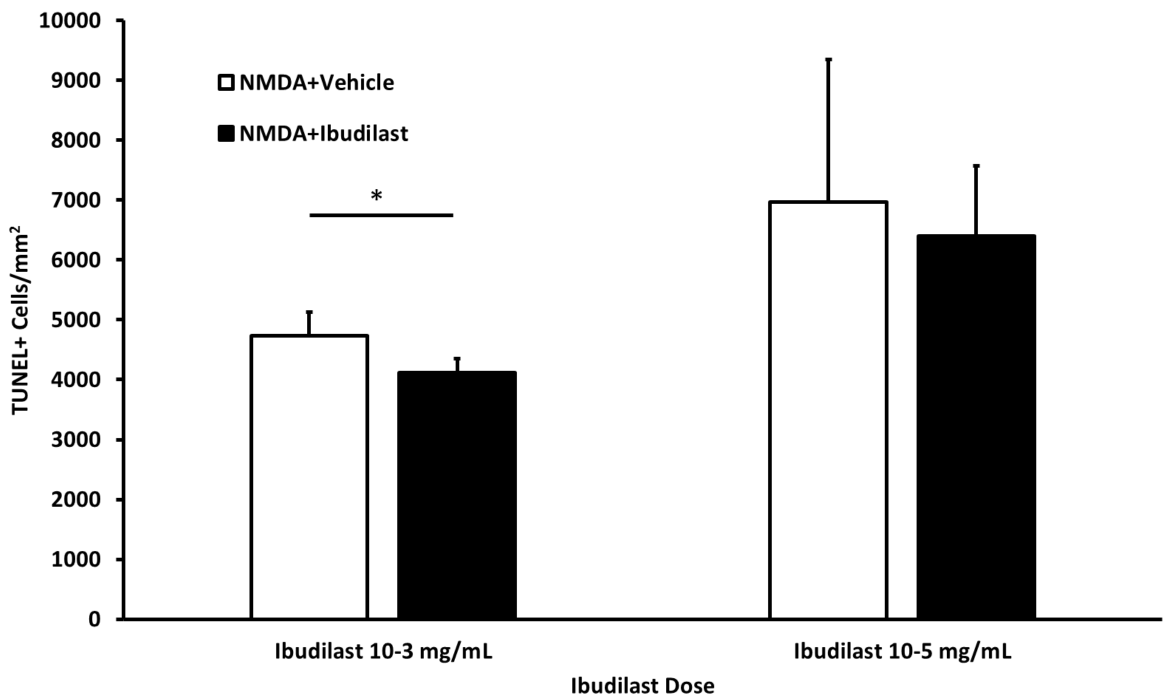

Figure 4. Ibudilast at 10^{-3} mg/mL significantly reduced TUNEL response in retina cells. Experiments with a fresh stock of NMDA where both eyes were injected with NMDA. The left eye received either ibudilast at 10^{-3} mg/ml or 10^{-5} mg/ml while the right eye control received vehicle (sterile water). In earlier experiments where NMDA was only injected in the drug treated eye, we had found 10^{-5} mg/ml was the most promising dose. However, with a fellow eye NMDA control we found that 10^{-5} mg/ml did not achieve significance ($p=0.592$). A higher dose of 10^{-3} mg/ml, did achieve significance ($p=0.025$) leading us to reevaluate the higher ibudilast drug dosages.

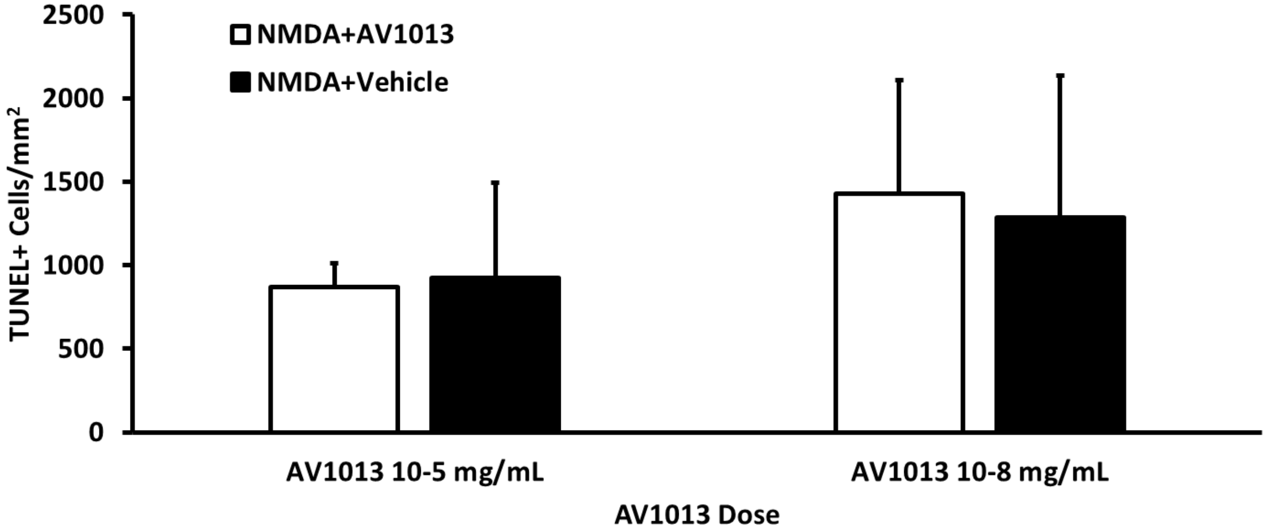

Figure 5. AV1013 does not reduce TUNEL response after NMDA damage. AV1013 was tested at 10^{-5} and 10^{-8} mg/mL to match preliminary ibudilast dose testing. Treatment with 10^{-5} mg/mL did not reduce TUNEL compared to NMDA + vehicle (867.2±142.0 vs 924.6±570.7, respectively, p=0.84). Treatment with 10^{-8} mg/mL also did not have an effect compared to NMDA + vehicle (1430.4±675.8 vs 1288.1±848.8, p=0.82).

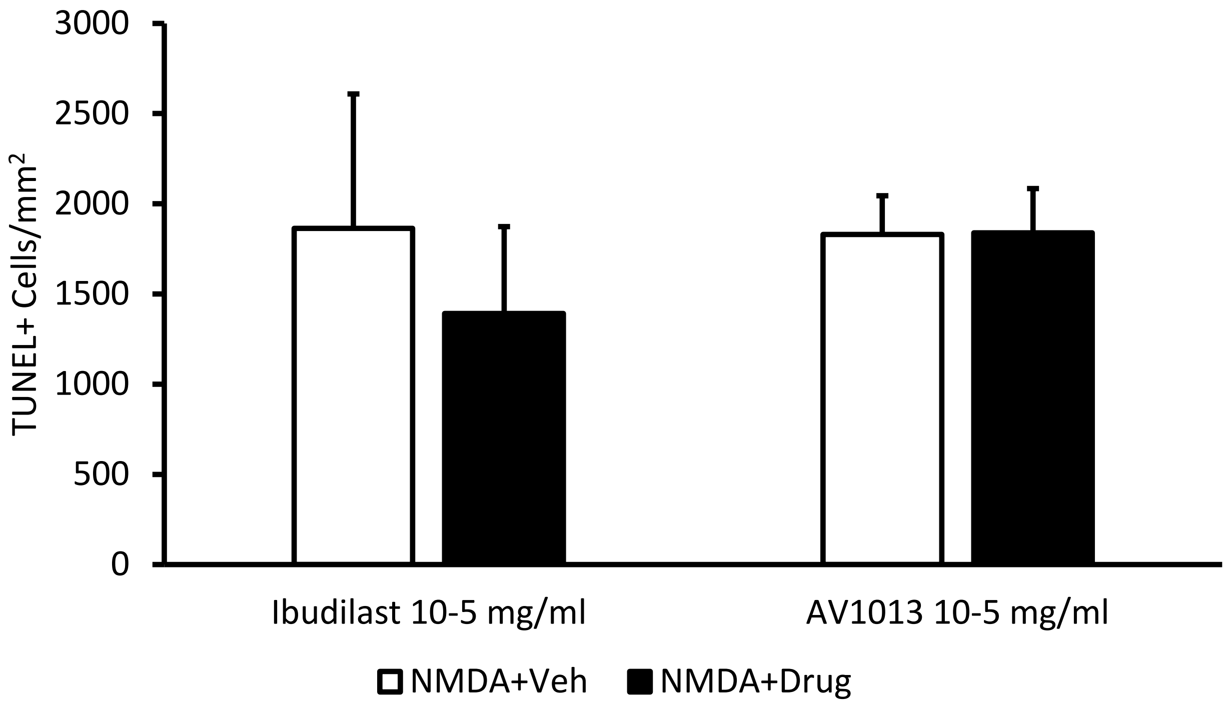

Figure 6. Dose timing studies with ibudilast and AV1013 at 10^{-5} mg/ml. NMDA was injected at time T=0. Drugs (ibudilast or AV1013) were injected two hours following the NMDA injection at time T=2hr. While neither drug showed a significant difference with the fellow eye vehicle control (ibudilast, p=0.225, AV1013, p=0.948), ibudilast showed a trend towards a lower cell death count.

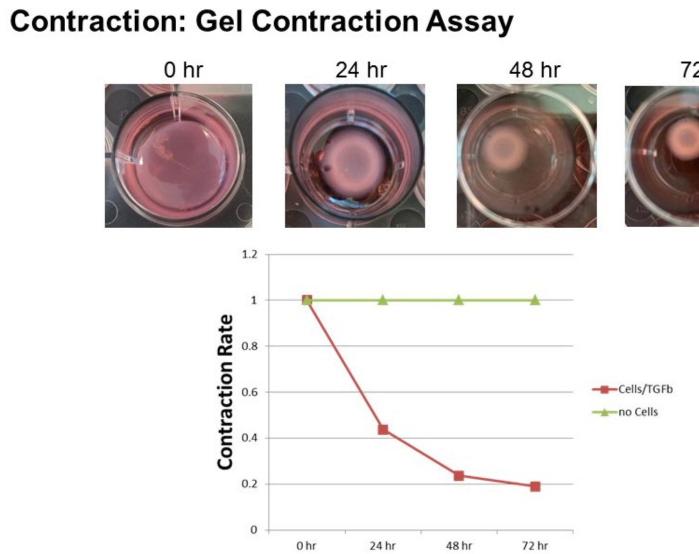

Figure 7. Gel Contraction Assay. ARPE-19 gel contraction assays show TGFβ-induced gel contraction and kinetics.

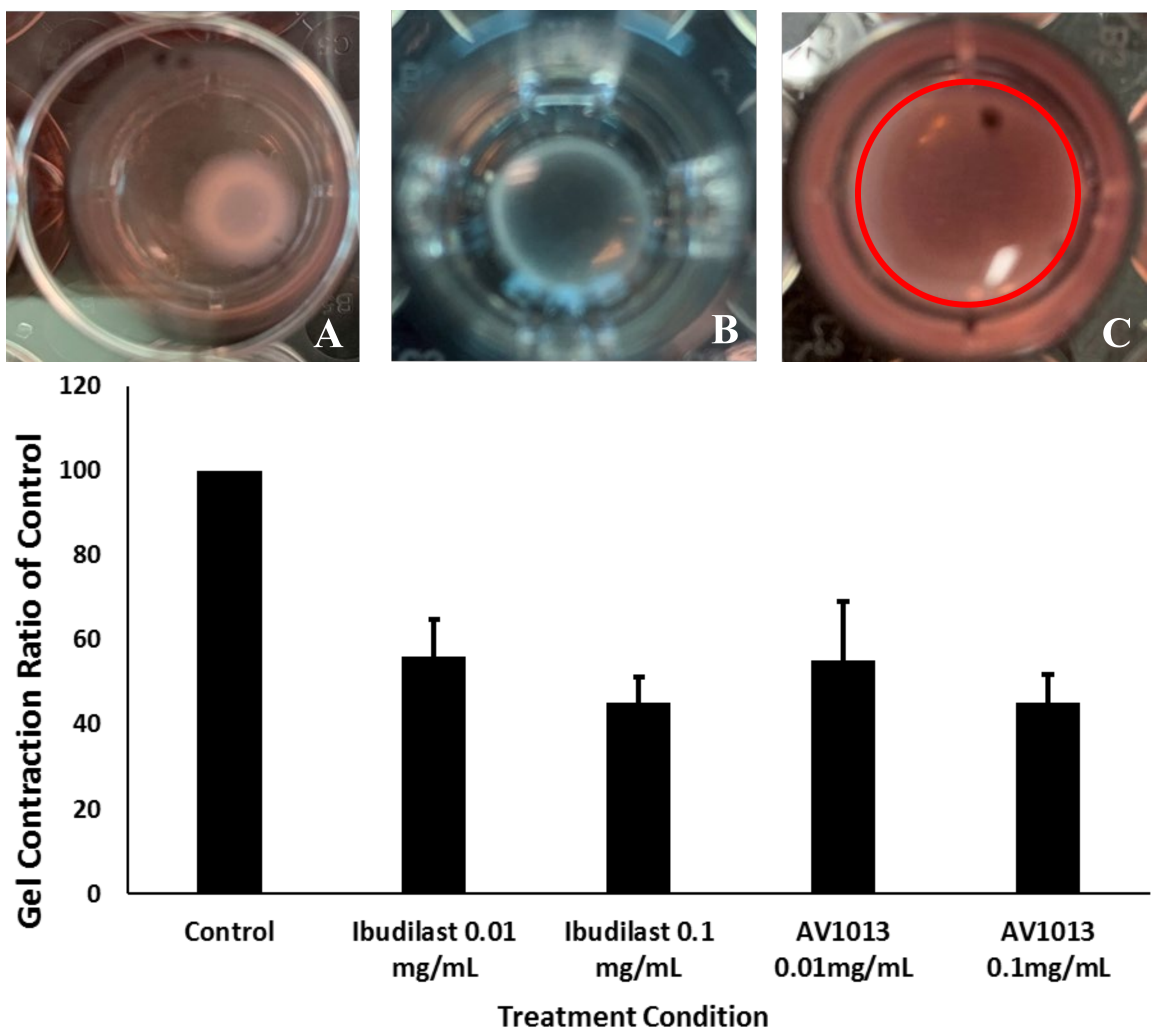

Figure 8. Ibudilast and AV1013 significantly block gel contraction. ARPE-19 gel contraction assays show representative results of (A) Control with maximal contraction (B) Ibudilast 0.01mg/ml, (C) Ibudilast 0.1mg/ml. Below is quantitation of gel contraction which shows significant decrease in contraction compared with control

for ibudilast 0.01mg/ml (P=0.004), ibudilast 0.1mg/ml (P=1.2426 x E-06), AV1013 0.01mg/ml (P=0.12), and AV1013 1mg/ml (P=3.96221E-07).

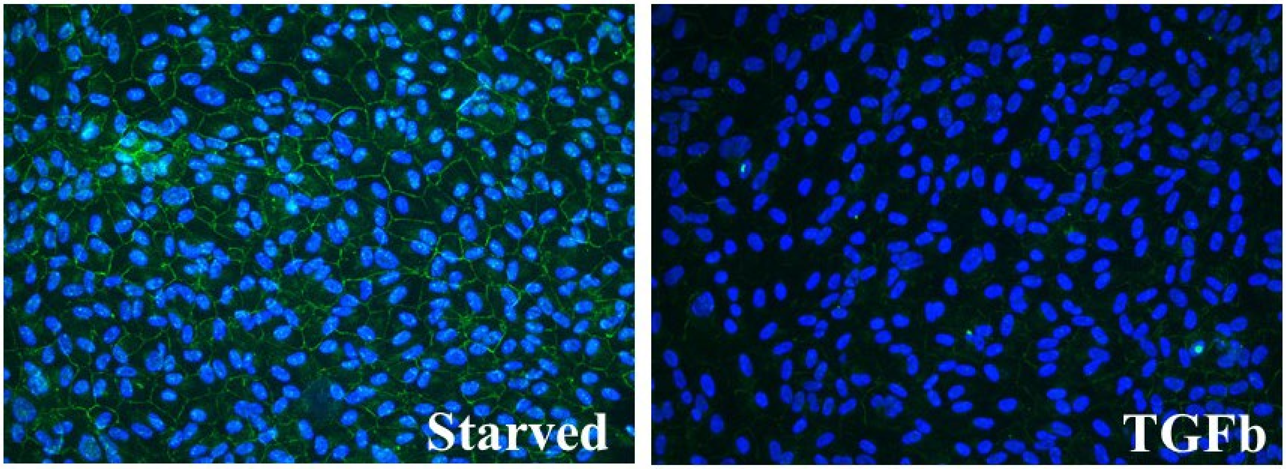

Figure 9. EMT is induced in the TGFβ ARPE-19 *in vitro* PVR model: Epithelial marker ZO-1 is decreased in TGFβ treated ARPE-19 cells.

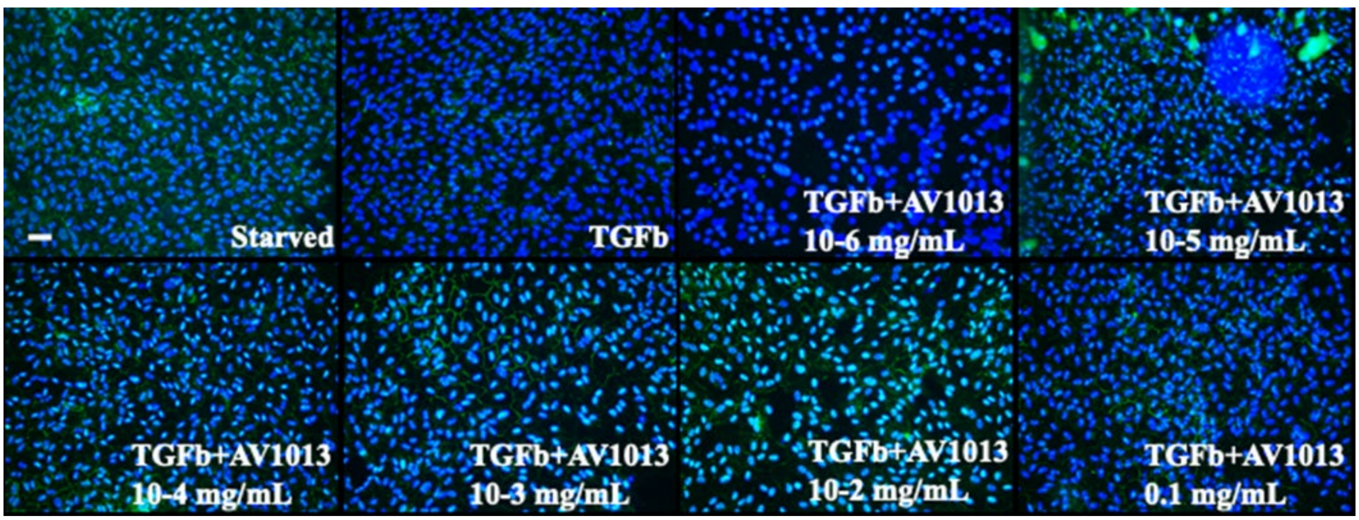

Figure 10. MIF inhibition by AV1013 blocks loss of ZO-1. Phosphodiesterase inhibitor activity is not essential for blocking EMT in the *in vitro* PVR model. ZO-1, green. DAPI, blue. Scale bar 50μm.

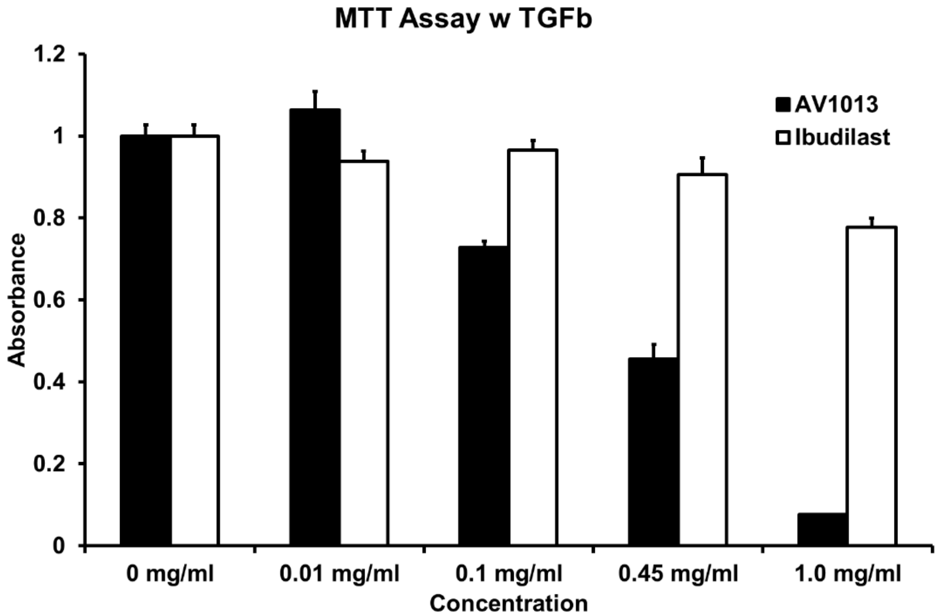

Figure 11. Proliferation of TGFβ treated ARPE-19 is blocked by MIF inhibitor AV1013 more than ibudilast by MTT assay. CPSI-1306 did not reduce proliferation (data not shown).

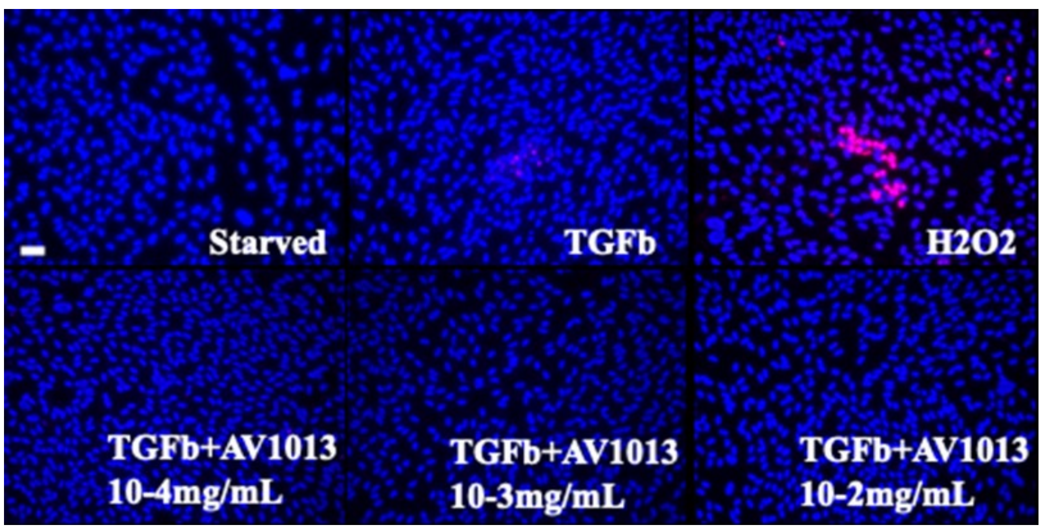

Figure 12. MIF inhibition does not induce apoptosis in ARPE-19 cells. H_2O_2 was used as a positive control to induce cell death. TUNEL, red. DAPI, blue. Scale bar 50μm.

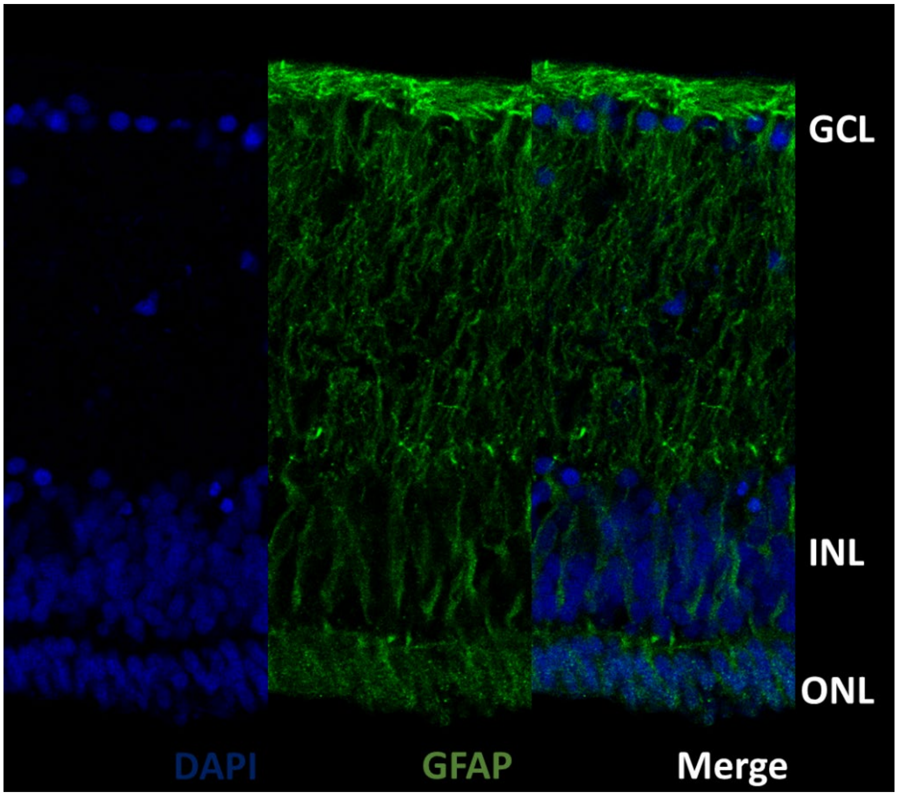

Figure 13. GFAP immunofluorescence in the chick retina. An anti-GFAP antibody was titrated in the chick retina in both NMDA treated and vehicle control eyes. This GFAP pattern (green) is seen in an NMDA treated eye stained with a 1:1000 working concentration of antibody. DAPI, blue. GFAP, green.

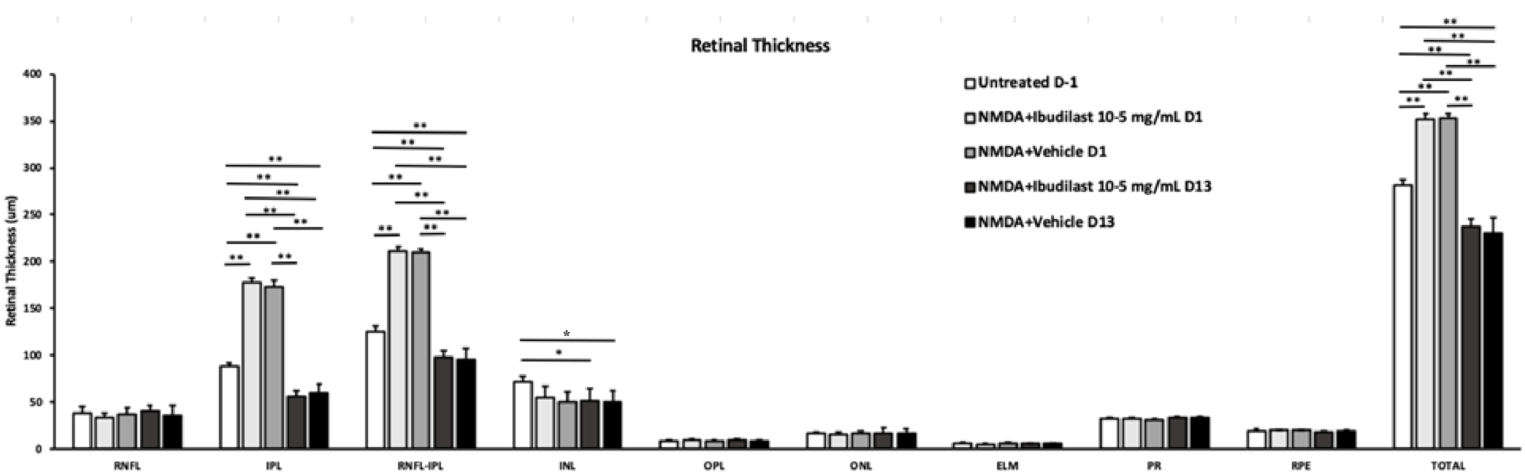

Figure 14. OCT data show 10-5 mg/mL ibudilast did not significantly save retinal thickness loss from NMDA damage. This experiment suggests that the new NMDA stock is more potent, since 10-5 mg/mL has previously shown more neuroprotection. There is greater loss of INL thickness compared to previous normative studies. Larger measurements at D1 conditions are likely due to swelling from intraocular injections, which was also greater than in prior studies. Bars indicate values are significantly different. '*' indicates a p value of <0.05, and '**' indicates a p value of <0.0001.

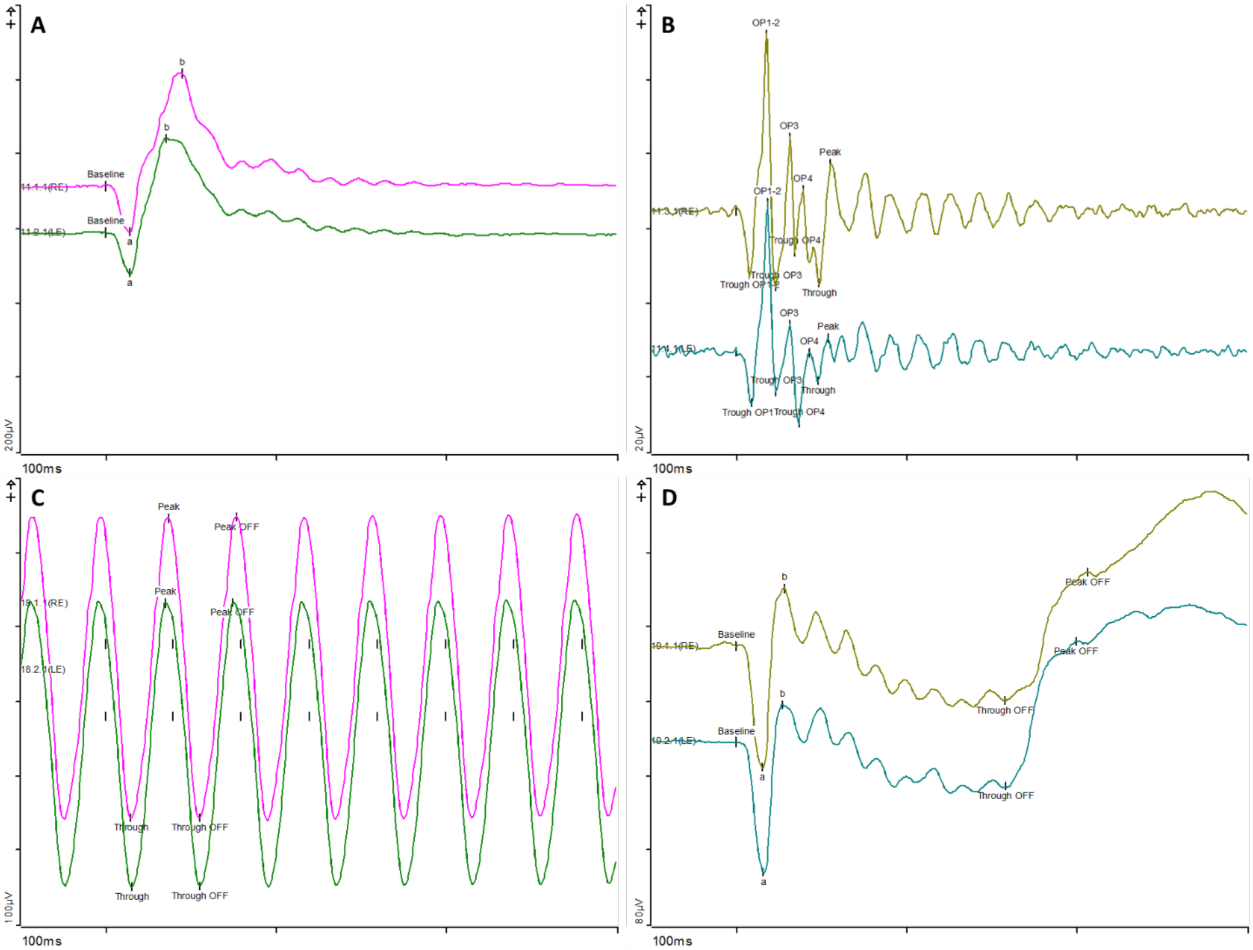

Figure 15. Representative ERG waveforms with new Celeris ERG system. (A) Flash ERG at 5 cd.s/m^2 showing a- and b-waves, (B) Flash ERG oscillatory potentials at 5 cd.s/m^2 showing OP1-2, OP3, OP4, and OP5, (C) 25Hz flicker waveform at 5 cd.s/m^2 showing two trough to peak measurements, and (D) ON/OFF waveform at 5 cd.s/m^2 showing the ON bipolar cell responses and the OFF bipolar cell response.

What opportunities for training and professional development has the project provided?

This project has continued to provide ample opportunity for undergraduates to learn new lab skills under the supervision of senior lab members. Undergraduates gained hands-on experience in histology, immunohistochemistry, microscopy, and data analysis. Some volunteers have aided in TUNEL analysis and INL thickness analysis. The project introduced them to the rigors of pre-clinical and translational research, and continues to provide opportunities to teach about experimental design. The project also continues to provide senior lab members the opportunity to teach and mentor aspiring scientists. Dr. Cebulla and Tyler Heisler-Taylor continue to mentor Master's Optometry student Richard Wan on data analysis, manuscript writing, and experimental design for his master's thesis. Elizabeth Urbanski is primarily responsible for training and supervising the undergraduates, with assistance from Sumaya Hamadmad and Dr. Cebulla.

Dr. Julie Racine continues to collaborate with the lab on ERG experiments, and she has been instrumental in mentoring Tyler in many aspects of visual electrophysiology, particularly data interpretation. Collaborations with other labs that were made possible due to the ERG have continued.

Lab members have abstracts on the project published in Investigative Ophthalmology and Visual Science that were accepted for presentation at ARVO 2020 before its cancellation. These poster presentations were adapted into narrated powerpoint presentations that were presented virtually at OSU's eARVO.This virtual conference was a collaboration and brought together many vision researchers and physicians from across the university to share exciting results in basic, translational, and clinical vision research.

Professional Development Activities:
A manuscript is in preparation.

How were the results disseminated to communities of interest?

At OSU's eARVO, two lab members presented data from the project. Sumaya presented on the in-vitro experiments relating to wound healing mechanisms in retinal pigment epithelial cells, and how MIF inhibitors impacted these mechanisms. Elizabeth presented on the ibudilast-associated reduction in TUNEL response after NMDA damage. Tyler presented chick ERG data at the OSU Ophthalmology Research Day conference, comparing data from untreated versus NMDA damaged eyes that was acquired as part of the ERG normative database. Abstracts for these presentations were published in the ARVO journal Investigative Ophthalmology and Visual Science.

What do you plan to do during the next reporting period to accomplish the goals?

In the next year, we will aim to complete dose testing in both the NMDA and RD models. RD methodology has been taught, and lab members have done practice RDs to gain experience in this difficult technique before beginning dose testing experiments in this model. Preliminary RD dose testing studies are planned and will commence soon. We will also aim to complete drug timing studies and acquire SD-OCT and ERG data to complete Task 1. Cell studies are progressing and will be completed soon. We will also continue antibody optimization and validation to continue making progress on subtask 2b. Additionally, we will complete pending data analysis from previous experiments, including the dose timing experiments and the recent NMDA stock comparison experiment.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The chick is an excellent model for vision research and the studies performed herein will expand the utility of the model for other vision scientists. The normative database studies on retinal structure and function (particularly with spectral domain optical coherence tomography (SD-OCT) imaging and electrophysiology studies) in normal and damage responses will expand knowledge of the chick eye as it relates to ophthalmic research. By understanding long-term effects of retinal detachment and excitotoxic damage on the chick eye, researchers will be able to extrapolate data from chicks used in research to gain further insight into what happens in the human eye under the same conditions. Chick and human eyes have two important similarities – a cone-driven retina, and the presence of a central high-density photoreceptor region (macula and area centralis), the point in the retina where visual acuity is highest. Having these features makes the chick eye an especially good model for the human eye, and a favorable alternative to mouse models of eye diseases. Mice dominate animal research for their well-understood genetics, and the ease and diversity of genetic manipulation available. However, mice also lack a macula and have a rod-driven retina as well as very low visual acuity. These differences from the human eye make mice a less-than-ideal model for studying ocular diseases and retinal pathologies. Continued study of normative measurements will allow for further understanding of the chick model of retinal damage. Further development and understanding of the chick retinal damage model may lead to its increased use in ophthalmic research as a fitting model for the human eye.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Given the necessary troubleshooting with our supply of NMDA, we reevaluated how we are storing the drug. With the first vial of NMDA, we diluted the full amount to the stock concentration of 0.1M in sterile saline, aliquoted the solution, and stored the aliquots at -20C until use. When we noticed more variability and ordered the new vial of NMDA, we changed protocols. NMDA powder was stored at 4C until reconstitution, and a small amount of 0.1M stock would be prepared fresh when preparing for an experiment. Keeping the NMDA in powder form until needed did not have any apparent effect on the potency of the drug as we predicted. Upon reviewing all collected TUNEL data, measuring small quantities of NMDA powder may have caused unexpected fluctuations in dosing. Going forward, whole vials of NMDA will be diluted and aliquoted to remove variability in the stock concentration. We expect that variability in TUNEL response will decrease to some degree due to this change.

Actual or anticipated problems or delays and actions or plans to resolve them

Due to university COVID-19 restrictions, the lab was closed from mid-March to mid-June. We were unable to finish data acquisition or conduct any experiments during that time. Upon reopening the lab, we adapted to scheduling lab work so as to maintain social distancing, especially in our procedure room. Undergraduates were also not allowed back in lab. Given this, lab work took longer to complete and took careful planning of use of the lab space. We continue to adhere to these adaptations, which marginally slow our efficiency.

Post-doc Mohd Hussain Shah's contract ended September 30th of 2020. Due to ongoing COVID-19 response measures by the university, we expect a delay in being able to hire a new post-doc. Progress on the project will be slightly slower than originally planned while we have reduced manpower. To compensate, undergraduates receiving pay or research credit will take an increased role in data acquisition and analysis under supervision.

After our last chick order for 2020 is received, we will have used 466 chicks of the 470 approved on our protocol. In order to resume animal work in early 2021, an amendment to our animal protocol is necessary to increase the allotted number of chicks. When the last chick experiment of the year is complete, we will submit an amendment for IACUC and ACURO approval. This may delay animal work for early 2021 until the amendment is approved. Meyer hatchery also does not offer hatchling chicks after the first week of November, so no new chick experiments will occur for the remainder of the year.

Changes that had a significant impact on expenditures

As in year 1 of the project, we came in under-budget for year 2. We still do not have a graduate student receiving pay from the project funds, and our paid undergraduates were not allowed in the lab due to COVID-19 restrictions. While the costs of PPE and some commonly used lab reagents have increased in recent months, we managed to use less materials than anticipated which saved on costs. We also did not conduct any lab work for a period of three months while the lab was shut down due to the university's restrictions. Since we plan to request a one year no cost extension at a later date, we have tried to be economical in our expenditures in order to fund a fourth year of the project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to Report.

Books or other non-periodical, one-time publications.

Elizabeth G Urbanski; Tyler N Heisler-Taylor; Richard Wan; Mohd Hussain Shah; Sumaya Hamadmad; Andrew J Fischer; Abhay Satoskar; Colleen M Cebulla. MIF Inhibitor Ibudilast Prevents Retinal Cell Death in Chick Excitotoxic Retinal Damage Model. Investigative Ophthalmology & Visual Science June 2020, Vol.61, 2478. Abstract. Published.
Acknowledgement of Federal Support: Yes

Sumaya Hamadmad; Alana Reese; Elizabeth Urbanski; Tyler Heisler-Taylor; Colleen M Cebulla. Differential effects of MIF inhibitors on an *in vitro* PVR model. Investigative Ophthalmology & Visual Science June 2020, Vol.61, 3054. Abstract. Published.
Acknowledgement of Federal Support: Yes

Tyler N Heisler-Taylor; Richard Wan; Hailey Wilson; Elizabeth G Urbanski; Mohd Hussain Shah; Julie Racine; Colleen M Cebulla. Effects of age on chick light adapted ERGs with and without inner retinal excitotoxic damage. Investigative Ophthalmology & Visual Science June 2020, Vol.61, 2238. Abstract. Published.
Acknowledgement of Federal Support: Yes

Other publications, conference papers and presentations.

Elizabeth G Urbanski; Tyler N Heisler-Taylor; Richard Wan; Mohd Hussain Shah; Sumaya Hamadmad; Andrew J Fischer; Abhay Satoskar; Colleen M Cebulla. MIF Inhibitor Ibudilast Prevents Retinal Cell Death in Chick Excitotoxic Retinal Damage Model. PowerPoint presentation by Elizabeth Urbanski at OSU eARVO 2020.
Acknowledgement of Federal Support: Yes

Sumaya Hamadmad; Alana Reese; Elizabeth Urbanski; Tyler Heisler-Taylor; Colleen M Cebulla. Differential effects of MIF inhibitors on an *in vitro* PVR model. PowerPoint presentation by Sumaya Hamadmad at OSU eARVO 2020.
Acknowledgement of Federal Support: Yes

Tyler N Heisler-Taylor; Richard Wan; Hailey Wilson; Elizabeth G Urbanski; Mohd Hussain Shah; Julie Racine; Colleen M Cebulla. Effects of age on chick light adapted ERGs with and without inner retinal excitotoxic damage. PowerPoint presentation by Tyler Heisler-Taylor at Ophthalmology Research Day 2020.
Acknowledgement of Federal Support: Yes

- **Website(s) or other Internet site(s)**

  Nothing to Report.

- **Technologies or techniques**

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report.

- **Other Products**

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Dr. Colleen M Cebulla
No change.

Name: Dr. Andy J Fischer
Project Role: Co-investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Recommended new CD45 antibody, provided troubleshooting and feedback on updated immunostaining protocol

Name: Dr. Abhay Satoskar
No change.

Name: Dr. Julie Racine

No change.

Name: Tyler Heisler-Taylor
No change.
Funding Support: Ohio Lions Eye Research Foundation Norbert Peiker Eye Research Fellowship

Name: Mohd Hussain Shah
No change.

Name: Elizabeth Urbanski
No change.

Name: Richard Wan
No change.

Name: Sumaya Hamadmad
No change.

Name: Hailey Wilson
No change.

Name: Bayan Shalash
No change.

Name: Krupa Patel
No change.

Name: Mohamed Soumakieh
Project Role: Undergraduate Research Volunteer
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Recorded clinical exam data during animal experiments, completed TUNEL staining and microscopy, performed literature review.

Name: Yushin Jeng
Project Role: Undergraduate Research Volunteer
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Recorded clinical exam data during animal experiments, completed TUNEL staining and microscopy, performed literature review.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Colleen M Cebulla
Status: Previously Active, Now Closed
Title: Evaluation of an OCA2 enhancer variant as a modifier of the phenotype of BAP1-tumor predisposition syndrome
Grant Number: R21CA219884
Role: Co-Investigator
Sponsoring Organization: NCI/NIH
Dates Active: 06/18/2018-05/31/2020

Status: Active
Title: Full Field OCT for Cellular Level Structural and Functional Retinal Imaging
Grant Number: R01EY031098 (MPIs: Doble, Zawadzki) 9/01/2020- 08/31/2024

Sponsoring Organization: NEI/NIH
Role: Co-Investigator
Dates Active: 9/01/2020- 08/31/2024

Andrew J Fischer
Status: Pending
Title: Fatty acid binding-proteins and endocannabinoids in the retina; roles in glial reactivity and reprogramming of Muller glia into progenitor cells
Grant Number: RO1 EY032141-01
Role: PI
Sponsoring Organization: NIH/NEI
Dates Active: 02/01/2021 – 01/31/2026

What other organizations were involved as partners?

Nationwide Children's Hospital
Columbus, OH
Dr. Julie Racine continues to collaborate with and advise the lab on ERG experiments. She has also generated several protocols using Diagnosys software for our experiments.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Nothing to Report.

QUAD CHARTS: See attached.

9. APPENDICES:

See attached.