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TITLE: Macrophage Migration Inhibitor (MIF) Therapeutics for Neuroprotection and Prevention of Scar in Traumatic Retinal Detachment

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14. ABSTRACT Retinal detachment (RD) is one of the gravest military-relevant injuries to the visual system caused by ocular trauma. Severe vision loss and blindness result from the death and degeneration of the photoreceptors as well as from excessive retinal scar formation, a disorder known as proliferative vitreoretinopathy (PVR), which occurs at elevated rates following ocular trauma. Blast injury is also a common component of battlefield trauma and a major molecular mechanism is excitotoxic damage with N-Methyl-D-aspartic acid (NMDA) receptor activation. There is currently inadequate mitigation and treatment of these types of retinal damage following ocular trauma. Inflammation plays a critical role in these vision-threatening processes following RD. This proposal stems from our recently published work which identified macrophage migration inhibitory factor (MIF) as a key player in retinal injury associated with RD and unpublished work on NMDA-mediated retinal damage. MIF is a pro-inflammatory cytokine with a key role in innate and acquired immune responses. In the murine RD model, systemic administration of the MIF inhibitor ISO-1 significantly blocked photoreceptor apoptosis, outer nuclear layer thinning, and retinal gliosis. Our work on a chick model, which has a cone-rich retina and similar damage responses to humans following RD, demonstrates that MIF is upregulated following NMDA-induced excitotoxicity. This elevation in MIF levels is associated with increases in cell apoptosis in the chick retina. Administration of intravitreal ISO-1 protected the retina from the NMDA-induced damage. These pre-clinical data have clinical relevance; elevated MIF levels have been detected in the vitreous and subretinal fluid of patients with RD developing PVR compared with those without PVR(13, 14), providing a rationale for future clinical therapy in high-risk RDs. Several MIF inhibitors are currently in clinical evaluation for a variety of other diseases, including rheumatoid arthritis, diabetes, and cancer. Ibudilast, an inhibitor of MIF has already been approved in Japan as an anti-inflammatory agent and is currently undergoing clinical trials in the US as a neuroprotective agent in amyotrophic lateral sclerosis and multiple sclerosis. CPSI-1306, a derivative of ISO-1, displays improved clinical properties over its predecessor.					
15. SUBJECT TERMS None listed.					
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1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Our long-term objective was to develop improved therapeutics, for local delivery to retinal detachment and penetrating or perforating injuries, which are both neuroprotective for photoreceptors and other retinal neurons and inhibit pathological gliotic responses that lead to vision loss. Since MIF promotes photoreceptor apoptosis and retinal gliosis and it is known to antagonize the anti-inflammatory effects of glucocorticoids, which are the standard pharmacologic treatment used in traumatized eyes, we hypothesized that clinically relevant MIF inhibitors, that target the inflammatory response, would yield benefit in retinal neuroprotection and scar prevention in traumatic retinal damage. We tested the ability of promising agents identified based on our preliminary data to enhance preservation of tissue form and function.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

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

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Task 1. Evaluation of clinically relevant MIF inhibitors, CPSI-1306, ibudilast/AV1013 in our *in vivo* retinal damage models (RD and NMDA) for neuroprotection of photoreceptors.

The clinically relevant MIF inhibitors CPSI-1306, ibudilast, and AV1013 were evaluated in our *in vivo* retinal damage models (RD and NMDA) for their ability to be neuroprotective of photoreceptors. This task utilized electrophysiology, spectral domain optical coherence tomography (SD-OCT), fundus imaging, and TUNEL/Immunohistochemistry (IHC) to assess the ability of MIF inhibitors to be protective of retinal neurons during RD- and NMDA-induced retinal damage. First an appropriate dosage was determined in a chick model. This included dose escalation studies in untreated animals with a single intravitreal injection to find a toxic and maximal therapeutic dose. Dose ranging studies were then conducted in retinal damage models to find the optimal dose for neuroprotection. Finally, drug timing studies were performed to test the impact of timing on the neuroprotective benefit.

Timeframe: months 1-18. To be performed at The Ohio State University College of Medicine by Dr. Cebulla (PI), Dr. Bongsu Kim (Post-Doc), Tyler Heisler-Taylor (Graduate Student), Sumaya Hamadmad (Research Assistant), Dr. Fischer (Co-I), Dr. Racine (electrophysiology, Co-I), and Dr. Satoskar (Co-I).

Subtask 1a: Dose escalation studies in untreated animals to find toxic and maximal therapeutic dose.

Dose escalation studies for the MIF inhibitors were performed using a single intraocular injection in untreated chick eyes to find toxic and maximal therapeutic doses. This task involved weighing the chicks, performing ocular examinations on days 1, 3, 7, 14, 21, and 28 days post injection, and recording intraocular pressure (IOP) measurements. Eyes were examined with ophthalmoscopy to evaluate for infection or inflammatory response (graded using the Nussenblatt scale). Electroretinogram (ERG) and SD-OCT with color fundus imaging of the

retina was also performed on a subset of chicks and compared with histology in a masked fashion. This experiment was carried out on a limited number of untreated chicks (n=4/group).

Timeframe: months 1-6. To be performed at The Ohio State University College of Medicine by Dr. Cebulla (PI), Dr. Bongsu Kim (Post-Doc), Tyler Heisler-Taylor (Graduate Student), Dr. Sumaya Hamadmad (Research Assistant), Dr. Fischer (Co-I), Dr. Racine, electrophysiologist (Co-I), and Dr. Satoskar (Co-I).

Completed: Y2 Q4

Subtask 1b: Dose range finding studies for MIF inhibitors in retinal damage models.

Dose ranging studies for MIF inhibitors, delivered by a single intraocular injection, in retinal damage models were performed. Retinal damage models included RD and NMDA models. This task included pilot studies performed on a limited number of animals (n=4/group) to find optimal doses of administration to block retinal neuronal cell death when assessed by TUNEL, a cell death analysis. RD TUNEL analysis was performed at a day 3 time-point, while NMDA TUNEL analysis was performed at a day 1 time-point for the pilot studies. Following the initial studies, another independent experiment with larger numbers (n=10/group) was carried out to confirm the results using histology, SD-OCT/fundus imaging, and ERG at a later time-point (day 14 for the RD model, and day 7 for NMDA).

NCE 2 Edits: Dose range finding studies for the MIF inhibitor ibudilast were performed in a murine RD model using a limited number of animals to serve as a pilot study. Mice received a single intraocular injection to find the optimal dose with neuroprotective effect. Retinas were evaluated via TUNEL assay and compared to controls. Additionally, the route and dosing were optimized.

Timeframe: months 1-18. To be performed at The Ohio State University College of Medicine by Dr. Cebulla (PI), Dr. Andy Fischer (Co-I), Dr. Satoskar (Co-I), Dr. Bongsu Kim (Post-Doc), Dr. Sumaya Hamadmad (Research Assistant) and Tyler Heisler-Taylor (Graduate Student).

Completed: Y5Q4

Subtask 1c: Drug timing studies in retinal damage models to test the impact of timing on the neuroprotective benefit of MIF inhibitors.

Drug timing studies in retinal damage models were performed to test the impact of timing on the neuroprotective benefit of MIF inhibitors. RD and NMDA damage models were treated with the optimized MIF inhibitor dose 2-6 hours after damage initiation. TUNEL staining and retinal thickness measurements were taken to evaluate the impact of MIF inhibitors on retinal neuroprotection hours following injury.

NCE 2 Edits: Drug timing studies to test the impact of timing on the neuroprotective benefit of ibudilast in both a murine RD model and a chick NMDA model have been performed. Subjects were treated with ibudilast 2 hours following the initiation of retinal damage. Retinas were evaluated using TUNEL assay and retinal layer thickness was evaluated to determine the neuroprotective impact of MIF inhibition following injury.

Timeframe: months 4-18. To be performed at The Ohio State University College of Medicine by Dr. Cebulla (PI), Dr. Andy Fischer (Co-I), Dr. Bongsu Kim (Post-Doc), Dr. Sumaya Hamadmad (Research Assistant), and Tyler Heisler-Taylor (Graduate Student).

Completed: Y4Q4

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 using *in vitro* and *in vivo* models.

Timeframe: months 6-36. To be performed at The Ohio State University College of Medicine by Dr. Cebulla (PI), Tyler Heisler-Taylor (Graduate Student), Dr. Bongsu Kim (Post-Doc), Dr. Fischer (Co-I), Sumaya Hamadmad (Research Assistant), Dr. Satoskar (Co-I) and Diana Summitt (Veterinary assistant).

Subtask 2a: Test the hypothesis that MIF inhibitors block gliosis and pathological wound healing responses *in vitro*. To 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 were incubated with TGF β to mimic PVR conditions. The effect of clinically relevant MIF inhibitors (CPSI-1306/ Ibudilast/AV1013) were 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 have been impacted by the MIF inhibitor therapeutics and role of phosphodiesterase inhibition.

Timeframe: months 6-30. To be performed at The Ohio State University College of Medicine by Dr. Cebulla (PI), Tyler Heisler-Taylor (Graduate Student), Dr. Bongsu Kim (Post-Doc), and Sumaya Hamadmad (Research Assistant).

Completed: Y4Q2

Subtask 2b: Test the hypothesis that MIF inhibitors block gliosis and pathological wound healing responses *in vivo*.

To test the hypothesis that MIF inhibitors block gliosis and pathological wound healing responses *in vivo*, doses of MIF inhibitors that were shown to block TUNEL staining were used to evaluate the effect on retinal gliosis in RD (murine) and NMDA (chick) damage models. Retinas were evaluated with four IHC stains; glial fibrillary acidic protein (GFAP) to detect gliosis, Aquaporin 4 (AQP4) to detect Müller glia and astrocyte changes, CD45 to detect microglia accumulation, and proliferating cell nuclear antigen (PCNA) to evaluate cell proliferation of Müller progenitor cells. Retinas were evaluated at day 14 (RD) and day 7/10 (NMDA). Further studies were then carried out in a rabbit PVR model to determine if intravitreal drug administration blocked PVR formation.

NCE 2 Edits:

RD and NMDA models: Ibudilast, which was shown to block TUNEL staining, was used to evaluate the effect of MIF inhibition on retinal gliosis in our chick NMDA and murine RD retinal damage models. The development of gliosis was evaluated using GFAP and aquaporin 4 immunostaining. CD45/ Iba1/ F4/80 were used to detect microglia accumulation, and PCNA was used to evaluate the proliferation of Muller glia progenitor cells labeled with Sox 2 or 9 markers at day 7-10 in NMDA chick models, and at day 14 in RD murine models.

Rabbit PVR model:

Rabbit PVR drug study: The MIF inhibitor Ibudilast was then used to evaluate if intravitreal drug administration blocks PVR formation in a rabbit model. The retinal status was evaluated in a masked fashion with an indirect ophthalmoscope on days 1, 3, 7, 14, 21, and 28. PVR was graded according to the Fastenberg scale of classification: stage 0, no disease; stage 1, epiretinal membrane; stage 2, vitreoretinal traction without retinal detachment; stage 3, localized retinal detachment (one to two quadrants); stage 4, extensive retinal detachment (two to four quadrants without complete detachment); and stage 5, complete retinal detachment. Fundus images were taken and SD-OCT was performed. On day 28, rabbits were euthanized and their eyes enucleated. PVR eyes were hemisected and imaged using a dissecting microscope prior to fixation, washing, and embedding. A subset of eyes were flash frozen as pairs for future analysis.

Timeframe: months 12-36. To be performed at The Ohio State University College of Medicine by Dr. Cebulla (PI), Dr. Andy Fischer (Co-I), Dr. Mitch Phelps (Co-I), Dr. Bongsu Kim (Post-Doc), Tyler Heisler-Taylor (Graduate Student), and Dr. Sumaya Hamadmad (Research Assistant).

Completed: Y5Q4

Task 3. Determine the ocular pharmacokinetics of clinically relevant MIF inhibitors and their effects on IOP in rabbits.

Methods: in vivo studies in untreated chicks will be performed in parallel to Aim 1 to evaluate retinal drug levels after a single intravitreal treatment. The MIF inhibitor drug showing the most promise in Aims 1/2 will be further evaluated in rabbits for pharmacokinetic properties. The plasma, vitreous, and retinal drug levels will be analyzed at different timepoints with assays developed by the Pharmacanalytical Core Facility at OSU. ERG and SD-OCT will be used to assess potential retinal toxicity. Systemic organ toxicity will be evaluated by necropsy by the Veterinary Core facility.

Timeframe: months 1-36. To be performed at The Ohio State University College of Medicine by Dr. Cebulla (PI), Dr. Mitch Phelps (Co-I), Dr. Bongsu Kim (Post-Doc), Dr. Fischer (Co-I), Dr. Satoskar (Co-I), Dr. Sumaya Hamadmad (Research Assistant), Tyler Heisler-Taylor (Graduate Student), and Diana Summitt (Vet Tech).

Subtask 3a: Pharmacokinetic study. A screening pilot study using chick, performed in parallel to Aim 1, was performed to evaluate drug levels in the retina.

In vivo studies in untreated chicks were performed to evaluate retinal drug levels after a single injection. The OSU Pharmacanalytical Core Facility developed an assay to analyze the plasma, vitreous, and retinal drug levels. ERG and SD-OCT were additionally used to assess the potential retinal toxicity, while the Veterinary Core Facility evaluated systemic organ toxicity via necropsy.

The MIF inhibitor that showed the greatest protective ability in the chick pilot studies was further evaluated in a rabbit pharmacokinetic study.

Timeframe: months 1-6. To be performed at The Ohio State University College of Medicine by Dr. Cebulla (PI), Dr. Andy Fischer (Co-I), Dr. Mitch Phelps (Co-I), Tyler Heisler-Taylor (Graduate Student), Dr. Bongsu Kim (Post-Doc), Dr. Sumaya Hamadmad (Research Assistant), and Diana Summitt (Veterinary assistant).

Completed: Y4Q3

Subtask 3b: Pharmacokinetic study. An extended pilot study using chick, performed in parallel to Aim 1, was performed to evaluate retinal drug levels after one (or two) intravitreal treatment of MIF inhibitor in untreated animals using an extended pilot study.

Methods: Untreated chicks (n=3/group) will be treated with one (or two if needed) intravitreal drug injections at 1-2 doses and retinal and vitreous drug levels will be evaluated at two timepoints (day 1 and day 3).

Timeframe: months 3-12. To be performed at The Ohio State University College of Medicine by Dr. Cebulla (PI), Dr. Andy Fischer (Co-I), Dr. Mitch Phelps (Co-I), Dr. Bongsu Kim (Post-Doc), Dr. Sumaya Hamadmad (Research Assistant), Tyler Heisler-Taylor (Graduate Student), and Diana Summitt (Veterinary assistant).

Completed: Y5Q1

Subtask 3c: Rabbit pharmacokinetics study: The MIF inhibitor drug showing the most promise in Aims 1/2 will be further evaluated in rabbits for pharmacokinetic properties.

Rabbit pharmacokinetic study – The MIF inhibitor showing the most promise in the chick pilot studies was selected for further pharmacokinetic evaluation in rabbits. Untreated rabbits eyes were intravitreally injected with Ibudilast. Initially fellow eyes were to remain untreated, however to reduce the amount of rabbits required, both eyes were used and collected for drug level evaluation. Clinical examination and IOP measurements were performed prior to enucleation for tissue evaluation of drug levels. The plasma, vitreous, and retinal drug levels were analyzed at multiple time points with assays developed by the Pharmacanalytical Core facility at OSU.

ERG, SD-OCT, and fundus imaging was used to assess for retinal toxicity. The Veterinary Core Facility performed systemic organ toxicity via necropsy at the 24hr time point.

NCE 2 Edits: The MIF inhibitor drug showing the most promise in Aims 1/2 was selected for further evaluation in rabbits for pharmacokinetic properties. Untreated rabbit eyes (n=3) will be intravitreally injected with ibudilast with the dosing scheme established in Aim 1. Fellow eyes will be untreated. Clinical examination will be performed to evaluate inflammation and IOP will be evaluated 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 Pharmacanalytical Core Facility at OSU, led by Dr. Mitch Phelps. ERG and SD-OCT will be used in a subset to assess potential retinal toxicity. Systemic organ toxicity will be evaluated by necropsy at the final timepoint by the Veterinary Core facility.

Methods: Untreated rabbit eyes (n=3) will be intravitreally injected with MIF inhibitor with the dosing scheme established in Aim 1. Fellow eyes will be untreated. Clinical examination will be performed to evaluate inflammation and IOP will be evaluated 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 Pharmacanalytical Core Facility at OSU, led by Dr. Mitch Phelps. ERG and SD-OCT and fundus imaging will be used to assess potential retinal toxicity. Systemic organ toxicity will be evaluated by necropsy at the final hour timepoint by the Veterinary Core facility.

Timeframe: months 24-36. To be performed at The Ohio State University College of Medicine by Dr. Cebulla (PI), Dr. Phelps (Co-I), Dr. Racine (Co-I), Dr. Satoskar (Co-I), Dr. Bongsu Kim (Post-Doc), Sumaya Hamadmad (Research Assistant), Tyler Heisler-Taylor (Graduate Student), and Diana Summitt (Veterinary assistant).

Completed: Y5Q4

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

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.

Subtask 1a: Perform dose escalation studies in untreated animals to find toxic and maximal therapeutic dose.

(A) **Dose escalation studies** to identify a toxic and maximal therapeutic dose were performed with a single intraocular injection of CPSI-1306, ibudilast and vehicle (injected in fellow eye) in untreated chick eyes (n=4/group). Eyes were evaluated at 1, 7, 14, 21, and 28 days post injection. Animals were weighed, and received clinical exams with ophthalmoscopy to evaluate for infection or inflammatory response with slit lamp and/or operating microscope (graded with Nussenblatt scale), and IOP checks. ERG, and SD-OCT with color fundus imaging of the retina to evaluate retinal layers was performed in a masked fashion on a subset and compared with histology (taken at D1,3,7,14,21). The fellow eyes will be injected with vehicle.

(n=4/group * 2 drugs * 1 endpoint * ~5 doses * 1 model (untreated) = **40 chicks**)

(n=4/group * 2 drugs * 5 endpoint * ~1 doses * 1 model (untreated) = **40 chicks**)

- A. Preparation:** Particularly in the 1st and 2nd quarters we prepared numerous standardized forms and standard operating procedures (SOPs) for our experimental procedures. Additionally, we had to order a number of supplies for our experiments including chick brooders, a tonopen, a fundus imaging camera, a micro-injector, the Leica SD-OCT data station, and an isoflurane anesthesia machine.
- B. Normative Database:** During the 1st fiscal year, we created a normative database of undamaged control and NMDA-damaged chicks tested with multiple modalities [PMC6215716]. This included data from 1-week post-hatch to 5-weeks post-hatch. With this database, we were able to compare the time course of NMDA-damage to undamaged chicks.
- a. Clinical Examinations:** With the start of the long extended timepoint studies to assess for potential retinal toxicity, we began performing clinical examinations on the chicks including IOP measurements, weight, slit lamp exams, and fundus imaging. There was significant variability in the IOP measurements (data not shown). After consultation with our collaborators, the variability appears to be due to the style of tonometer. Therefore, we purchased a new style of tonometer (Icare Plus) which has a finer tip and yielded more consistent measurements. In the manuscript we reported a lack of difference in IOP (**Fig 1**) and weight (**Fig 2**) measurements. We also showed the difference in fundus appearance between NMDA-damaged and undamaged eyes (**Fig 3**)
 - b. ERG Testing:** We acquired a large database comparing undamaged and NMDA-damaged chicks out to 5-weeks post-hatch at days 1, 7, 14, 21, & 28 post-damage (**Fig 4**). We were able to note many significant differences between the two groups across the different timepoints and testing parameters including the a-wave, b-wave, sum of oscillatory potentials, 20hz flicker tests, and long-flash ON/OFF tests.
 - c. SD-OCT Testing:** We acquired a normative database of SD-OCT measurements similar to the normative ERG database to use in the comparison of retinal thicknesses of NMDA-damaged and undamaged eyes (**Fig 5**). Of particular note, we observed that an edema-like behavior formed in the inner plexiform layer (IPL) at 1 day post-damage (D1) that resolved by 7 days post-damage (D7). At that point, a hyper-reflective band had appeared in the inner nuclear layer (INL) and significant thinning had been noted that persisted over the remainder of the time course. To aid in the identification of retinal layers, we also established an internal layering guideline for chick retina (**Fig 6**).
- C. Max Dose Toxicity Experiments:** To establish the maximum tolerated dose for ibudilast, AV1013, and CPSI-1306, we performed intravitreal injections of the maximum water-soluble concentration of each drug into a chick. The drugs did not cause any clinical side effects compared to control in the slit-lamp or fundus examinations (data not shown). There were no changes in weight between control and any of the treatment groups (data not shown).
- a. Ibudilast:** We established that the maximum soluble concentration of ibudilast in sterile water is 4.5 mg/ml. Sterile water was used since it is an acceptable diluent for human intraocular medications per discussion with the institution pharmacist. The experimental design was injection of 20µl max dose ibudilast in the left eye and 20µl vehicle injection in the right eye. After establishing our experimental methods in one test chick, we assigned four chicks for analysis D1 after injection and eight chicks for analysis D21 after injection. The D1 chick retinas were stained with TUNEL to detect any cell death. At D1 no significant difference was found between the ibudilast-treated and vehicle-treated eyes (9 vs. 3 cells/mm², $p=0.55$) (**Fig 7**). The D21 chicks were stained with DAPI, and both the INL and total retinal thickness measurements were acquired. Neither the INL thickness ($p=0.64$), the total retinal thickness ($p=0.90$), nor the ratio of the INL/Total ($p=0.37$) were found to be significant (**Fig 8**). **We concluded that the maximum soluble concentration was non-toxic**, and no further doses would be required for this subtask. The D21 chicks were also measured with the ERG system and SD-OCT system. The ERG measurements were acquired at D10 and D14 and analyzed with two-way ANOVA with Tukey post-hoc testing using GraphPad Prism. **No significant differences were found between the MIF inhibitor drug-treated, vehicle-treated, and age-matched untreated groups.** Rarely, significant difference points were found within the different datasets, but none

of these differences were consistent across all flash intensity ranges and appeared to be due to noise (Figs 9, 10, 11). SD-OCT thickness measurements were acquired on D7 and D16 and showed no significant differences between the max dose ibudilast-treated, vehicle-treated, or age-matched untreated groups (Fig 12). Statistics were calculated using two-way ANOVA with Tukey post-hoc testing in JMP.

- b. **CPSI-1306:** We established that the maximum soluble concentration of CPSI-1306 in sterile water is 1.25 mg/ml. The D1 and D3 chicks were stained with TUNEL to detect any retina cell death. Neither timepoint displayed a significant difference between the max dose CPSI-1306-treated and vehicle-treated eyes (D1: 1.15 ± 0 cells/mm², $p=0.391$; D3: 39.30 ± 0 cells/mm², $p=0.368$) (Fig 7). In Day 28 chicks, DAPI staining showed no significant difference in retinal layer thickness between the CPSI-1306-treated and vehicle-treated eyes (Total: 123.34 ± 12.75 v 123.41 ± 8.87 ; INL: 39.37 ± 3.99 v 39.61 ± 3.82 , respectively) (Fig 7). ERG measurements were taken on three chicks at D8, D14, D21, and D28 post-injection (Figs 9, 10, 11) and were analyzed with two-way ANOVA with Tukey post-hoc testing using GraphPad Prism. No significant differences were noted between the max dose CPSI-1306 treated eyes and controls (Figs 9, 10, 11). SD-OCT measurements were taken on three chicks at D8, D14, D21 and D28 post-injection (Fig 12). Statistics were calculated using two-way ANOVA with Tukey post-hoc testing in JMP. No significant differences between CPSI-1306-treated, vehicle-treated, or untreated eyes were detected. We found no evidence of toxicity for the maximum soluble CPSI-1306 concentration of 1.25 mg/ml and concluded no further doses needed testing for this subtask.
- c. **AV1013:** After continuing in vitro 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 for the comparison. TUNEL was evaluated for 4.5 mg/mL AV1013 compared to vehicle control (18.8 ± 17.6 and 2.4 ± 3.4 , respectively, Fig 13); the difference was not significant ($n=3/\text{group}$, $p=0.42$). 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 was not toxic to the retina and was unlikely to cause any adverse effects in the eye.

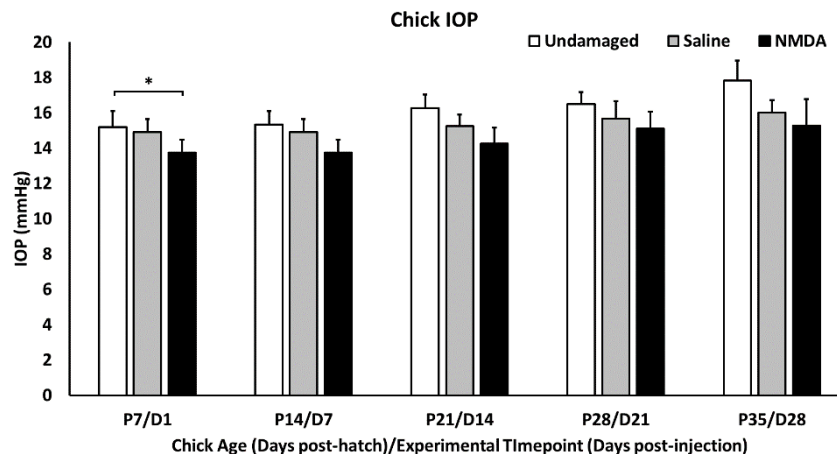


Fig 1: IOP measurements. Chick IOP measurements on undamaged chicks and NMDA-damaged chicks (OD: saline, OS: NMDA) taken weekly starting at post-hatch day 7 or post-injection day 1 till post-hatch day 35 or post-injection day 28.

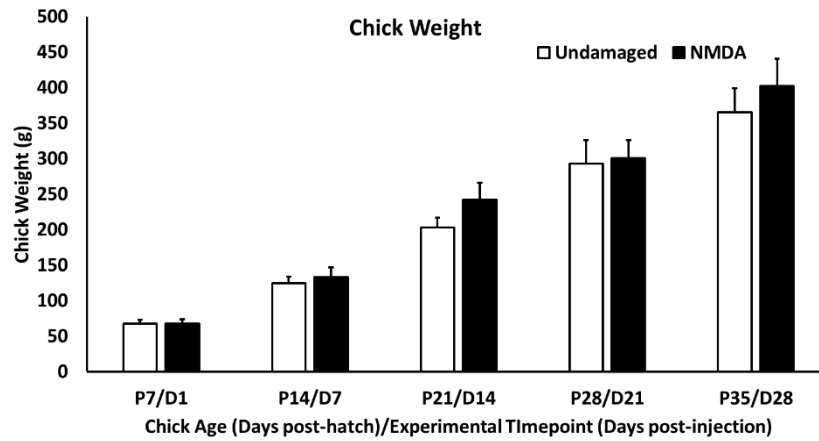


Fig 2: Weight measurements: A normative database of undamaged and NMDA-damaged chicks were weighed weekly up until sacrifice at post-hatch day 35 (P35). The two treatment groups were typically weighed within one day of each other except for the P21/D14 timepoint where the NMDA-damaged chicks were measured three days after the undamaged chicks due to experimental constraints (n=10/group).

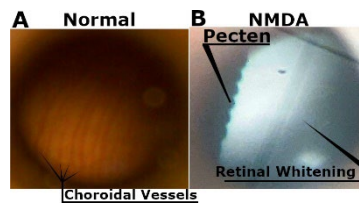


Fig 3: Representative fundus imaging. Choroidal vessels are visible in healthy chick eyes (A). While in NMDA-damaged chick eyes they can no longer be observed, instead we see the retina assume a homogenous white color potentially due to a lack of perfusion (B). The sharp and clear presence of the pecten rules-out media opacity in the NMDA-damaged eyes.

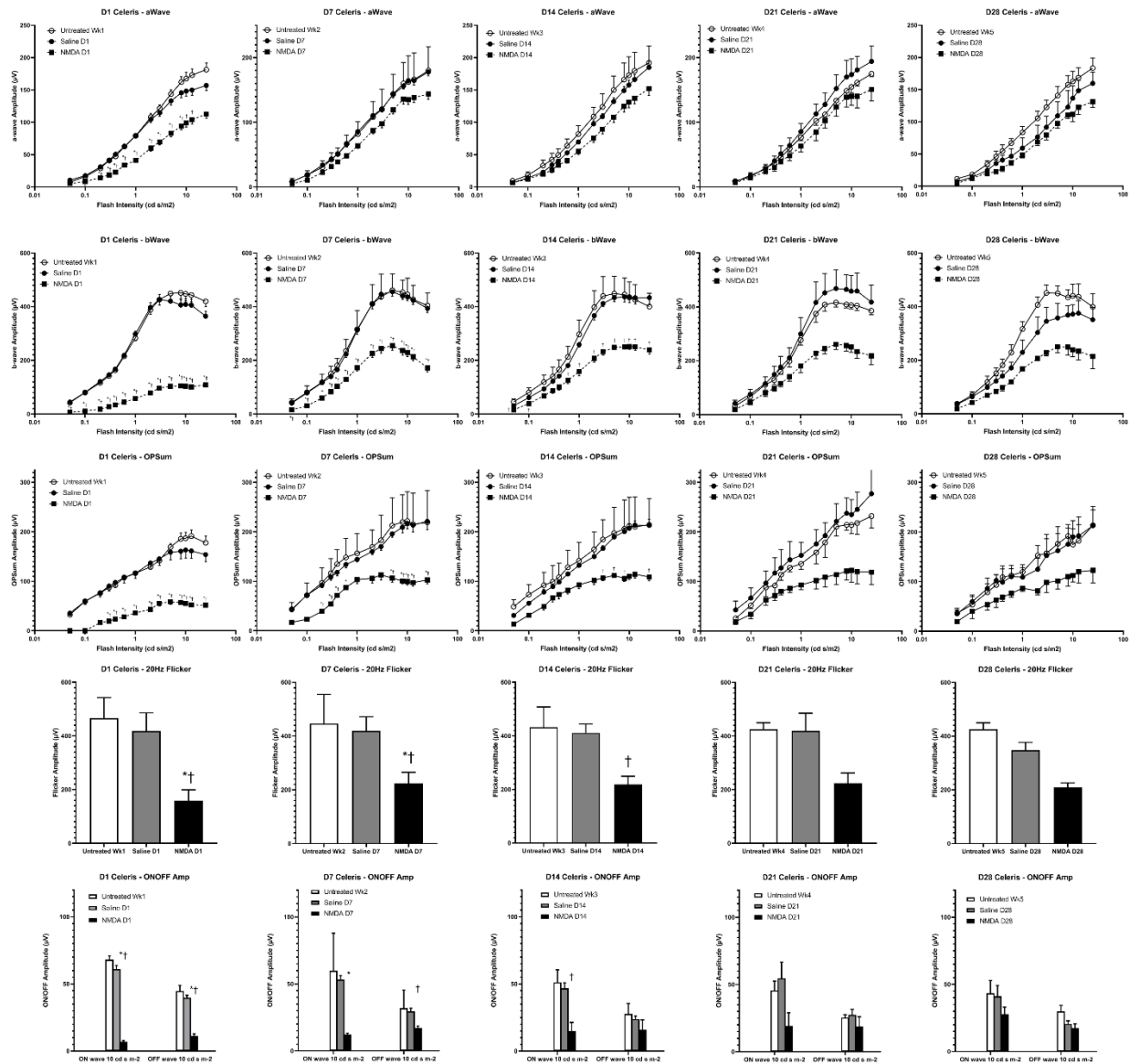


Fig 4: ERG results of NMDA-damaged, vehicle-treated, and age-matched undamaged controls.

Undamaged chicks (n=10 P7 to P21, n=5 P28 to P35), NMDA-damaged chick eyes (OS), and saline vehicle-injected fellow chick eyes (OD) (n=10, P7/D1 to P14/D7, n=8, P21/D14, n=6, P28/D21, n=5, P35/D28) were examined with the Celeris ERG system. The data is organized by time (columns) and parameter (rows). From left to right, the columns represent one day post-injection (D1) and 7 days post-hatch (P7), D7/P14, D14/P21, D21/P28, and D28/P35. From top to bottom, the rows represent a-wave amplitudes, b-wave amplitudes, the amplitudes of the sum of oscillatory potentials (OPSum), flicker amplitudes at 20Hz, and ON/OFF bipolar cell response amplitudes. * indicates significance with vehicle (saline) controls, † indicates significance with undamaged controls

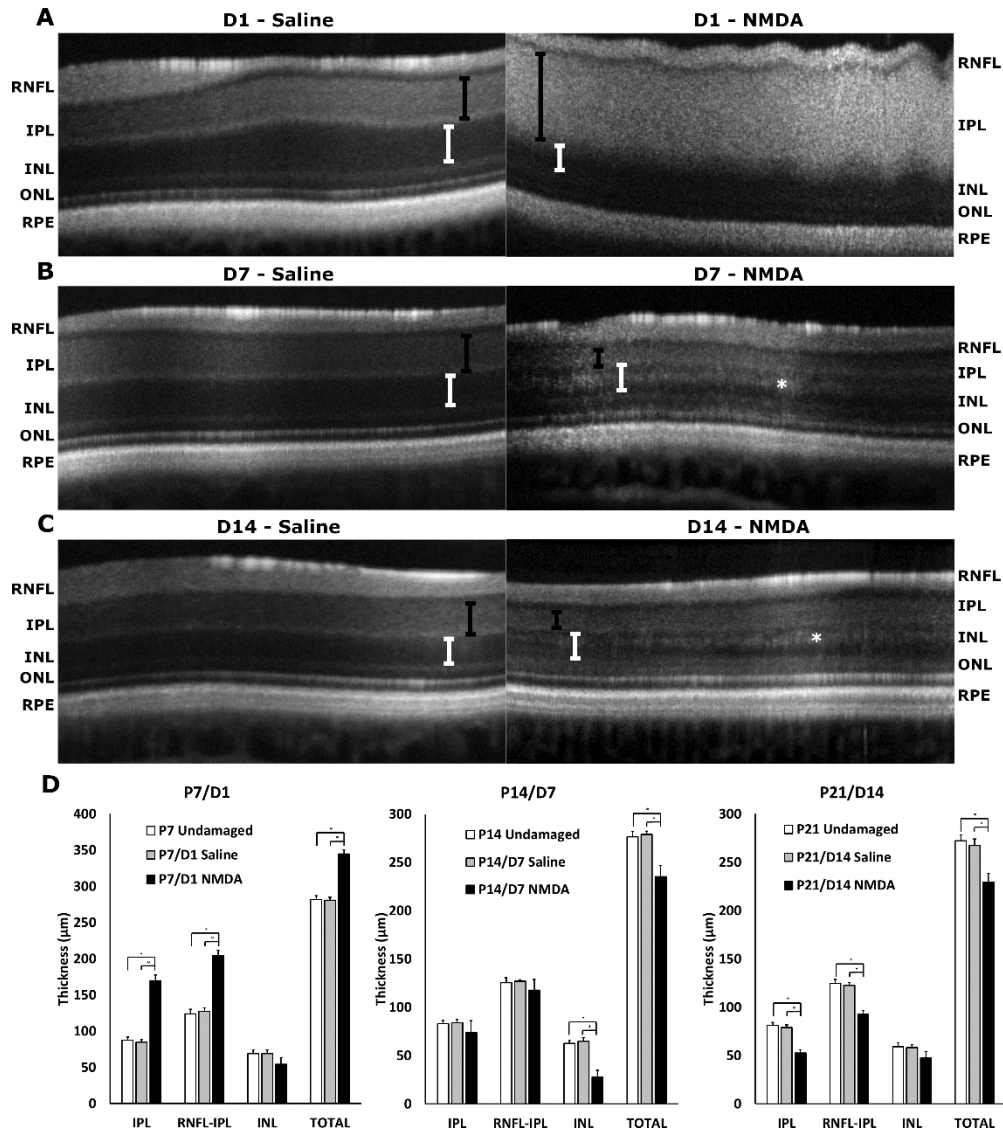


Fig 5: SD-OCT analysis of NMDA-treated and corresponding control eyes. Representative b-scans of D1 vehicle (saline, left) and D1 NMDA (right) showing edema in IPL (A), D7 vehicle (saline, left) and D7 NMDA (right) (B), and D14 vehicle (saline, left) and D14 NMDA (right) (C). (D) Analysis of retinal thicknesses of the IPL, RNFL-IPL combined, INL, and total thickness between NMDA and saline (vehicle) treated eyes at P7/D1 to P21/D14 post injection and their respective age-matched untreated controls. Black brackets show IPL thickness, white brackets show INL thickness, asterisk denotes hyperreflective layer. Error bars are shown as standard deviation. Abbreviations: RNFL - retinal nerve fiber layer, IPL - inner plexiform layer, INL - inner nuclear

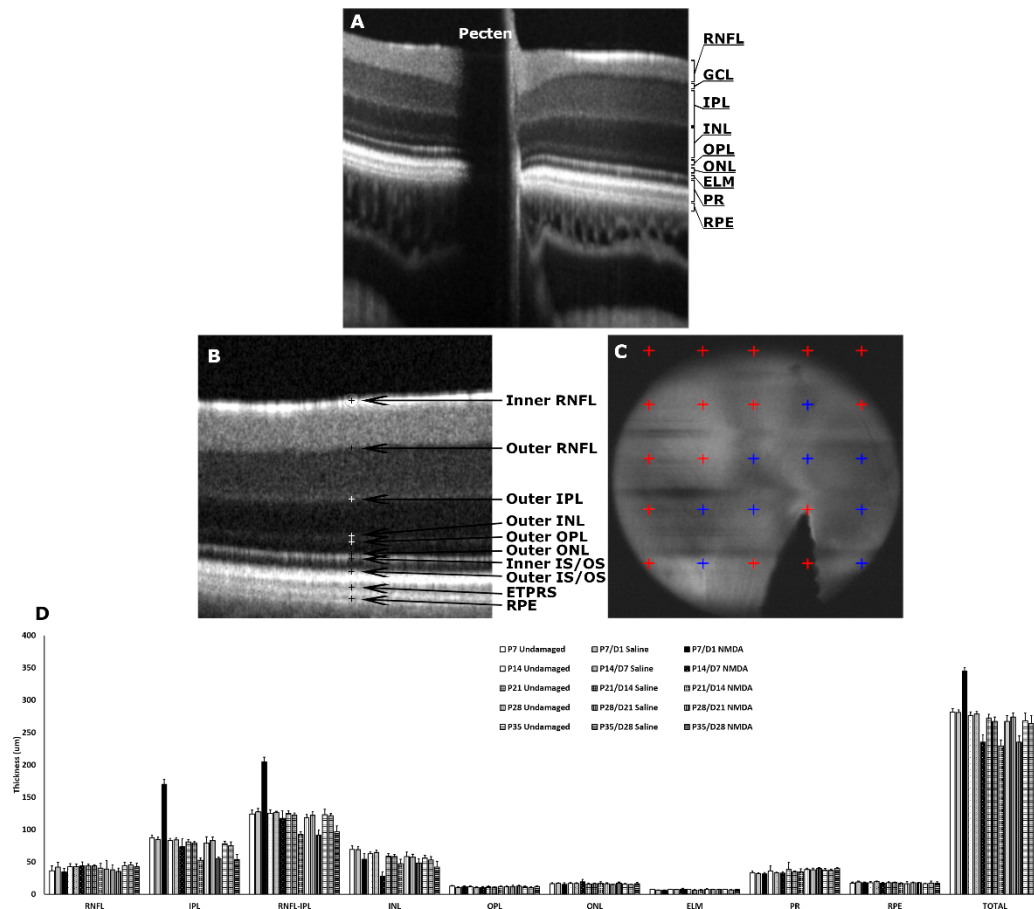


Fig 6: SD-OCT marker positions and fundus projection: (A) Representative capture of chick retinal layers with the pecten shown in the center. (B) Layer identifiers used by the InVivoView Diver software to measure retinal thicknesses. (C) Representative fundus capture from volumetric OCT scan. Blue crosses represent where measurements were taken. (D) Measurements of all retinal thicknesses of the RNFL, IPL, RNFL-IPL, INL, OPL, ONL, ELM, PR, RPE, and full retina layers between NMDA and saline (vehicle) treated eyes at P7/D1 to P35/D28 post injection and their respective age-matched untreated controls. Error bars are shown as standard deviation. Abbreviations: RNFL - retinal nerve fiber layer, GCL – ganglion cell layer, IPL - inner plexiform layer, INL - inner nuclear layer, OPL - outer plexiform layer, ONL - outer nuclear layer, ELM – external limiting membrane, PR – photoreceptors, IS - inner segment, OS - outer segment, ETPRS - end tip of photoreceptors, RPE - retinal pigment epithelium.

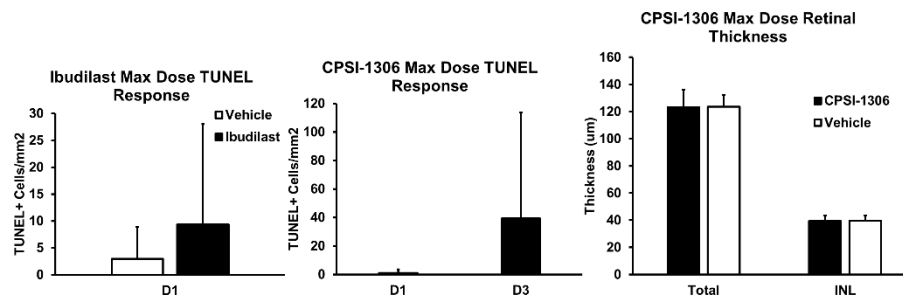


Figure 7. Max Dose MIF Inhibitor Toxicity TUNEL & Histology Results in undamaged retinas. Ibudilast at its max dose of 4.5mg/ml displayed no significant difference in the number of TUNEL positive cells per mm² of retina between the ibudilast and vehicle (sterile water) treated eyes at D1 post-injection (left). CPSI-1306 at its max dose of 1.25mg/ml displayed no difference in the number of TUNEL positive cells per mm² of retina between the CPSI-1306 and vehicle (sterile water) treated eyes at D1 and D3 post-injection (center). Vehicle eyes for the CPSI-1306 treatments possessed no TUNEL positive cells and therefore do not appear on the graph. Additionally CPSI-1306 treated eyes were measured at D28 post-injection for any changes in INL or total retinal thickness (right) on DAPI nuclear counterstained sections. No significant difference was detected.

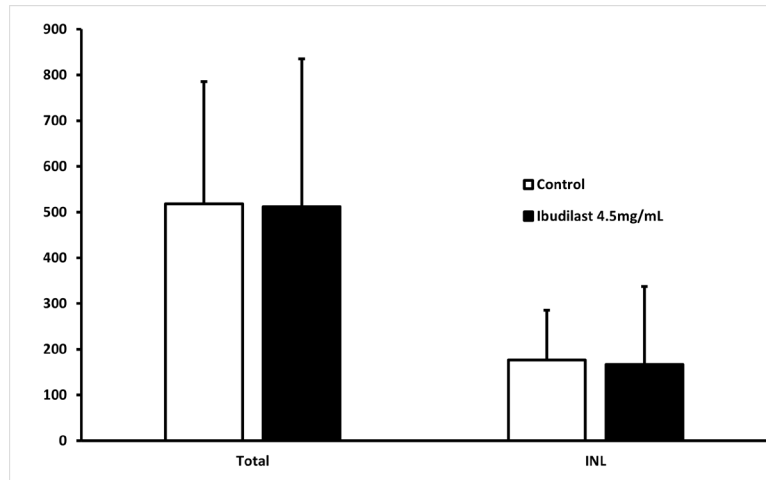


Figure 8. Max ibudilast dose retinal thickness results. Ibudilast at its max dose of 4.5mg/ml displayed no significant difference between the INL thickness, the total retinal thickness, or the ratio of the two as determined by histology and DAPI staining.

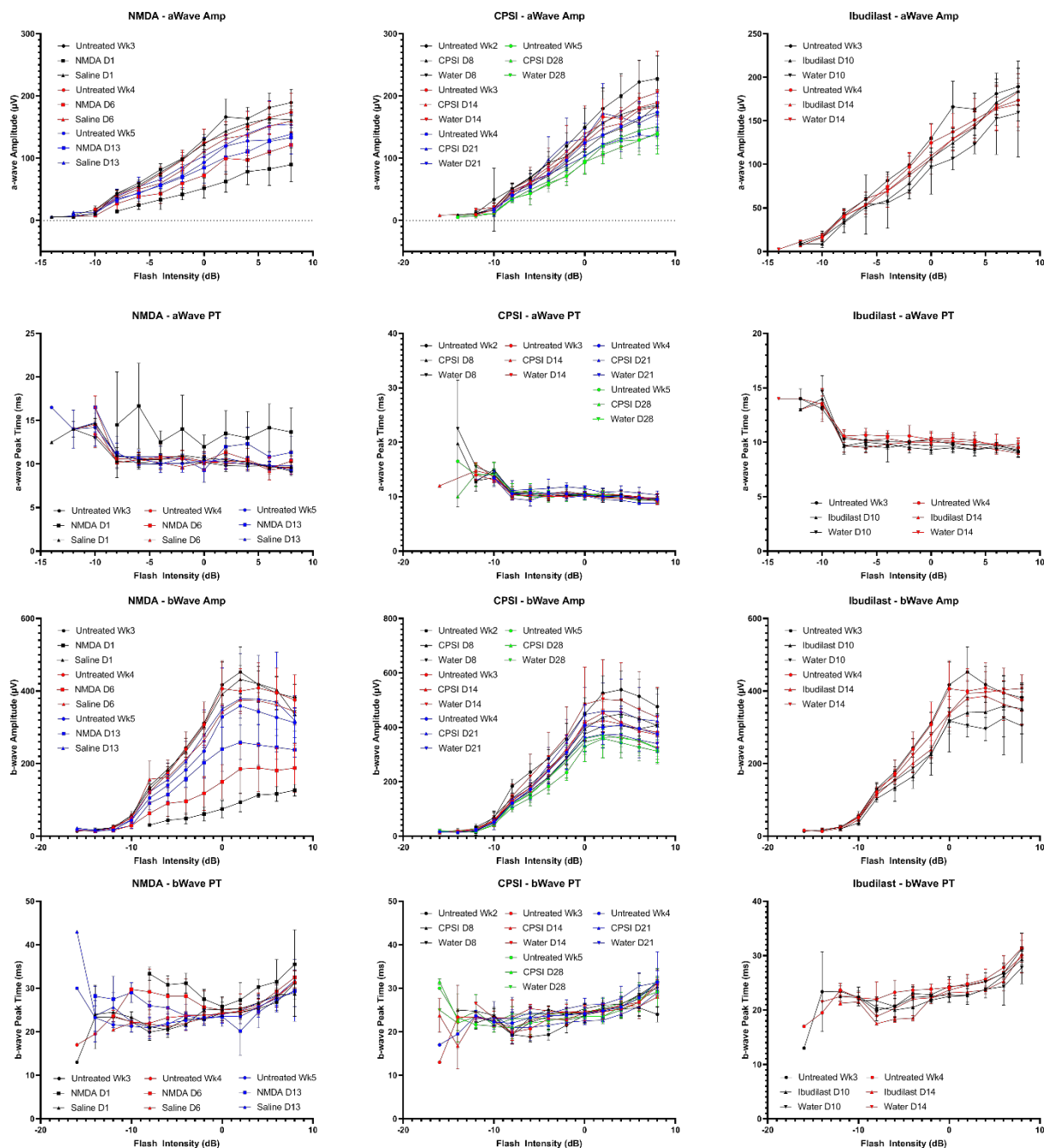


Figure 9. ERG A-wave and B-wave amplitudes and peak times (UTAS machine). NMDA damaged (left column), max dose CPSI-1306 undamaged (middle column), and max dose ibudilast undamaged (right column) treated and control chick ERG measurements were collected on at least three chicks per group. NMDA treated chicks were measured at D1, D6, and D13 post-injection and compared to their vehicle treated eye (sterile saline) and age-matched untreated counterparts. CPSI-1306 treated chicks were measured at D8, D14, D21, and D28 and compared to their vehicle treated eye (sterile water) and age-matched untreated counterparts. Ibudilast treated chicks were measured at D10 and D14 and compared to their vehicle treated eye (sterile water) and age-matched untreated counterparts. A-wave amplitudes (row 1), peak times (row 2), B-wave amplitudes (row 3), and peak times (row 4). As a positive control, NMDA-treatment reduced ERG amplitudes compared to saline. In contrast, the MIF inhibitors in undamaged eyes did not significantly impact the ERG amplitudes or peak times. More variability with the UTAS was noted and later studies switched to the Celeris ERG system.

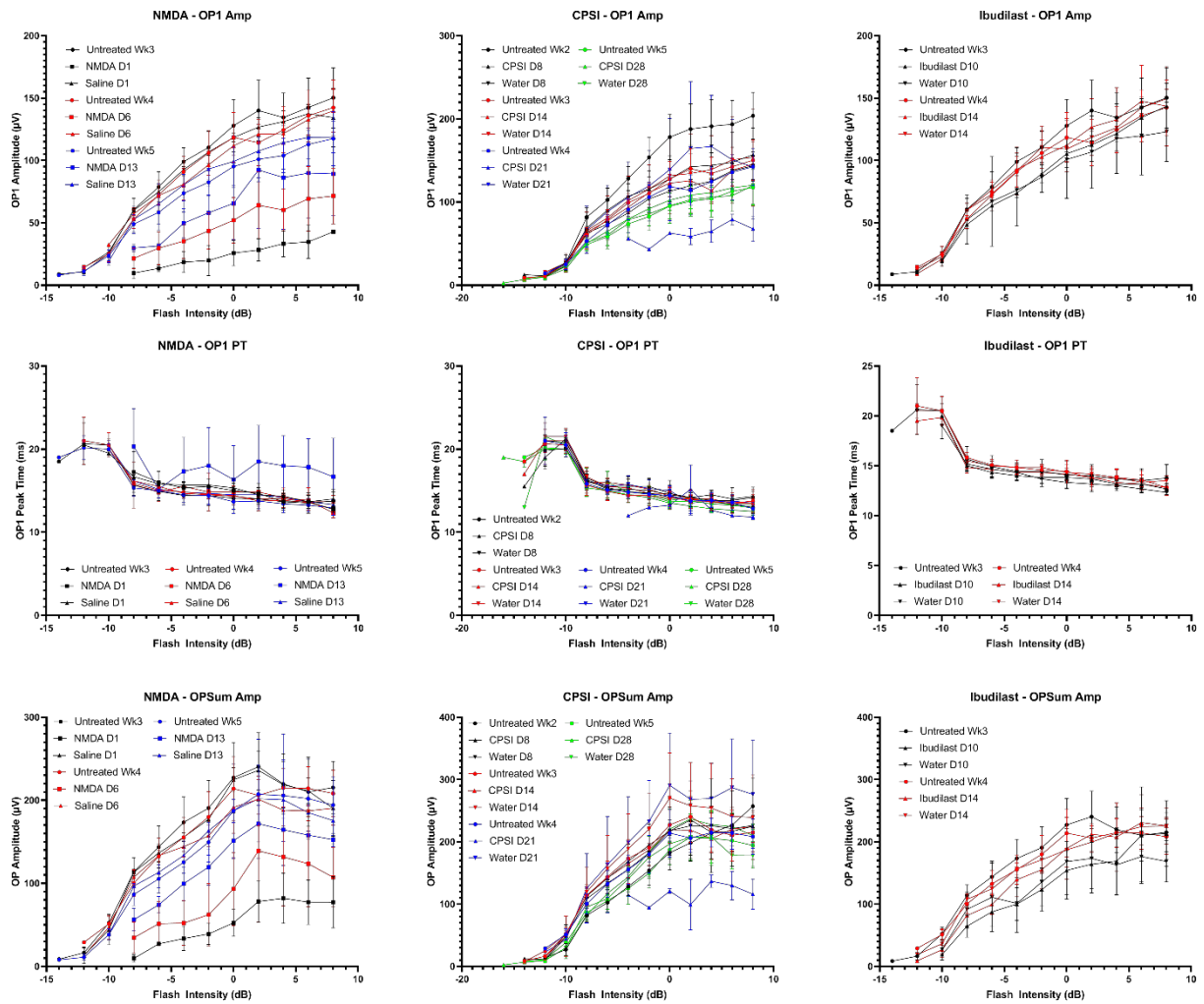


Figure 10. ERG Oscillatory potential (OP) measurements (UTAS). NMDA damaged (left column), max dose CPSI-1306 undamaged (middle column), and max dose ibudilast undamaged (right column) chick ERG measurements were collected on at least three chicks per group. NMDA treated chicks were measured at D1, D6, and D13 post-injection and compared to their vehicle-treated eye (sterile saline) and age-matched untreated counterparts. CPSI-1306 treated chicks were measured at D8, D14, D21, and D28 and compared to their vehicle-treated eye (sterile water) and age-matched untreated counterparts. Ibudilast treated chicks were measured at D10 and D14 and compared to their vehicle-treated eye (sterile water) and age-matched untreated counterparts. First OP wave amplitudes (row 1) and peak times (row 2) and the overall sum of the measure OP waves (row 3) are shown. As expected, NMDA amplitudes were decreased compared to vehicle controls. The CPSI OPSum Amplitudes at D21 were noted to be significantly lower than vehicle controls ($p < 0.0001$ at the highest intensity), however, the D28 amplitudes were not significantly different from controls ($p = 0.98$), suggesting that other factors may have influenced the findings. Moreover, the UTAS ERG system was found to have more variability in the waveforms and the future drug comparison studies were conducted using the Celeris ERG system, which reduced the variability.

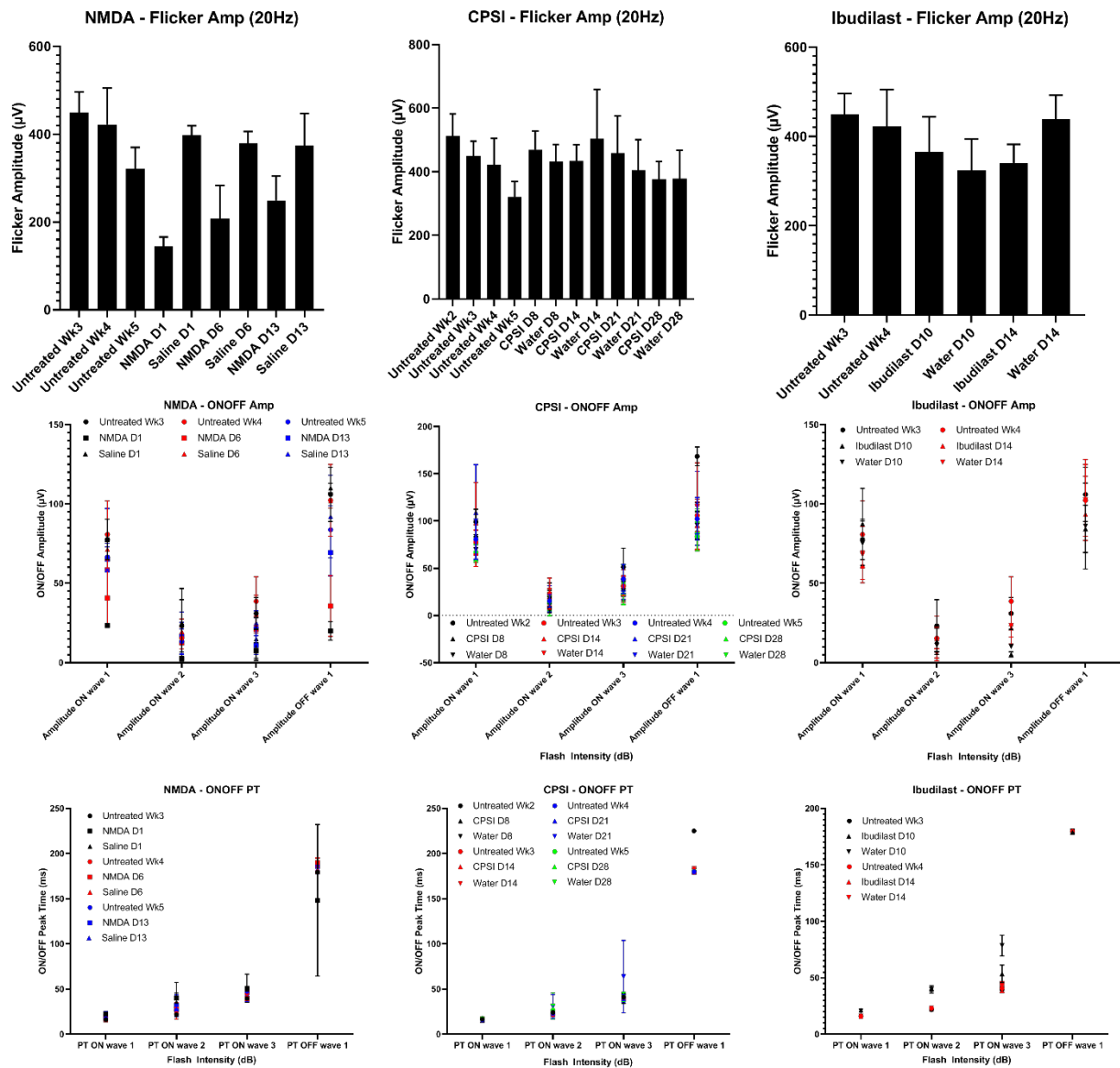


Figure 11. ERG Flicker and ON/OFF measurements (UTAS). NMDA damaged (left column), max dose CPSI-1306 undamaged (middle column), and max dose ibudilast undamaged (right column) treated chick ERG measurements were collected on at least three chicks per group. NMDA treated chicks were measured at D1, D6, and D13 post-injection and compared to their vehicle treated eye (sterile saline) and age-matched untreated counterparts. CPSI-1306 treated chicks were measured at D8, D14, D21, and D28 and compared to their vehicle treated eye (sterile water) and age-matched untreated counterparts. Ibudilast treated chicks were measured at D10 and D14 and compared to their vehicle treated eye (sterile water) and age-matched untreated counterparts. Flicker amplitudes at 20Hz (row 1), ON/OFF wave amplitudes (row 2), and ON/OFF wave peak times (row 3). Flicker amplitudes and peak to peak times were collected for 15, 20, and 25Hz, however no difference was observed in any of the peak to peak times and neither the 15Hz nor 25Hz differed greatly from the 20Hz measurement.

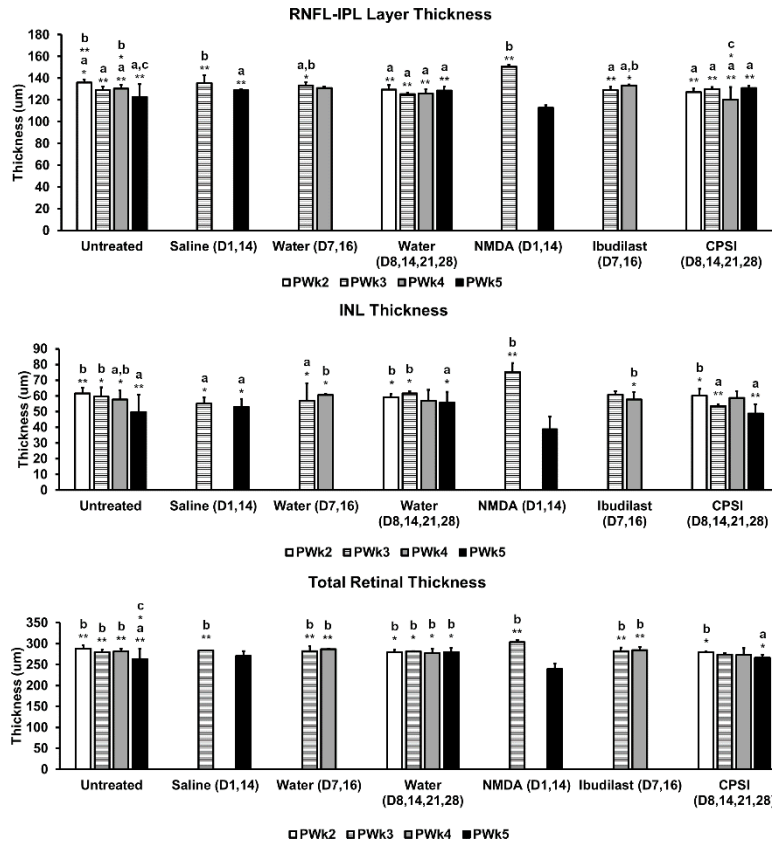


Figure 12. SD-OCT Thickness Measurements of Undamaged Controls, NMDA Damage, Max Dose Ibudilast, and Max Dose CPSI-1306. Measurements of the combined thickness of the retinal nerve fiber layer (RNFL) and the inner plexiform layer (IPL) (top), inner nuclear layer (INL, middle), and total retinal thickness (bottom). Post hatch week age of the chick is shown in the legend (PWk2-5). From left to right, untreated normative database chick eyes, sterile saline treated vehicle eyes (D1 and D14 post-injection), sterile water treated vehicle eyes (D7 and D16 post-injection), sterile water treated vehicle eyes (D8, D14, D21, and D28 post-injection), NMDA treated eyes (D1 and D14 post-injection), max dose ibudilast treated eyes (D7 and D16 post-injection), and max dose CPSI-1306 treated eyes (D8, D14, D21, and D28 post-injection). Max dose ibudilast and max dose CPSI-1306 were not significantly different from vehicle treated and untreated controls. NMDA treatment significantly increased the D1 inner retinal thickness (retinal swelling) followed by significantly decreased retinal thickness at D14. ‘a’ indicates significant difference from NMDA D1 values, ‘b’ indicates significant difference from NMDA D14, ‘c’ indicates significant difference from Untreated PWk2, ‘*’ indicates significance $p < 0.05$, and ‘**’ indicates significance $p < 0.01$. Statistics were calculated using two-way ANOVA with Tukey post-hoc testing in JMP.

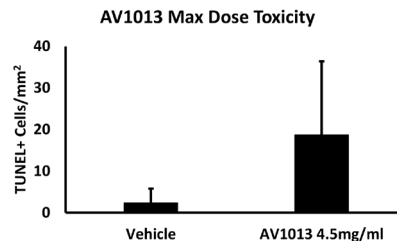


Figure 13. Maximal dose of AV1013 does not cause retinal toxicity. AV1013 at 4.5 mg/mL (18.8 ± 17.6) did not cause significant increase in TUNEL response in retinal cells when compared to vehicle control (2.4 ± 3.4 , $n=3/\text{group}$, $p=0.42$). This suggests AV1013 is not toxic to retinal cells and will not cause adverse effects.

Subtask 1b: Perform dose range finding studies for MIF inhibitors in retinal damage models.

(B) Dose range finding studies of CPSI-1306 and ibudilast/AV1013 after single intraocular injection in a limited number of animals (n=4/group) as a pilot studies were performed to find the optimal dose with neuroprotective effect by measuring the inhibition of photoreceptor apoptosis by TUNEL assay in day 3 (RD) and day 1 (NMDA) compared to controls. If the murine RD model is used, route and dosing will be optimized, including intraocular injection, intraperitoneal injection, oral administration.

(n=4/group * 2 drugs and vehicle (injected in the fellow eye) * 1 endpoint (D1) * ~5 doses * 1 model (NMDA) = 40 chicks)

(n=4/group * 3 drugs and vehicle * 1 endpoint (D3) * 3 doses * 1 model (RD) = 36 chicks)

(n=4/group * 3 drugs and vehicle * 1 endpoint (D3) * 3 routes of administration * 3 doses * 1 model (RD) = 108 mice)

AV1013, the analog of ibudilast that lacks phosphodiesterase inhibitor function, will be used in subsequent studies at the same concentration found for ibudilast. Studies will aim to find a no-effect dose range and mean effective dose. Side effects will be recorded. Clinical exams with indirect ophthalmoscopy or slit lamp to evaluate for infection or inflammatory response, intraocular pressure (IOP) checks, and weight will be performed. TUNEL assay will be performed in a masked fashion. Following the initial pilot study, an independent experiment with larger numbers (n=10) will be used to confirm the results and evaluate the effect of AV1013 as well as ibudilast and CPSI-1306. TUNEL staining of the retina to evaluate retinal apoptosis will be performed and compared at day 3 (RD) or day 1 (NMDA) in a masked fashion. The fellow eyes will be untreated (RD) or treated with vehicle (NMDA).

(n=10/group * 4 drugs and vehicle * 1 endpoint (D3) * 1 dose * 1 model (RD) = 40 chicks and/or mice)

(n=10/group * 3 drugs and vehicle * 1 endpoint (D1) * 1 dose * 1 model (NMDA) = 30 chicks)

Subsequent experiments of longer duration to better evaluate effective neuroprotective doses to preserve ONL thickness in the RD model (day 14) and inner retinal thickness for the NMDA model (day 7) will be performed (n=10/group). Clinical examinations, IOP, weight, will be recorded. A subset of animals undergoing histologic analysis will be analyzed with ERG and SD-OCT / fundus imaging (n=10 animals per group). Retinas will be also evaluated in a masked fashion with ONL thickness analysis.

(n=10/group * 4 drugs and vehicle * 1 endpoint (D14) * 1 dose * 1 model (RD) = 40 chicks and/or mice)

(n=10/group * 3 drugs and vehicle * 1 endpoint (D7) * 1 dose * 1 model (NMDA) = 30 chicks)

A. Dose testing for ibudilast, CPSI-1306, and AV1013:

- a. **NMDA Damage Model:** We performed a study to find the optimal neuroprotective dose of ibudilast in the NMDA damage model between concentrations of 4.5mg/ml and 10^{-10} mg/mL to find the minimum effective dose across a full dose response curve. While TUNEL assays were performed across this range of ibudilast concentration, the doses from 10^{-5} to 1.0 mg/ml had their TUNEL responses characterized as ratios with respect to their fellow eye controls (NMDA+vehicle) (10^{-5} mg/ml: n=3, 1.025 ± 0.127 , p=0.1891; 10^{-3} mg/ml: n=12, 0.8047 ± 0.4288 , p=0.0040; 0.1 mg/ml: n=6, 0.7362 ± 0.2559 , p=0.0002; 1.0 mg/ml: n=13, 0.7422 ± 0.4997 , p=0.0014; Steel-Dwass with Control; **Fig 14**). As AV1013 is an analogue of ibudilast, it was determined that the dose response should behavior similarly. We performed some initial dose ranging studies between 10^{-8} mg/ml and 1.0 mg/ml for CPSI-1306; however, we subsequently determined it was not a viable candidate to pursue (**Fig 15**).
- b. **Retinal Detachment:** We began creating chick retinal detachments (RD) to optimize our technique. However, inconsistencies in results and low cell death numbers in controls led us to turn to our established murine RD model. Due to the species change, the injection paradigm also required change. With the left eye receiving the RD surgery and the fellow eye remaining undamaged as a control, we delivered daily intraperitoneal injections of 120 microliters of ibudilast at a 2.0mg/ml concentration, which produced a reliably solubilized maximal drug concentration. We created RDs in n=13/group for Day 3 and n=13/group for the Day 14

timepoints. Due to failures of the RD or significant hemorrhage/cataract, the successful eyes for histology were Day 3, n=12 ibudilast, n=11 vehicle and Day 14, n=11 ibudilast and n=12 vehicle. The sectioned tissue was found to be adequate for analysis in a subset of these eyes (reported below) despite multiple re-staining attempts, possibly due to two simultaneous freezer failures, which may have affected the tissue quality in some of the eyes.

B. Early-stage cell death assessment:

- a. **NMDA:** Chick NMDA studies utilizing ibudilast showed a significant drop in cell death via TUNEL assay ($p=0.0037$, **Fig 14**). Both eyes of the chicks were damaged with NMDA, then the left eye was treated with ibudilast 1.0mg/ml (1672 ± 807.8 TUNEL+ cells/mm²) while the right eye was given the vehicle (sterile water, 2900 ± 865.5 TUNEL+ cells/mm²). This represented a nearly 40% reduction in cell death. The ibudilast analogue, AV1013, was unable to prevent cell death (NMDA+Vehicle: 2496 ± 699.1 TUNEL+ cells/mm² vs NMDA+AV1013: 2117 ± 1329 TUNEL+ cells/mm², $p=0.2519$, **Fig 14**). Similarly, CPSI-1306 was unable to show a reduction in cell death (**Fig 16**).
- b. **RD:** TUNEL assay results at 3-days post RD damage were unable to show any significant difference in dying photoreceptors between mice treated with ibudilast (n=6, 1711.97 ± 996.92 TUNEL+ cells/mm²) or vehicle injections (n=3, 1266.72 ± 679.07) ($p=0.5145$, **Fig 17**).

C. Retinal Thickness Evaluations:

- a. **NMDA:** All three MIF inhibitors were utilized to assess the effectiveness of each drug to prevent the loss of retinal layer thickness due to cell death as measured through nuclear counterstain IHC. These were compared to the untreated fellow eye control and undamaged controls. No significant differences in INL or IPL thickness were detected between the treatment groups compared to controls (**Fig 18**).
- b. **RD:** The thickness of the ONL and total retinal thickness were compared in RD damaged mice with ibudilast or vehicle IP injections. Neither the ONL (RD+Vehicle: 54.69 ± 11.17 μ m vs RD+ibudilast: 51.43 ± 9.92 μ m, $p=0.6040$) nor the total retinal thickness measurements (RD+Vehicle: 131.83 ± 18.51 μ m vs RD+ibudilast: 119.30 ± 16.87 μ m, $p=0.2485$) were significantly different (**Fig 19**).

D. OCT Evaluation: A highly sensitive method to compare retinal layer thickness differences is through SD-OCT measurements. This technique captures cross-sections of the retina in live subjects in real time. Focusing on a comparison between untreated NMDA damage, AV1013 NMDA treatment, and ibudilast NMDA treatment, we were able to show that ibudilast was able to restore the retinal thickness compared to controls ($p=0.0260$) while AV1013 was not (**Fig 20A-D**). SD-OCT measurements were also captured with NMDA+CPSI-1306 treated eyes (**Fig 21**), however, no significant differences could be detected.

E. ERG Evaluation: Electroretinograms were acquired on chicks with NMDA+ MIF inhibitor (ibudilast, AV1013, or CPSI-1306) treatment compared to fellow eye NMDA+vehicle controls (**Figs 20, 22, 23, 24, 25, 26**). Light adapted full field ERG measurements were taken from with flash intensities ranging from 0.05 to 25 cd*s/m². At each of these flash intensities, we measured the a-wave, the b-wave, and the oscillatory potentials. Additionally, we measured the 20Hz flicker and long flash ERGs. No recovery was noted for AV1013 or CPSI-1306 treatment in any of the metrics: a-wave (**Fig 22**), b-wave (**Fig 23**), the sum of the oscillatory potentials (**Fig 24**), the 20hz flicker test (**Fig 25**), and the long flash test for ON & OFF bipolar cells (**Fig 26**). Only ibudilast treatment showed significance from untreated NMDA-damage eyes in restoring the b-wave (**Fig 20**). Moreover, unlike the other two MIF inhibitors, ibudilast improved the waveforms of the sum of the oscillatory potentials, although not enough to reach statistical significance (**Fig 20, 24**). Finally, the ON bipolar cell response was nearly restored to undamaged levels with ibudilast treatment and waveforms significantly improved compared to NMDA (**Fig 20I**, $p<0.05$); the other two drugs did not restore responses. (**Fig 20, 26**). In summary, ibudilast, but not AV1013 and CPSI-1306, could preserve some retinal functional responses, particularly for the retinal bipolar cells during NMDA damage.

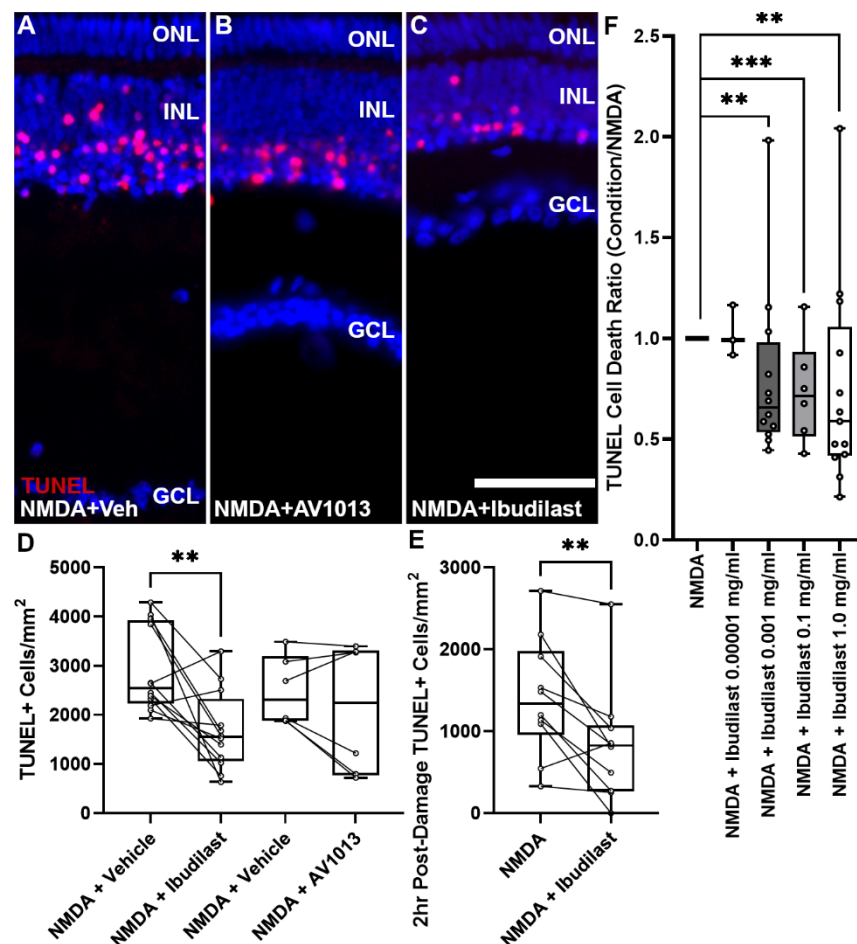


Fig 14. Ibudilast protects against neuronal cell death during excitotoxic damage, while analog AV1013 does not. Day 1 TUNEL-positive neurons were significantly reduced in the inner nuclear layer after pre-treatment (3A,C) with intravitreal injection of ibudilast 1mg/mL compared with vehicle in the fellow eye (n=12 chicks/group, $p=0.0037$). Ibudilast also significantly reduced the density of TUNEL positive cells when injected 2 hours following NMDA damage (3E). AV1013 was not preventing neuronal cell death (Figure 3B,D). Observed a dose-dependent decrease in TUNEL positive cells in the INL with ibudilast treatment (Fig 3F).

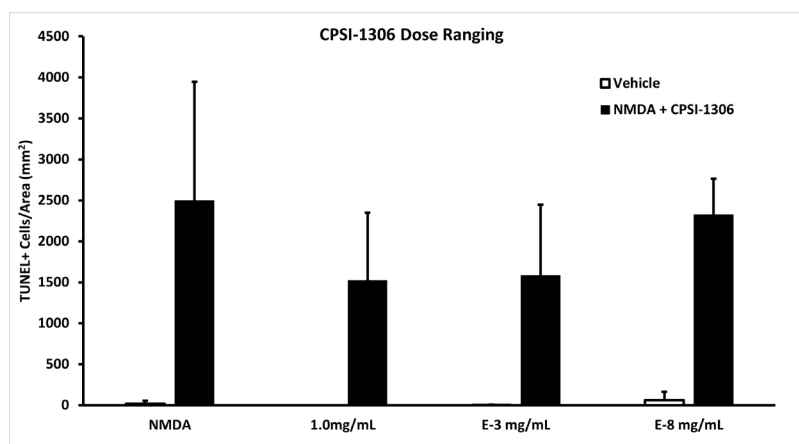


Fig 15. CPSI-1306 dose ranging study. CPSI-1305 TUNEL response in NMDA damaged chick eyes did not show any statistical significance between any dose and the untreated NMDA controls.

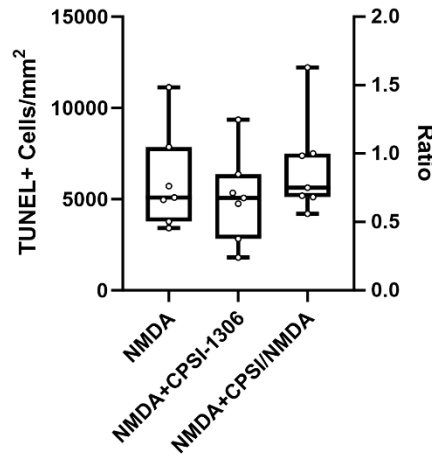


Fig 16. CPSI-1306 Chick NMDA study. CPSI-1306 was unable to prevent cell death in the chick NMDA damage model. Neither the paired analysis of NMDA vs NMDA+CPSI-1306 ($p=0.3822$) nor the ratio of paired eyes ($p=0.3750$) were significant. One more reason CPSI-1306 did not move forward in our experiments.

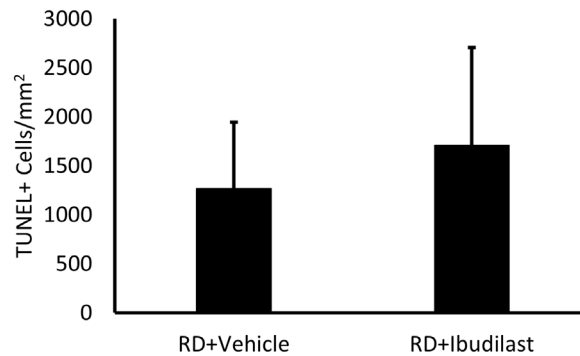


Fig 17. Murine RD with daily ibuprofen treatment was unable to restore retinal neurons. A limited study to investigate the potential of ibuprofen to prevent neuronal death was conducted in the murine retinal detachment model. No significant difference was identified.

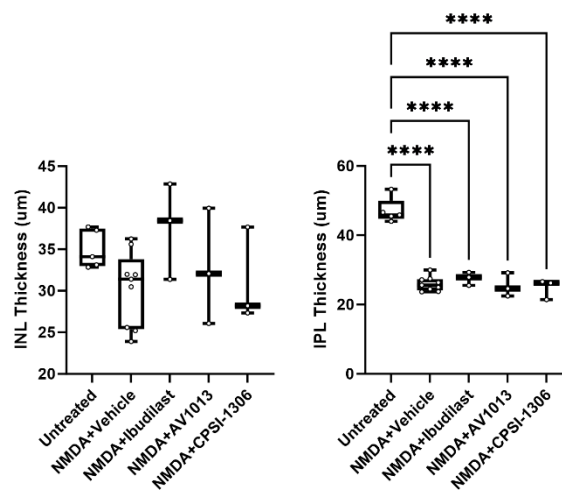


Fig 18. Chick NMDA retinal layer thickness analysis of three MIF inhibitors. Histologic frozen section slides with DAPI nuclear counterstain were analyzed to measure the thickness of the INL, IPL, and total retinal thickness at day 13 post NMDA. The INL and IPL thicknesses were compared between the group as well as the ratio of each layer with the total retinal thickness as a form of normalization. Statistics were calculated via a 1-way ANOVA with Tukey multiple comparison correction.

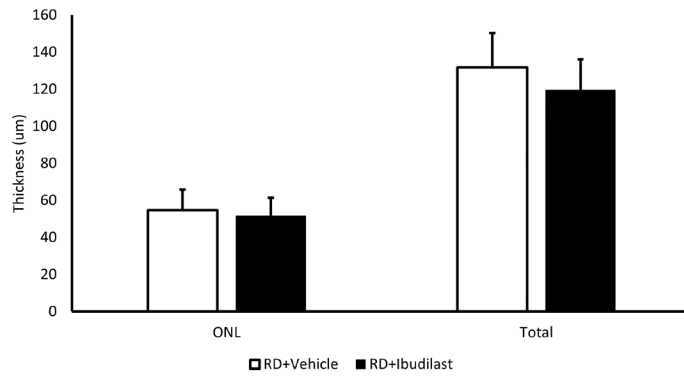


Fig 19. Retinal layer thickness analysis of RD mice with ibudilast treatment. Ibudilast was unable to preserve retinal layer thickness in the ONL or the total retinal thickness in the murine RD model compared to vehicle controls on histologic analysis of DAPI stained slides at Day 14 post treatment.

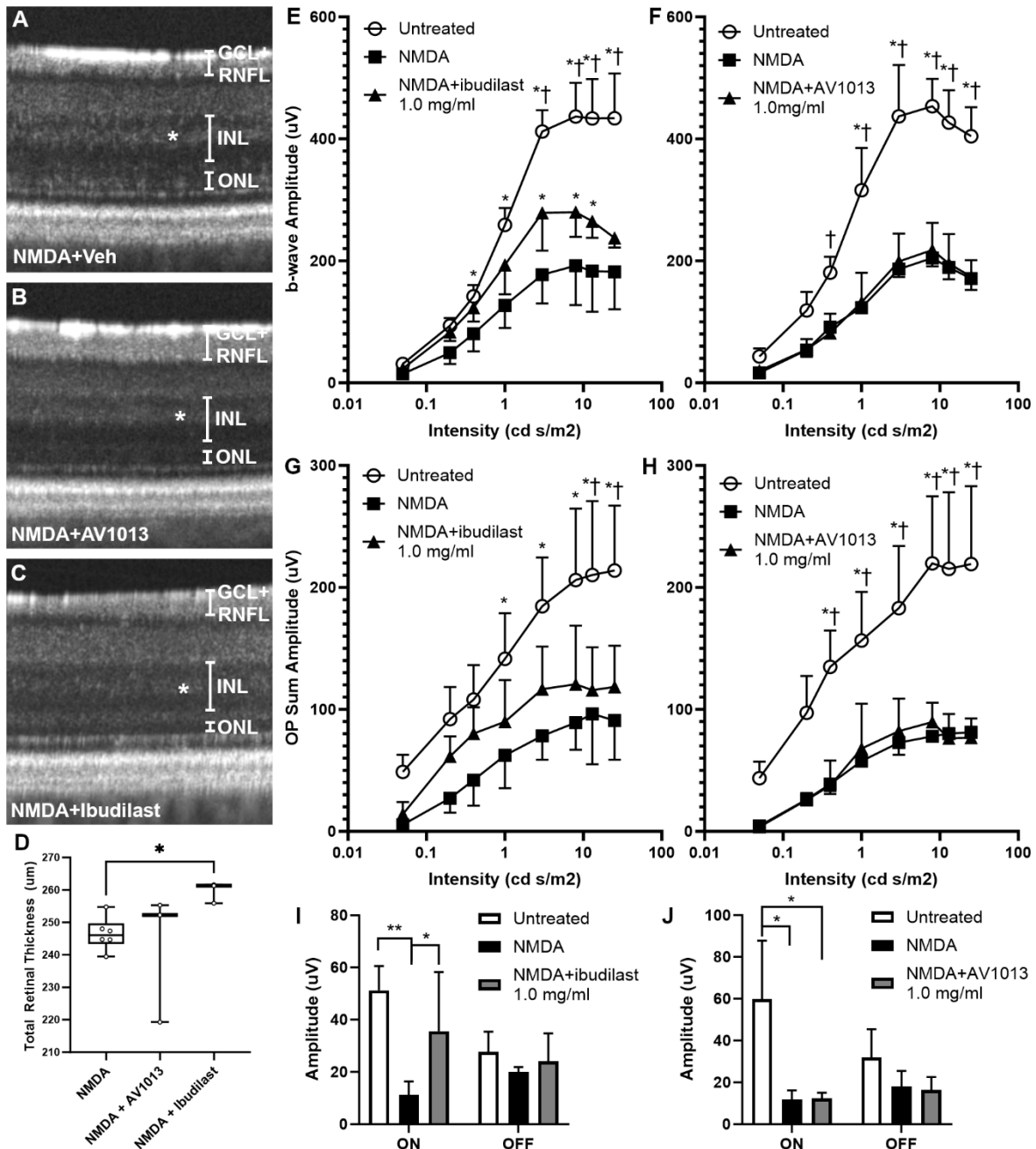


Fig 20: MIF inhibition with ibudilast preserves NMDA-damaged chick retinal thickness on SD-OCT and bipolar cell function by ERG. Intravitreal injection of ibudilast 1mg/mL given at the time of NMDA+vehicle significantly preserved retinal thickness on SD-OCT at day 10 compared to controls (n=3 chicks/group, $p=0.0260$, Dunn's multiple comparison test) (A,C,D). NMDA+AV1013 was unable to preserve retinal thickness ($p>0.9999$) (B,D). Light adapted (LA) ERG (n=3/group) showed significant preservation of the b-wave with ibudilast during NMDA damage (E), while oscillatory potentials, a measure of amacrine cell function, showed improvement with ibudilast treatment (G) vs AV1013 treatment (F,H). The long-flash ERG ON responses were significantly preserved with ibudilast, indicating the preservation of ON bipolar cells (I). AV1013 treatment was unable to show any functional recovery of the ON bipolar cells (J). * indicates $p<0.05$ with NMDA damaged eyes, † indicates $p<0.05$ with NMDA+Drug injection eyes.

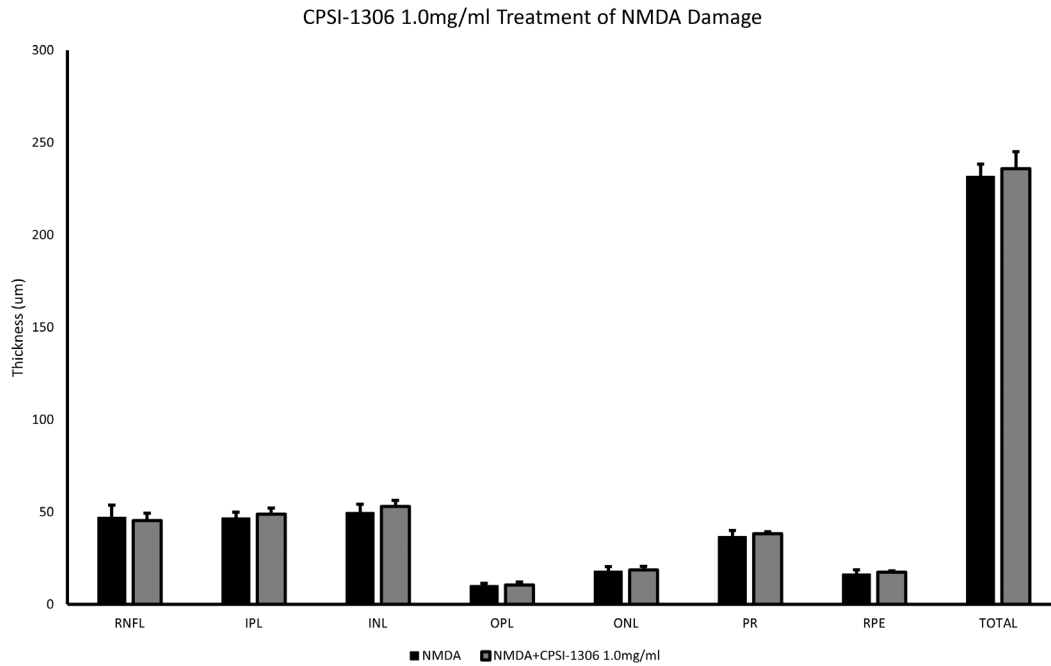


Fig 21. OCT thickness measurements of CPSI-1306 treated NMDA damaged chicks. OCT retinal layer thickness measurements acquired from chicks treated with NMDA+vehicle (right eye) and NMDA+CPSI-1306 (left eye). No significant differences were found.

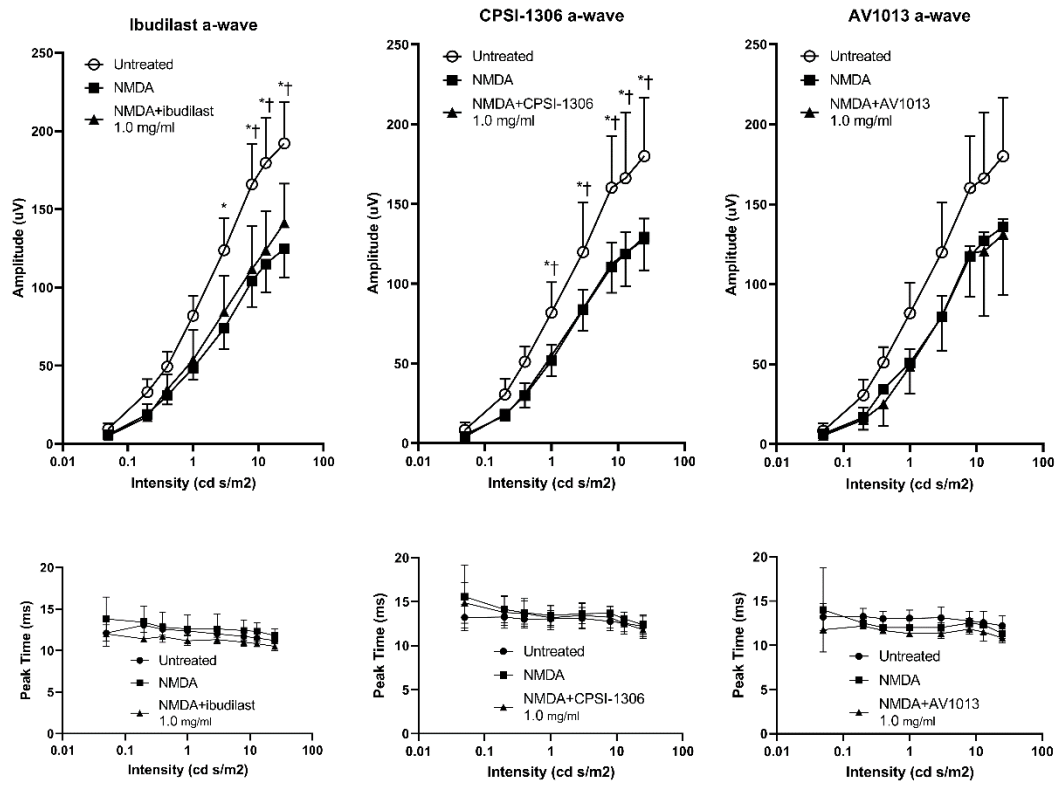


Fig 22. MIF inhibitor a-wave results. The resulting amplitudes and peak/implicit times from light-adapted ERG scans of undamaged, NMDA+vehicle damaged, and NMDA+Drug injection chicks. * indicates $p < 0.05$ with NMDA damaged eyes, † indicates $p < 0.05$ with NMDA+Drug injection eyes.

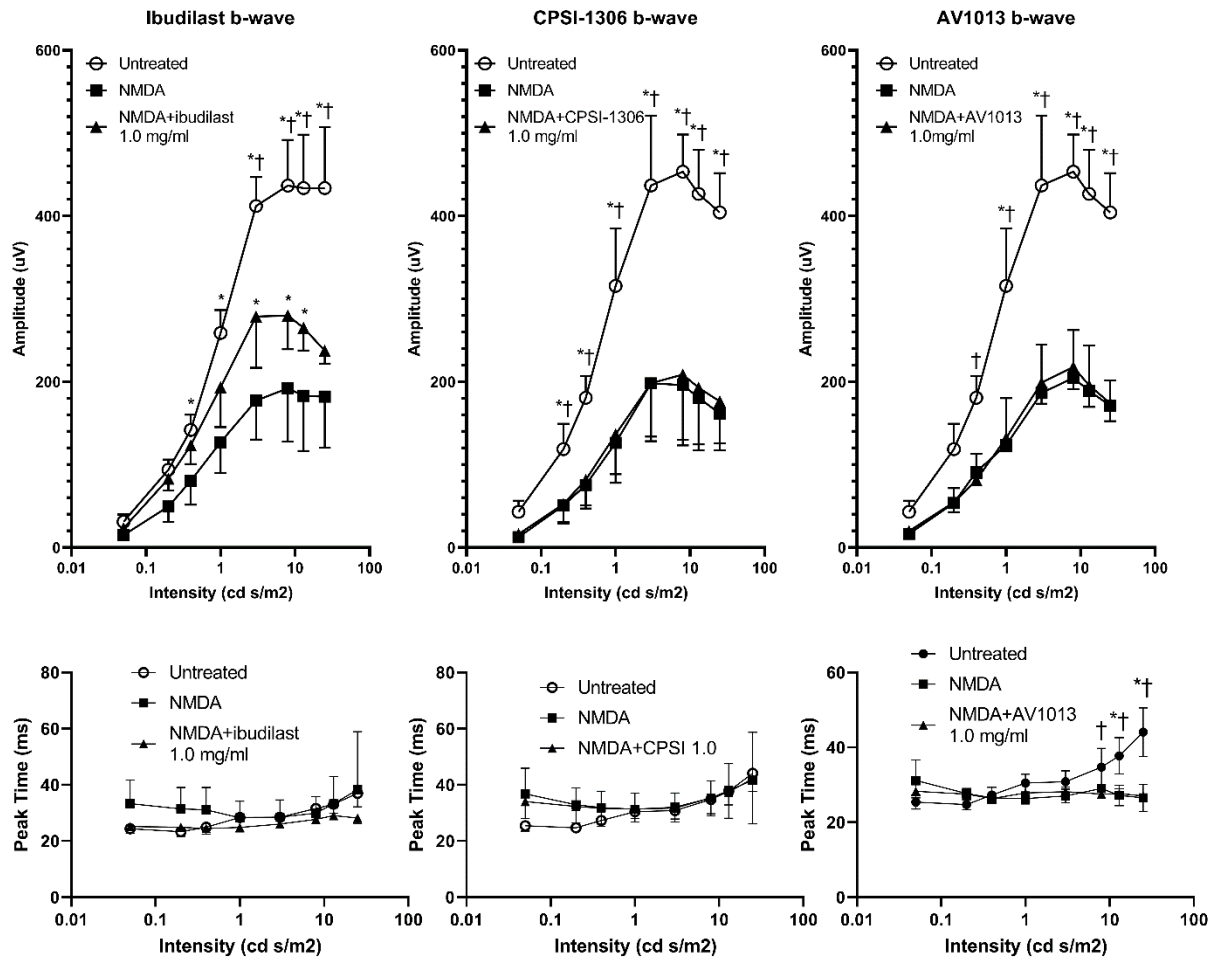


Fig 23. MIF inhibitor b-wave results. The resulting amplitudes and peak/implicit times from light adapted-ERG scans of undamaged, NMDA damaged, and NMDA+Drug injection chicks. * indicates $p < 0.05$ with NMDA damaged eyes, † indicates $p < 0.05$ with NMDA+Drug injection eyes.

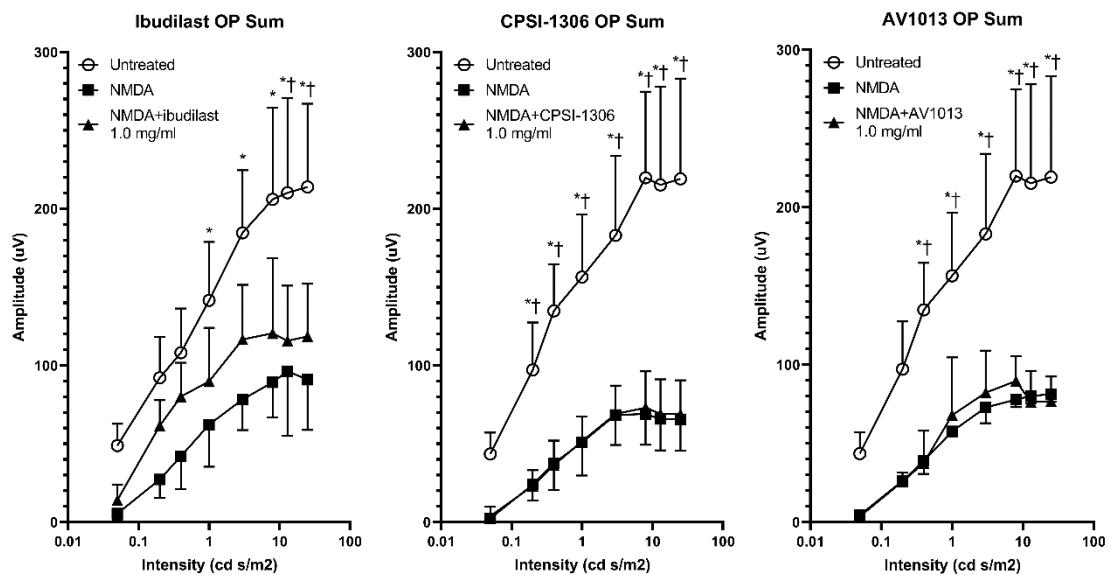


Fig 24: MIF inhibitor oscillatory potential results. The resulting amplitudes from oscillatory potential scans of undamaged, NMDA damaged, and NMDA+Drug injection chicks. * indicates $p<0.05$ with NMDA damaged eyes, † indicates $p<0.05$ with NMDA+Drug injection eyes.

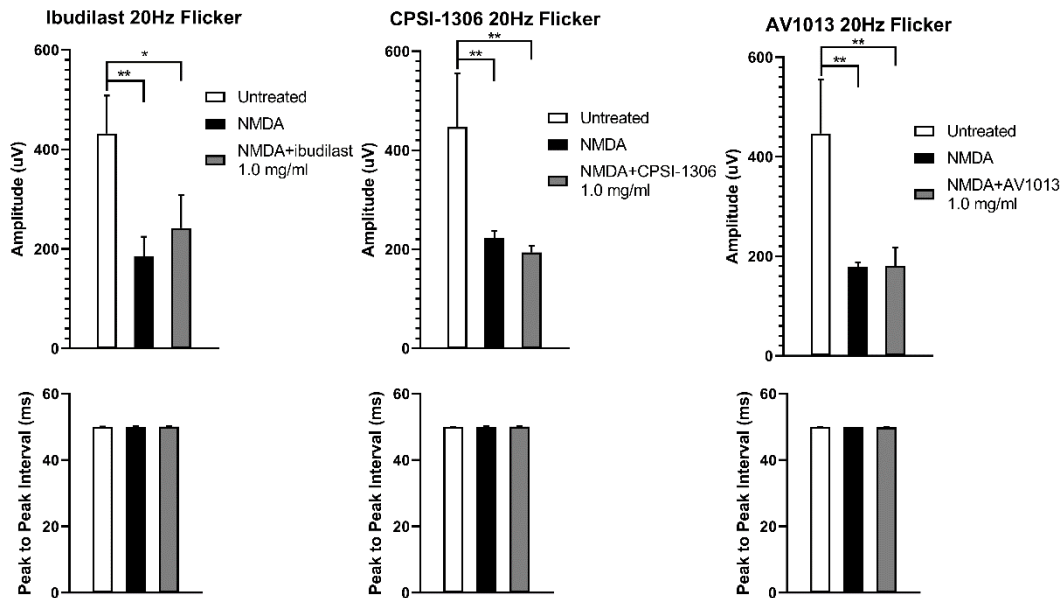


Fig 25: MIF inhibitor flicker ERG results. The resulting amplitudes and peak to peak times from 20Hz flicker scans of undamaged, NMDA damaged, and NMDA+Drug injection chicks. * indicates $p<0.05$, ** indicates $p<0.001$

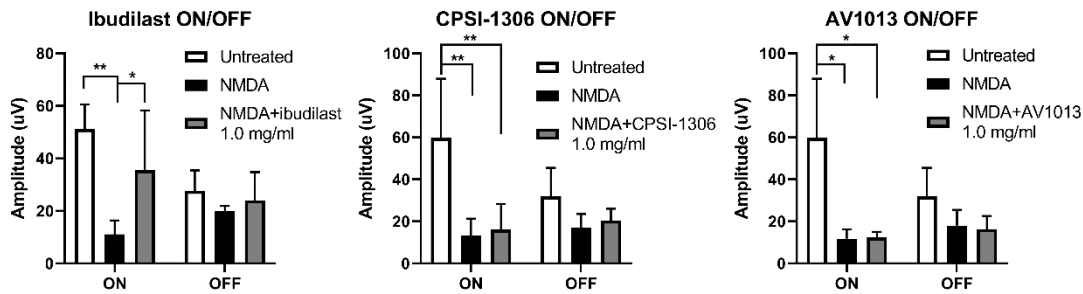


Fig 26: MIF inhibitor ON/OFF, long flash ERG results. The resulting amplitudes from long flash, ON/OFF bipolar cell scans of undamaged, NMDA+vehicle damaged, and NMDA+Drug injection chicks. * indicates $p<0.05$, ** indicates $p<0.001$

Subtask 1c: Drug timing studies in retinal damage models to test the impact of timing on the neuroprotective benefit of MIF inhibitors.

(C) Drug Timing Studies in retinal damage models were performed to test the impact of timing on the neuroprotective benefit of MIF inhibitors. Animals were treated with the optimized MIF inhibitor dose 2-6 hours following the initiation of retinal damage to assess the effect on TUNEL staining and inner retinal or outer nuclear thickness measurements to evaluate the impact of MIF inhibitors on retinal neuroprotection following injury.

(n=10/group * 4 drugs and vehicle * 1 endpoint (D3-TUNEL) * 1 dose * 1 model (RD) = 40 chicks and/or mice)

(n=10/group * 3 drugs and vehicle * 1 endpoint (D1-TUNEL) * 1 dose * 1 model (NMDA) = 30 chicks)

(n=10/group * 2 drug and vehicle * 1 endpoint (D14- ONL thickness) * 1 dose * 1 model (RD) = 20 chicks)

A. Drug timing studies:

- a. **NMDA:** Drug timing studies were carried out in NMDA damaged chicks (n=10), with ibudilast at 1.0 mg/ml being delivered two hours *after* damage was initiated. This is to simulate the fastest feasible time between a patient's injury (such as battlefield blast injury) and when they could be treated at a medical facility. With this delayed treatment, ibudilast was still able to reduce cell death (NMDA: 1412 ± 722.6 TUNEL+ cells/mm² vs. NMDA+ibudilast: 830.3 ± 712.7 TUNEL+ cells/mm², p=0.0059) (**Fig 14E**). Further analysis was carried out on subjects 9 days post injury that had received the treatment dose 2 hours post-damage. The IPL, INL, ONL, and total retinal thicknesses were compared between chicks treated with ibudilast and their untreated fellow eyes. Similar to previous retinal thickness measurements, even with an early reduction in dying cells, an effect on the later retinal thickness could not be detected compared with controls (IPL: p=0.6107, INL: p=0.9387, ONL: p=0.4071, Total: p=0.8577) (**Fig 27**).
- b. **RD:** Ibudilast treatment of RD murine eyes was not able to achieve significance under treatment at the time of injury. As such, further experiments to test the drugs efficacy with later delivery were not conducted.

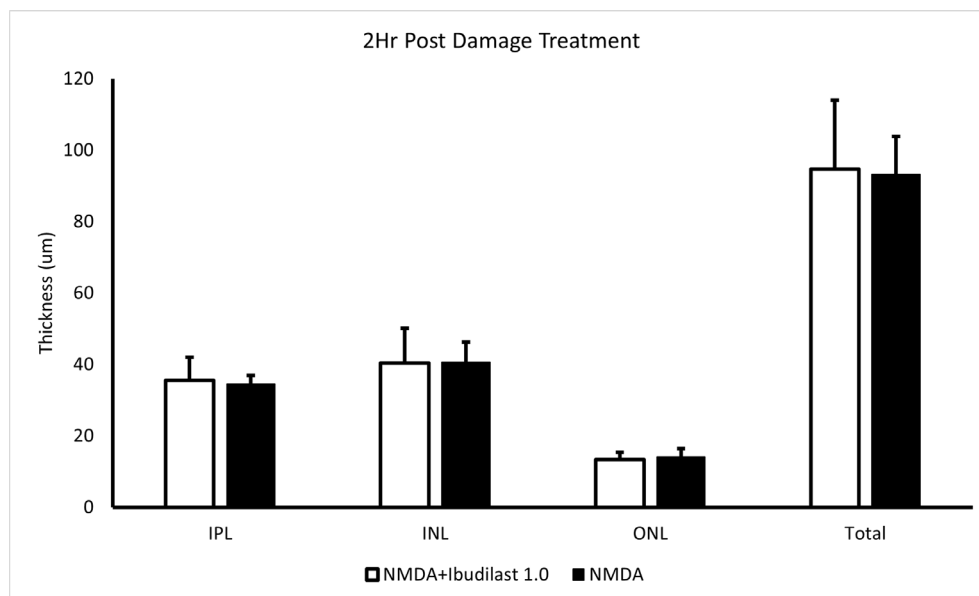


Fig 27. Drug timing retinal thickness unchanged with ibudilast treatment of chick NMDA damage. Paired retinal thickness measurements show no significant improvements in retinal layer thickness with ibudilast compared with vehicle injected 2 hours after NMDA damage.

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.

Subtask 2a: Test the hypothesis that MIF inhibitors block gliosis and pathological wound healing responses *in vitro*.

To 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 were incubated with TGFβ to mimic PVR conditions. The effect of clinically relevant MIF inhibitors (CPSI-1306/ Ibudilast/AV1013) were 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 have been impacted by the MIF inhibitor therapeutics and role of phosphodiesterase inhibition.

- a. **Cell Line Validation:** We initially proposed working on two cell lines: ARPE-19 and MIO-M1, which model human retinal pigment epithelium cells and human Müller glia, respectively. To

verify these cell lines, we ran short tandem repeat (STR) profiling to compare with published results in the literature and confirm the identity of the cell types. Our ARPE-19 cell line matched the established profile perfectly (**Table 1**). While the MIO-M1 cell line does not have a published STR profile, the characterizing literature reports the cell line originated from a female patient with an XX karyotype. However, the STR profile results showed the presence of both X and Y chromosomes, suggesting a male source (**Fig 28**). After discussion with several collaborators, we discontinued all further work with the MIO-M1 cell line.

- b. **Scratch Assay:** We conducted a series of scratch assays with the ARPE-19 cell line testing the effects of ibudilast and CPSI-1306 on wound closure and migration in the presence of TGF β with and without exogenous MIF (**Fig 29**). We found no statistical significance between the conditions, implying the drugs do not impede ARPE-19 migration in the context of a scratch assay. We later evaluated migration with transwell experiments, which evaluate migration in response to a chemotactic source. In that assay we did discover that ibudilast and AV1013 reduced cell migration in the *in vitro* PVR model (see below).
- d. **MTT Assay:** We performed MTT assays on the ARPE-19 cell line, treated with TGF β to mimic PVR-like conditions. MTT measures cell proliferation, an important aspect of PVR development. Cells were treated with different concentrations of CPSI-1306, ibudilast, and AV1013, the ibudilast analogue that lacks phosphodiesterase inhibitor activity (**Fig 30**). CPSI-1306 showed no significant effect on cell proliferation ($p \geq 0.475$), while ibudilast and AV1013 significantly reduced the cell proliferation (ibudilast: 0.45mg/ml v control, $p=0.046$, AV1013: 0.45mg/ml v control, $p<0.0001$) (**Fig 30**). The drug doses used mimic the *in vivo* dose based on dilution of the drug in the vitreous. For example, the 0.45mg/ml *in vitro* dose is equivalent to the 4.5mg/ml *in vivo* dose. TUNEL assay was also performed, which showed no toxicity from the drugs (see Cell Toxicity below), suggesting that ibudilast and AV1013 inhibited cell proliferation without inducing cell toxicity.
- e. **Gel Contraction Assay:** Another aspect of wound healing and PVR is cell contraction. We performed gel contraction assays with three-dimensional collagen gels. Using a Cell Biolabs CytoSelect 24-well Cell Contraction Assay kit, we measured the rate of contraction over a period of 72 hours. We calculated a contraction ratio by measuring the area of the gel and dividing it by the initial area at time 0. This initial experiment was performed on ARPE-19 cells with and without TGF β to confirm that the experimental model is working (no cell control ratio: 1.0 after 72hrs, cells control ratio: 0.283 after 72hrs, cells+TGF β ratio: 0.189 after 72hrs) (**Fig 31**). We then tested the effects of MIF inhibitors on gel contraction of ARPE-19 cells as our *in vitro* PVR model. Over a period of 24 hours, ibudilast, AV1013, and CPSI-1306 were all tested for their ability to influence contraction. 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 were calculated as a percent of control. MIF inhibitors were tested at 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 critical condition of PVR. Our findings indicate that MIF inhibitors were able to inhibit contraction in a dose dependent manner (ibudilast 0.01mg/ml: $p=0.004$, ibudilast 0.1mg/ml: $p=1.2426 \times 10^{-6}$, AV1013 0.01mg/ml: $p=0.12$, AV1013 0.1mg/ml: $p=3.96221 \times 10^{-7}$) (**Fig 32**).
- f. **EMT Assays:** A hallmark of PVR is the epithelial mesenchymal transition (EMT). EMT assays were performed with 0.01 and 0.1mg/mL ibudilast or 0.01 and 0.1mg/mL AV1013 with TGF β treated ARPE-19 cells and controls. Staining for epithelial and mesenchymal cell markers showed that ibudilast and AV1013 both blocked EMT. After 5 days of serum starvation (1% fetal bovine serum), ARPE-19 cells were either left untreated or treated with 10 ng/mL TGF β to induce EMT, with or without the indicated concentrations of AV1013 or ibudilast for 48 hours. Cells were fixed in 4% paraformaldehyde and stained for the two mesenchymal markers vimentin and smooth muscle actin (SMA) and epithelial cell marker zonula occludens (ZO-1) to determine level of EMT inhibition by MIF (**Figs 33, 34**).

- g. Cell Toxicity:** Since MIF inhibition decreased cell proliferation on the MTT assay, TUNEL staining was performed to rule out cell death caused by the MIF inhibitors (ibudilast, AV1013, CPSI-1306). Hydrogen peroxide (H₂O₂) at 1mM was used as a positive control for cell death; MIF inhibition did not increase cell death (**Fig 35**).
- h. Transwell migration assay:** Cell migration is an essential step in PVR scar formation. This is why we tested the ability of MIF inhibitors to influence ARPE-19 cell migration in our *in vitro* PVR model. Transwell assay was used and TGF β with or without serum was added to the lower chamber as the chemoattractant. The number of cells migrating from the upper to the lower chamber was quantified using a colorimetric assay. Excluding the CPSI-1306 data, which was discontinued after determining it was generally ineffective *in vivo*, the AV1013 and ibudilast conditions were all significantly lower than the control group (ibudilast 0.01 mg/ml: p=0.0015, ibudilast 0.1: p=0.0008, AV1013 0.01 mg/ml: p=0.0012, AV1013 0.1 mg/ml: p=0.0005) (**Fig 36**).

Results for Submitted Sample			ATCC Reference Database Profile	
Loci	Query Profile		Database Profile	
Amelogenin	X	Y	X	Y
CSF1PO	11		11	
D13S317	11	12	11	12
D16S539	9	11	9	11
D21S11	28	29	28	29
D5S818	13		13	
D7S820	9	11	9	11
TH01	6	9.3	6	9.3
TPOX	9	11	9	11
vWA	16	19	16	19

Table 1. STR profile results from submitted DNA of ARPE-19 matches the published STR profile.

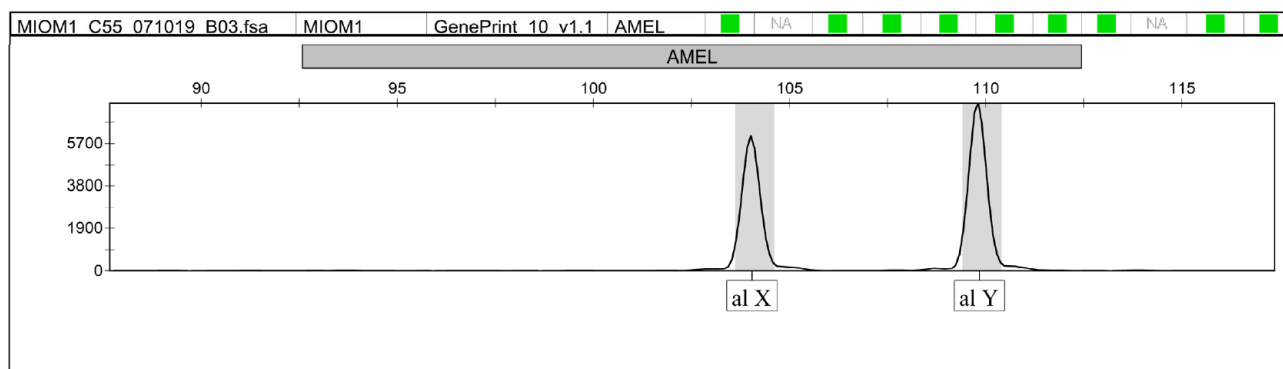


Fig 28. STR profiling of MIO-M1 shows X and Y chromosome-specific PCR products. This suggests the origin of the cell line is from a male donor, while the MIO-M1 cell line is noted to be obtained from an individual with two X chromosomes.

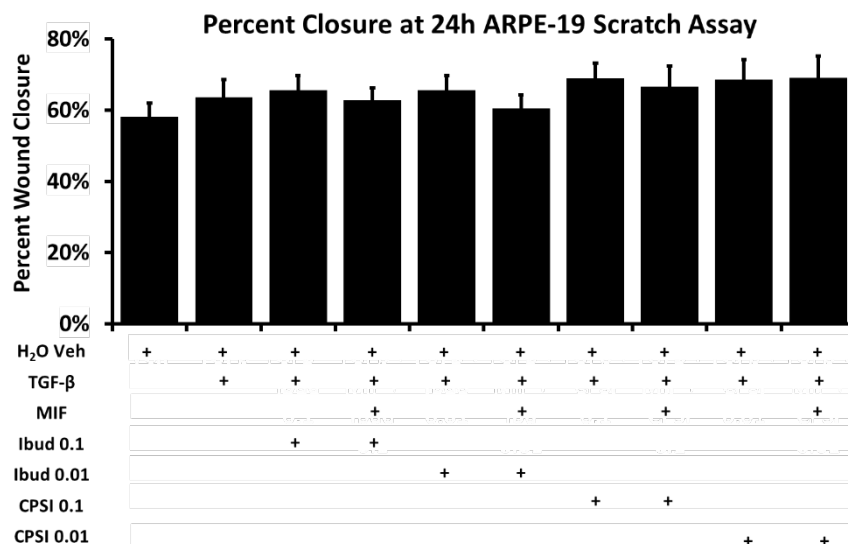


Fig 29. Scratch Assay Results. ARPE-19 scratch assay data shows no significant differences between experimental conditions ($p>0.05$). The addition of ibudilast (0.1 mg/ml and 0.01mg/mL) and CPSI-1306 (0.1 mg/ml and 0.01mg/mL) in the presence of MIF does not significantly increase the migration rate.

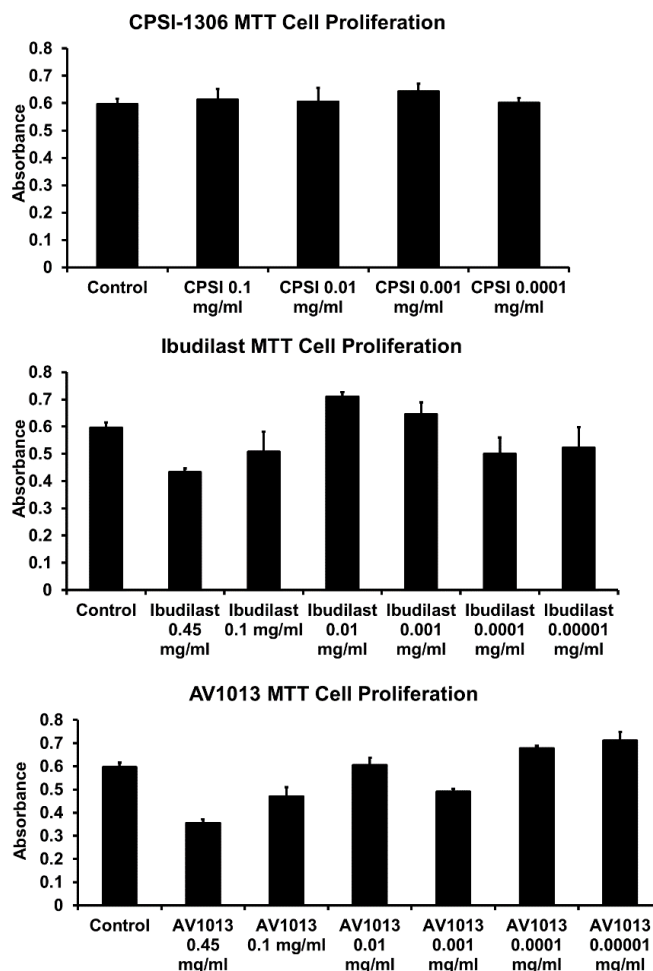


Fig 30. MTT Cell Proliferation Results. MTT absorbance results on CPSI-1306 (top), Ibudilast (middle), and AV1013 (bottom). CPSI-1306 showed no significant differences between the different doses. Ibudilast and AV1013 both significantly reduced cell proliferation (ibudilast: 0.45mg/ml v control, $p=0.046$, AV1013: 0.45mg/ml v control, $p<0.0001$).

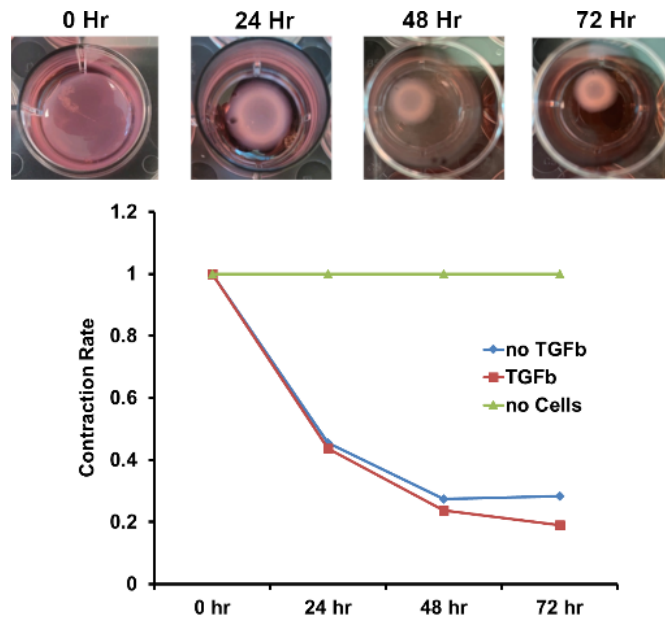


Fig 31. Gel Contraction Preliminary Results. A gel contraction assay was performed on ARPE-19 cells with and without TGF β . The test was successful in displaying contraction.

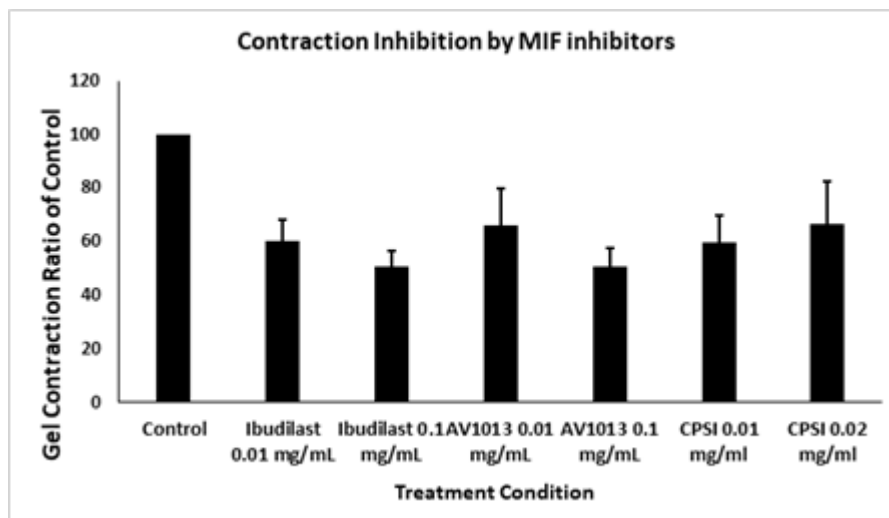


Fig 32. MIF inhibitors block collagen gel contraction in *in vitro* PVR model. ARPE-19 cells were treated with the indicated concentrations of MIF inhibitors while subjected to gel contraction studies in the *in vitro* PVR model. Results are shown as percent of control contraction (maximum gel contraction set at 100%). 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. Ibudilast and AV1013 both significantly reduced gel contraction (ibudilast 0.01mg/ml: $p=0.004$, ibudilast 0.1mg/ml: $p=1.2426 \times 10^{-6}$, AV1013 0.01mg/ml: $p=0.12$, AV1013 0.1mg/ml: $p=3.96221 \times 10^{-7}$).

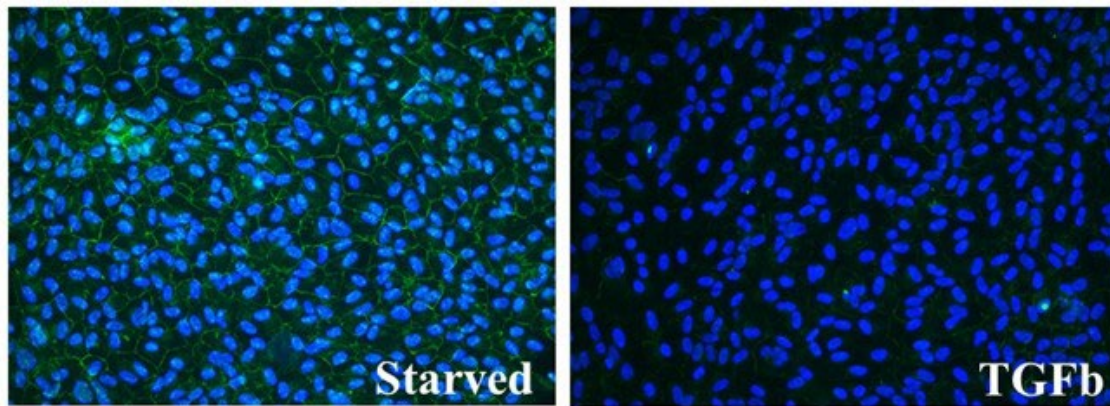


Fig 33. EMT is induced in the TGF β ARPE-19 *in vitro* PVR model: Epithelial marker ZO-1 (green immunofluorescence staining) is decreased in TGF β treated ARPE-19 cells.

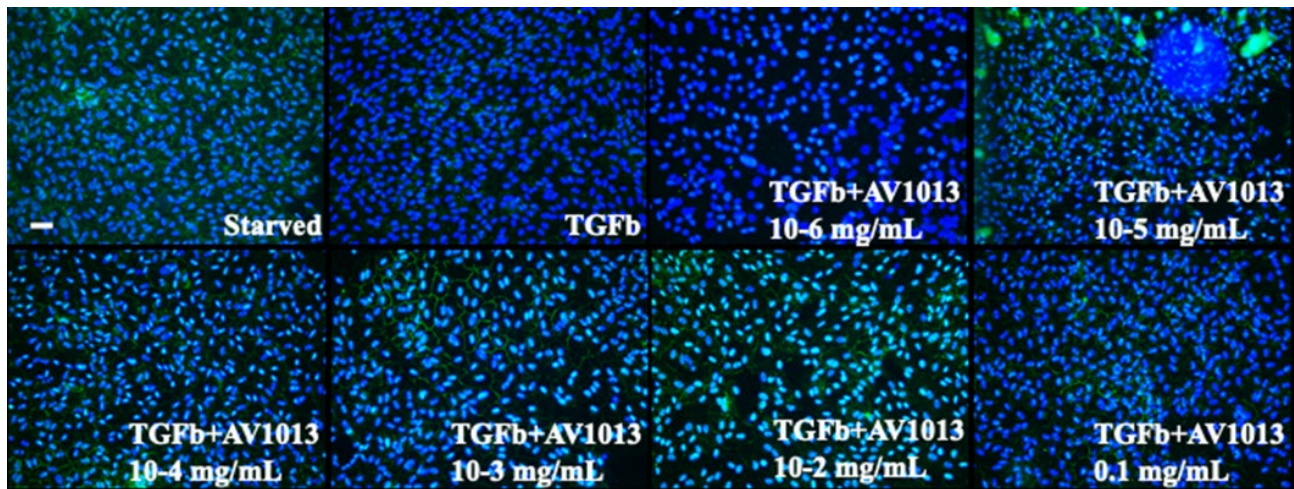


Fig 34. MIF inhibition by AV1013 blocks loss of ZO-1. Phosphodiesterase inhibitor activity is not essential for blocking EMT in the *in vitro* PVR model. ZO-1, green. DAPI, blue. Scale bar 50 μ m.

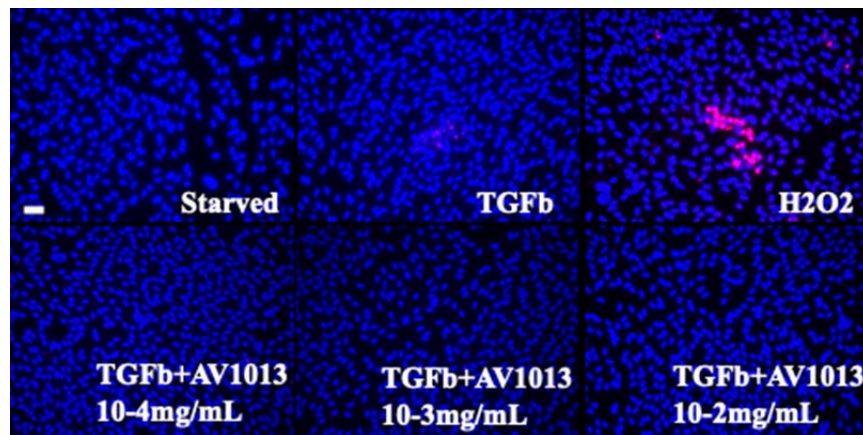


Fig 35. MIF inhibition does not induce apoptosis in ARPE-19 cells. H₂O₂ was used as a positive control to induce cell death. TUNEL, red. DAPI, blue. Scale bar 50 μ m.

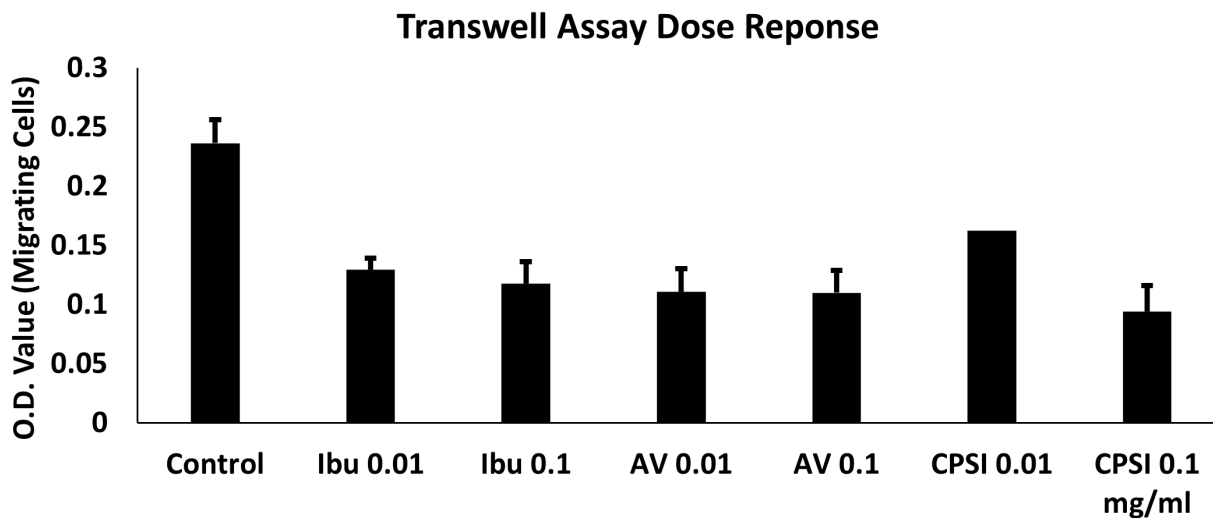


Fig 36. Transwell assay dose response chart showing the impact of MIF inhibition on cell migration. ARPE-19 cells were plated in the upper well of a transwell plate while TGFb was used as a chemoattractant in the lower chamber. MIF inhibitors were added to both chambers at the listed concentrations. Cells were allowed to migrate for 18 hours. Error bars represent standard error of the mean (SEM).

Subtask 2b: Test the hypothesis that MIF inhibitors block gliosis and pathological wound healing responses *in vivo*.

(A) RD and NMDA models: Doses of the intraocular MIF inhibitors shown to block TUNEL staining were used to evaluate the effect on retinal gliosis in our RD and NMDA retinal damage models. Retinas were evaluated for development of gliosis using GFAP and aquaporin 4 immunostaining to detect Muller glia and astrocytic changes, CD45 was used to detect microglia accumulation, and PCNA to evaluate cell proliferation of Muller glia progenitor cells, detected with Sox 2 or 9 markers, at day 14 in RD and day 7 in NMDA models. (n=10/group * 4 drugs and vehicle * 1 endpoint (D14) * 1 dose * 1 model (RD) = 40 chicks and/or mice) (n=10/group * 3 drugs and vehicle * 1 endpoint (D7) * 1 dose * 1 model (NMDA) = 30 chicks) If enough tissue is available from the experiment in Aim 1 (B), we may be able to use fewer animals for these studies.

(B) PVR model: MIF inhibitor testing with CPSI-1306 and ibudilast/AV1013 using paradigms developed in Aim 1 were performed in a well-established *in vivo* PVR model using the rabbit to determine if intravitreal drug blocks PVR formation.

Rabbit PVR model: The retinal status was evaluated in a masked fashion with an indirect ophthalmoscope approximately on days 1, 3, 7, 14, 21, and 28. PVR was graded according to the Fastenberg scale of classification: stage 0, no disease; stage 1, epiretinal membrane; stage 2, vitreoretinal traction without retinal detachment; stage 3, localized retinal detachment (one to two quadrants); stage 4, extensive retinal detachment (two to four quadrants without complete detachment); and stage 5, complete retinal detachment. SD-OCT/fundus imaging was performed on a subset of rabbits. On day 28, animals were euthanized, and eyes were enucleated (n=10 rabbits).

Rabbit PVR drug study: MIF inhibitor testing with ibudilast using the paradigms developed in Aim 1 was performed in an *in vivo* rabbit PVR model.

(n=10/group * 4 drugs and vehicle * 1 endpoint (D28) * 1 dose * 1 model (PVR) = 40 rabbits)

We will add additional rabbits for experiment troubleshooting this model (n= 20).

- A. **PVR Induction:** PVR was induced in 10 rabbits via an intraocular injection of C3F8 gas, followed by an injection of rabbit conjunctival fibroblast cells and platelet rich plasma (PRP) a week later. This experiment was performed to optimize the volumes of gas and PRP used for injection. Eyes were

evaluated using a slit lamp on D1 and D3 post injection of cells and PRP to monitor for infection. Exams utilizing an indirect ophthalmoscope to grade the PVR were performed weekly for four weeks after the injection of cells and PRP. Fastenberg grading demonstrated successful PVR development in the model, with optimized results using 50 microliters of gas and 50-100 microliters of cells and PRP. The volume used for the subsequent drug studies was 50 microliters of cells and PRP followed by 50 microliters of drug or vehicle. Ocular fundus photos were taken (**Fig 37**). After the final examination rabbits were euthanized and eyes were enucleated for future evaluation. A subset of enucleated eyes were examined under an operating microscope (**Fig 38**).

B. Immunohistochemical marker staining: We have utilized immunohistochemical marker staining to evaluate important factors for PVR development, including levels of gliosis (GFAP, AQP4), inflammation (CD45, Iba1), and proliferation (PCNA). Markers PCNA and CD45 required some troubleshooting. To ensure the best quality staining, tissue is stained as soon as possible after sectioning, and staining is done for PCNA and CD45 first to ensure full signal strength.

a. GFAP

- i. In the chick NMDA model, we used GFAP to evaluate the effect of AV1013 and ibudilast on gliosis immediately after damage at day 1 post-damage and at a longer timepoint at day 9 post-damage (n=6/drug). Treatment with Ibudilast significantly reduced GFAP expression (NDMA: 42.80 ± 11.71 vs. NDMA+ibudilast: 24.56 ± 9.957 , $p=0.0088$) 9 days after NMDA damage in the chick while AV1013 treatment acting in a more short-term fashion reducing gliosis at day 1 (NDMA: 44.26 ± 11.27 vs. NDMA+AV1013: 29.72 ± 12.14 , $p=0.0372$) (**Fig 39**).
- ii. In the murine RD model, retinas were stained with GFAP to evaluate the effect of ibudilast given via intraperitoneal injection on gliosis at day 3 (n=6/group) and day 14 (n=6/group). Assessments compared the mean GFAP intensity between the RD eye with ibudilast and vehicle treated mice at day 3 and day 14. Two-tailed t-tests were performed, finding no significant differences between groups at day 3 and 14 ($p=0.1302$ and $p=0.3284$, respectively) (**Fig 40**).

b. PCNA

- i. Ibudilast treated chick NMDA-damaged retinal tissue was assessed to quantify cell proliferation 1 day after damage with a PCNA antibody stain. Mean PCNA intensity was captured in the different layers (ONL, INL, IPL-GCL) and the full retina. The intensity and cell counts were assessed as raw measurements and as ratios with respect to the fellow control eye. None of the metrics examined resulted in significant differences between the control untreated eye and the ibudilast treated eye (**Fig 41**). PCNA is not expressed at levels high enough to quantify 9 days after NMDA damage.
- ii. Murine RDs were evaluated with PCNA at day 3 and 14. The mean PCNA intensity was evaluated between the RD eye of ibudilast vs vehicle treated mice. Two-sample t tests revealed no significant difference in PCNA between the two groups at either day 3 or 14 time points ($p=0.6158$ and $p=0.976$, respectively) (**Fig 42**).

c. AQP4

- i. NMDA damaged chicks were evaluated with an AQP4 antibody stain to detect for edema-like behavior and marker expression (n=5). Both the mean AQP4 expression and the area above a threshold (normalized against the retinal area and length) were assessed to detect differences in the GCL-RNFL region and the full retina. No differences were detected at the day 9 timepoint (**Fig 43**).
- ii. Murine RDs were evaluated with AQP4 to detect edema and protein expression are day 3 (n=6/group) and day 14 (n=6/group). The stain was analyzed using a two-tailed t-test for the mean AQP4 intensity and area above a threshold. The total retinal mean AQP4 expression for day 3 was significantly different where subjects treated with ibudilast showed an increase in AQP4 levels compared to controls ($p=0.0280$) (**Fig 44**). This difference lost significance at the day 14 time point (data not shown).

d. CD45

- i. We stained for CD45 to look for proliferating microglia in the chick NMDA model at day 1 and day 9 after damage with ibudilast and AV1013 treatment. At day 1, we assessed the mean count and morphology of the detected cells. Morphology was assessed by the form factor 1 (FF1) and the aspect ratio. No differences were detected between the vehicle treated control and either drug (**Fig 45**). At day 9, we again assessed the count and morphology but additionally measured in different regions of the retina since cells were detected in a wider distribution in the retina: photoreceptor outer nuclear layer to inner/outer segments (PR-IS/OS), INL to OPL, RNFL to IPL, and the total retina. The only statistically significant metric detected was that the count of CD45+ cells in the PR-IS/OS region appeared to decrease with AV1013 treatment ($p=0.0099$, **Fig 46**).

e. Iba1

- i. Murine RDs 14 days post-damage were stained with Iba1 and evaluated for the presence of microglia between RD eyes (left eye) and undamaged control (right eye) in mice treated with ibudilast or vehicle control. Iba1+ cells were detected and counted with normalization via the retinal area. No significant difference was detected between the RD eyes with and without ibudilast treatment (**Fig 47**).

C. Ibudilast treatment in rabbit PVR model: PVR was induced in 30 rabbits via an intraocular injection of C3F8 gas, followed by an injection of conjunctival fibroblast cells and platelet rich plasma (PRP) a week later. Rabbits received intravitreal injection of either MIF inhibitor ibudilast or saline, 5 minutes after PRP injection. Rabbits received a second ibudilast treatment 48 hours after PRP injection. Eyes were evaluated using a slit lamp on D1 and D3 post injection of cells and PRP to monitor for infection. Exams utilizing an indirect ophthalmoscope to grade the PVR were performed weekly for four weeks after the injection of cells and PRP. After final exams, rabbits were euthanized and eyes were enucleated. Eyes were dissected, gross images were taken using an operating microscope, and tissue was fixed for preservation. The eyes of 4 rabbits were flash frozen immediately following dissection for future dissection and evaluation. OCT images were also captured on a subset of these rabbits.

- a. Fastenberg Scale:** The Fastenberg grading scale is a metric developed to determine the severity of PVR cases with 0 being non-existent and 5 being the most severe. The grading was all performed by a clinical ophthalmologist. We found that the average grade for rabbits treated with ibudilast was significantly reduced compared to those treated with the saline vehicle at day 14 post-PVR induction ($p=0.0185$). This significant reduction in PVR grade disappeared by days 21 and 28 (**Fig 48**). This points towards ibudilast having a short-term effect. Coupled with our pharmacokinetic studies, which show a short half-life of ibudilast in the retina and vitreous, these results are consistent with ibudilast reducing PVR early after injection, then losing effectiveness as the drug is metabolized. Sustained inhibition of PVR likely requires reformulation of the drug for continued inhibition of fibrosis.
- b. SD-OCT captures:** OCT b-scans were acquired on rabbits after PVR induction. We were able to successfully capture images on most subjects ($n=3$), however the view was usually obscured by the PVR membranes in some form. This prevented the ability to take thickness measurements of the rabbit retina and make any statistical comparisons. Some examples of the b-scans and *en face* fundus images are presented below (**Fig 49**).
- c. Celeris Rabbit PVR ERG:** A subset of rabbits before and after PVR induction were selected to acquire ERG measurements on for functional assessment. A series of light adapted full field ERG tests were run at 1.0, 3.0, and 10.0 $\text{cd}^*\text{s}/\text{m}^2$, a 30Hz flicker was performed at 3.0 $\text{cd}^*\text{s}/\text{m}^2$, and series of dark-adapted ERG tests were run from 0.001 to 3.0 $\text{cd}^*\text{s}/\text{m}^2$. In the light adapted tests, the a-wave, b-wave, i-wave, and oscillatory potentials were measured. In the dark-adapted tests, the a-wave, b-wave, and oscillatory potentials were measured. We compared the ratio of each metric as the left (PVR) eye over the right (undamaged) eye for each subject ($n=3$). Throughout the many tests, two significant differences stand out. The first is a difference between the amplitude ratio of the pre-injury eyes (untreated OS / untreated OD) and the post-injury eyes (PVR OS / untreated OD) of the flicker test ($p=0.0429$). The other significant result is that in the light adapted 10.0 $\text{cd}^*\text{s}/\text{m}^2$ b-wave measurement, the PVR ratio was significantly

lower than the control 1.0 value ($p=0.0429$). This strongly indicates a functional loss within the inner retina, particularly the bipolar neurons (**Fig 50**).

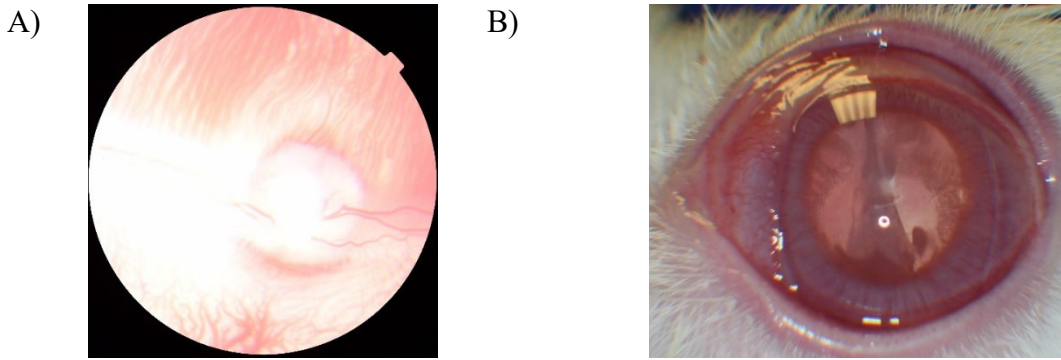


Fig 37. Fundus camera captures of undamaged retina (A) and membranes in PVR eyes (B).

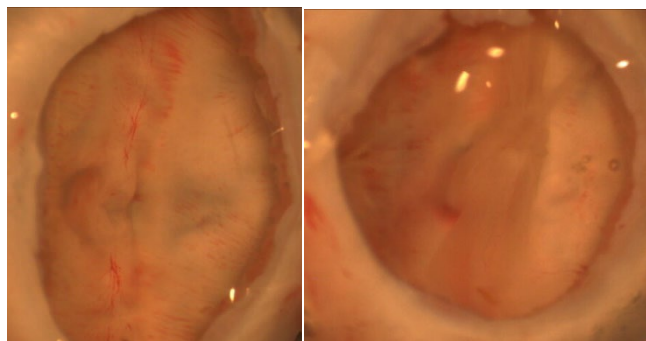


Fig 38. Operating microscope captures of undamaged retina (left) and PVR retina with epiretinal membranes (right).

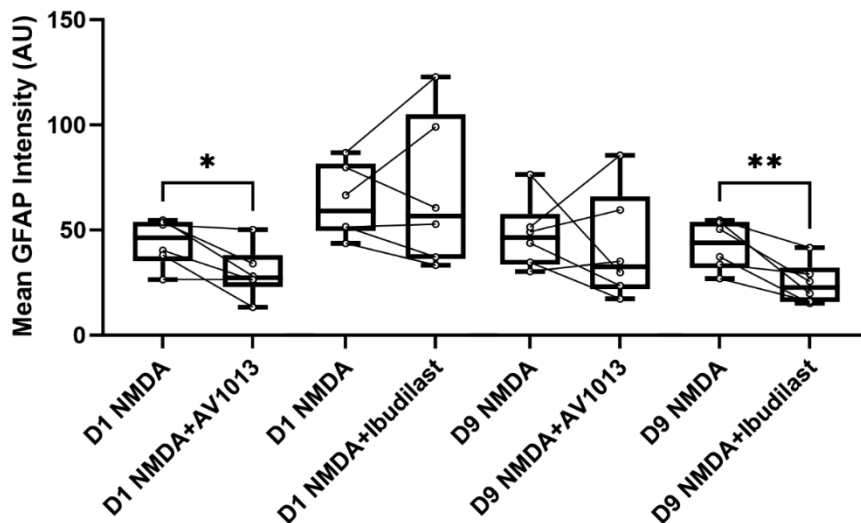


Fig 39. Mean GFAP intensity at day 1 and day 9 NMDA chicks. Measurements of mean GFAP intensity (AU) were compared between paired NMDA damaged eyes. Left eyes were either treated with ibutilast or AV1013 at 1.0 mg/ml while the right eye was left untreated as a control. Treatment with Ibutilast significantly reduced GFAP expression 9 days after NMDA while AV1013 treatment significantly reduced GFAP expression 24 hours after damage. Stats were calculated as paired t-tests. Treatment with Ibutilast significantly reduced GFAP expression (NMDA: 42.80 ± 11.71 vs. NMDA+ibutilast: 24.56 ± 9.957 , $p=0.0088$) 9 days after NMDA

damage in the chick while AV1013 treatment acting in a more short-term fashion reducing gliosis at day 1 (NDMA: 44.26 ± 11.27 vs. NDMA+AV1013: 29.72 ± 12.14 , $p=0.0372$). * indicates $p<0.05$, ** indicates $p<0.01$

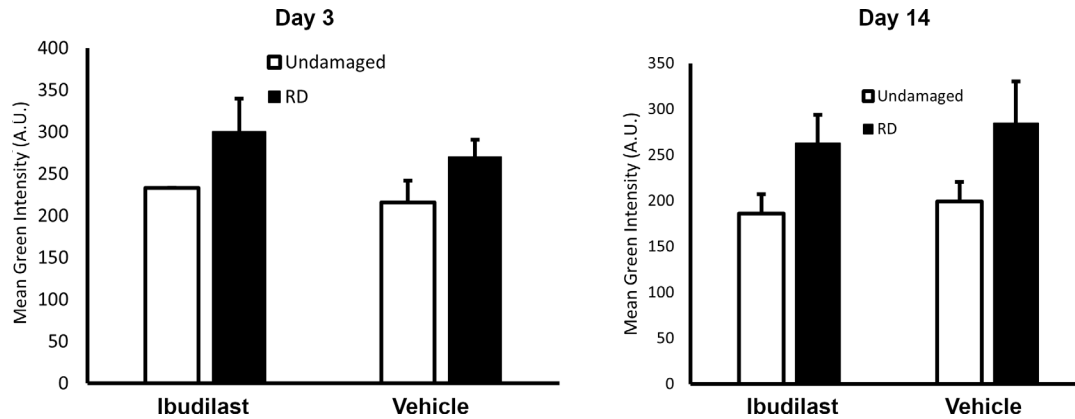


Fig 40. Mean GFAP intensity at day 3 and day 14 RD mice. Measurements of the mean green GFAP intensity values were taken in mice with ibudilast and vehicle treatment of RD eyes (left eye) and undamaged eyes (right eye) at day 3 and day 14. Statistics were calculated between RD eyes via student's t-test. No significant differences were detected between ibudilast treatment and controls.

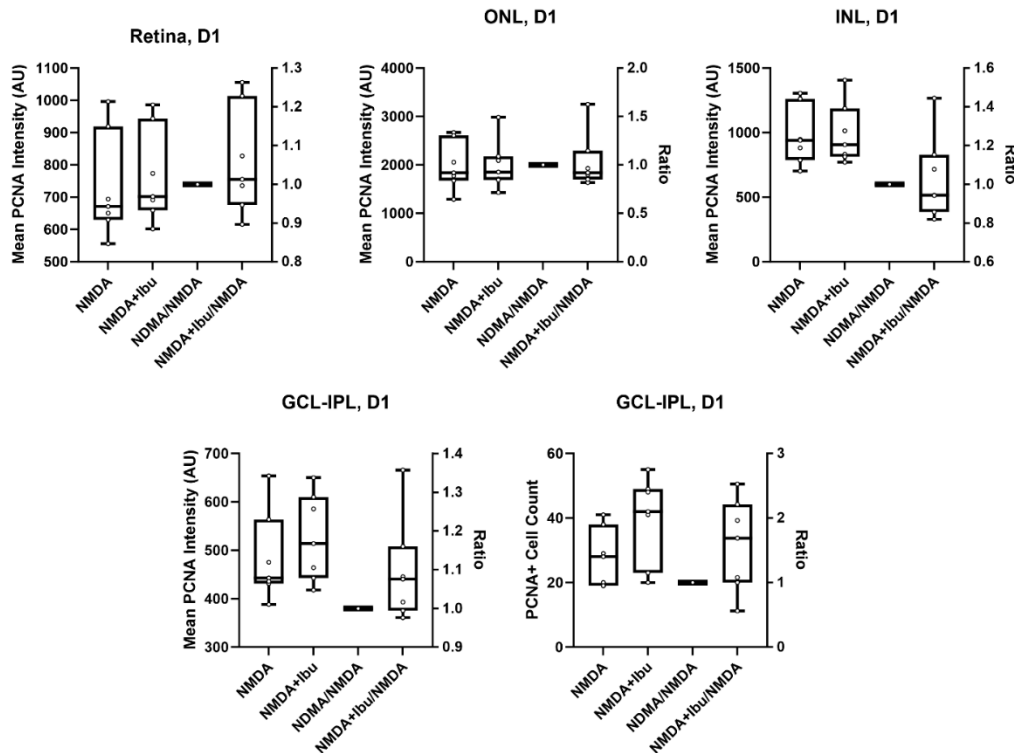


Fig 41. Day 1 mean PCNA intensity and cell count. Measurements of the mean PCNA intensity values were taken in chicks with NMDA damage in both eyes. The chicks received ibudilast treatment in the left eye and the right eye received the vehicle as a fellow eye control. PCNA intensity was measured in the following regions and compared between treatments: ONL, INL, IPL-GCL, and total retinal. Additionally, PCNA positive cells were counted in the inner retina (IPL-GCL). Raw measurement values (left y-axis) and the ratio of the treatment eye over the control eye (right y-axis) were compared. There was no significant difference between ibudilast treatment and control.

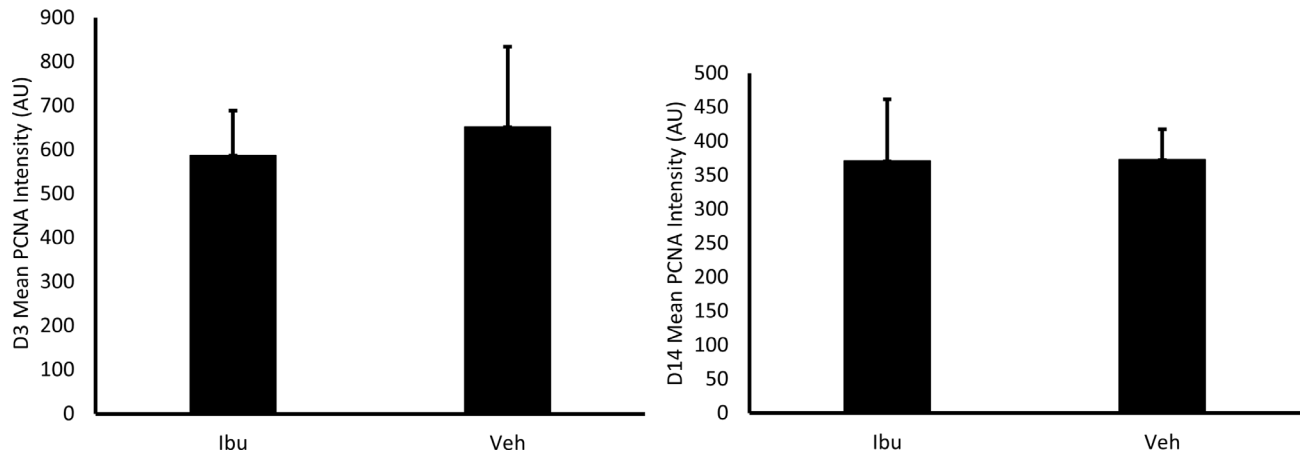


Fig 42. Ibuprofen treatment does not alter PCNA intensity measurements in the murine RD model. The mean PCNA intensity was measured in mouse RD eyes in groups that received ibuprofen treatment and vehicle control. Two-sample t-tests revealed no significant difference in PCNA between the two groups at either day 3 or 14 time points ($p=0.6158$ and $p=0.976$, respectively).

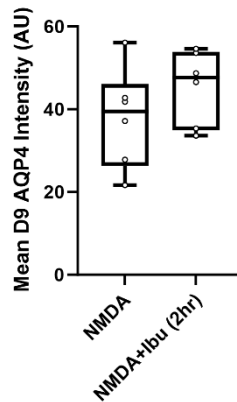


Fig 43. Ibuprofen treatment does not alter AQP4 levels in the chick NMDA model. The mean AQP4 intensity and the area above a threshold normalized by both the retinal area and retinal length were assessed between NMDA-damaged chick eyes. The left eye received ibuprofen treatment 2 hours after damage while the right eye served as a vehicle control. Statistics were calculated via a paired t-test. No significant differences with ibuprofen treatment were detected.

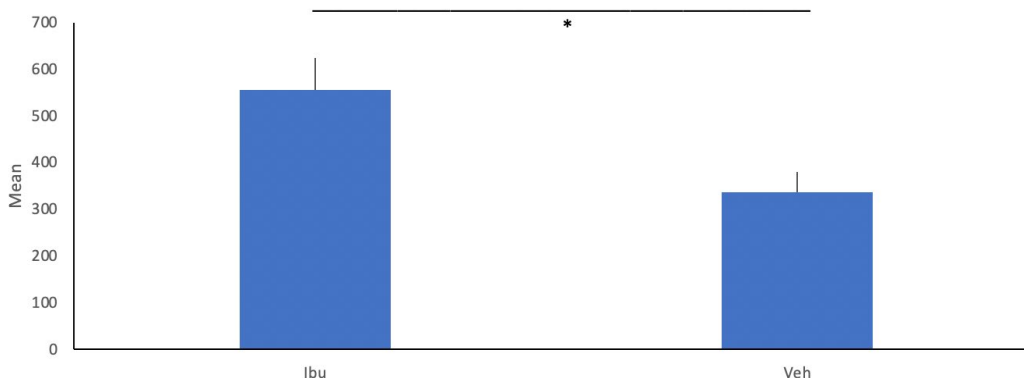


Fig 44. Ibudilast treatment of murine RD model increased AQP4 expression. Statistics were calculated via student's t-test. The total retinal mean AQP4 expression on immunostaining for day 3 RD was significantly different where subjects treated with ibudilast showed an increase in AQP4 levels compared to controls ($p=0.0280$) (**Fig 44**). This difference lost significance at the day 14 time point (data not shown).

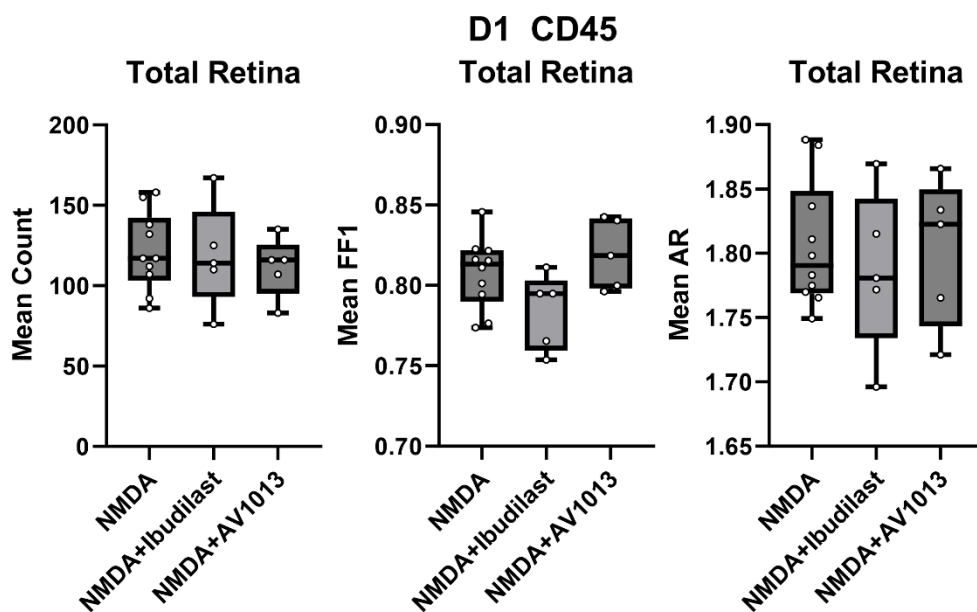


Fig 45. Neither ibudilast nor AV1013 affect the microglial accumulation or morphology at day 1. The accumulation and morphology of microglia were assessed via CD45 staining at day 1. The mean count, FF1, and aspect ratio were assessed between ibudilast, AV1013, and untreated NMDA-damaged chick eyes. Statistics were calculated via a 1-way ANOVA with Tukey multiple comparison adjustments. No differences were detected between the vehicle treated control and either drug

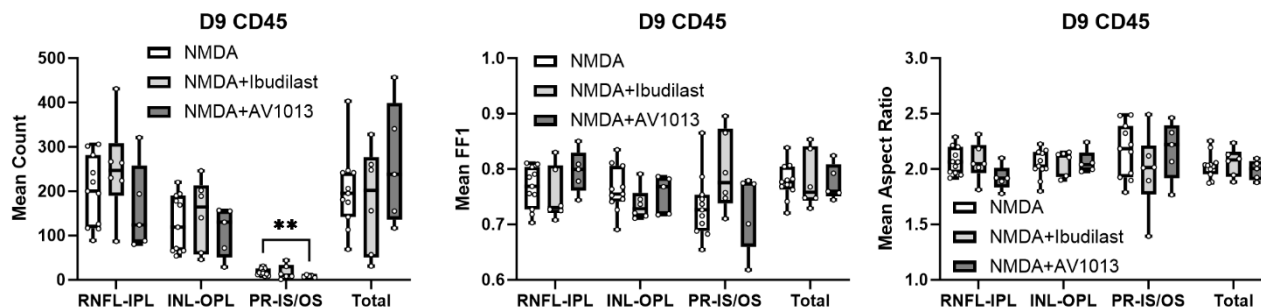


Fig 46. Effects of ibudilast and AV1013 treatment on CD45+ cells in the chick NMDA model.

The accumulation and morphology of microglia were assessed via CD45 staining at day 9 post damage. The mean count, FF1, and aspect ratio were assessed between ibudilast, AV1013, and untreated NMDA-damaged chick eyes. Statistics were calculated via a 2-way ANOVA with Tukey multiple comparison adjustments. The count of CD45+ cells in the PR-IS/OS region appeared to decrease with AV1013 treatment at day 9 ($p=0.0099$).

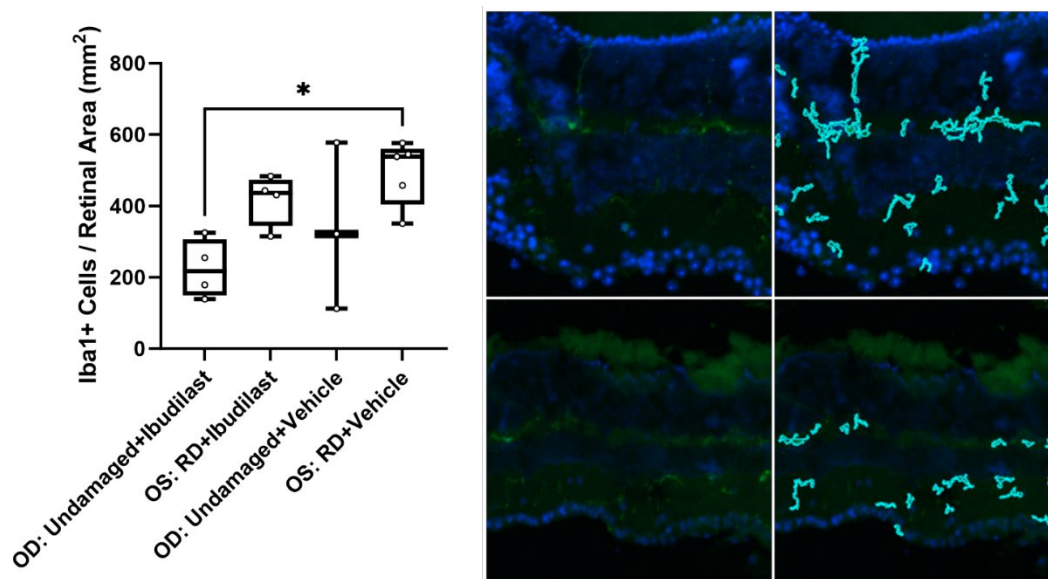


Fig 47: Ibudilast did not reduce the accumulation of Iba1+ cells in RD eyes. The accumulation of Iba1+ cells in the retina was assessed in the murine RD model between subjects treated with ibudilast and those receiving the vehicle control. Cells were detected utilizing a NIS-Elements macro. Counts were normalized with the total retinal area. Statistics were calculated with a 1-way ANOVA with Tukey's multiple comparison adjustment.

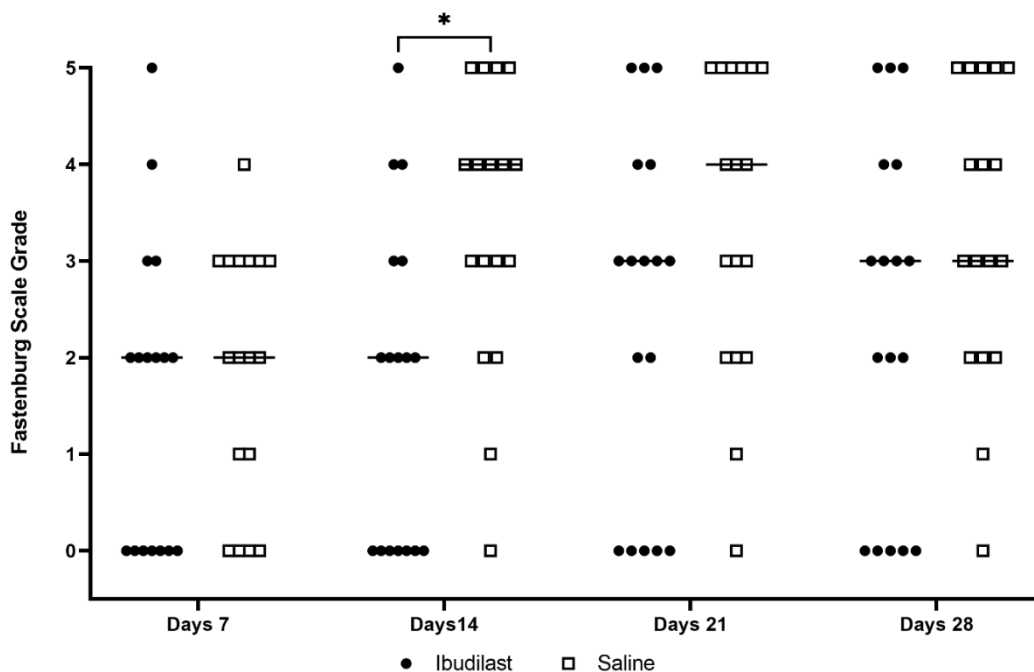


Fig 48: PVR drug study subjects plotted by their weekly Fastenburgh Scale Grade. Fastenburgh grading was performed on PVR rabbits receiving ibudilast or vehicle control treatments. A higher score indicates a more severe form of the disease. We found that the average grade for rabbits treated with ibudilast was significantly reduced compared to those treated with the saline vehicle at day 14 post-PVR induction ($p=0.0185$). This significant reduction in PVR grade disappeared by days 21 and 28.

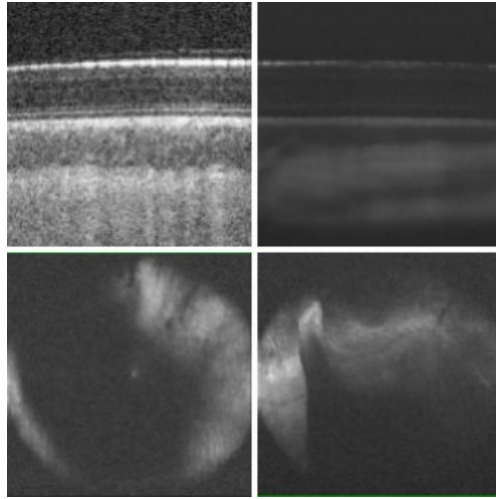


Fig 49. Example PVR rabbit OCT b-scan and fundus images. Two example b-scans that were able to be acquired and fundus images showing the level of obfuscation and distortion present in the PVR rabbit eyes.

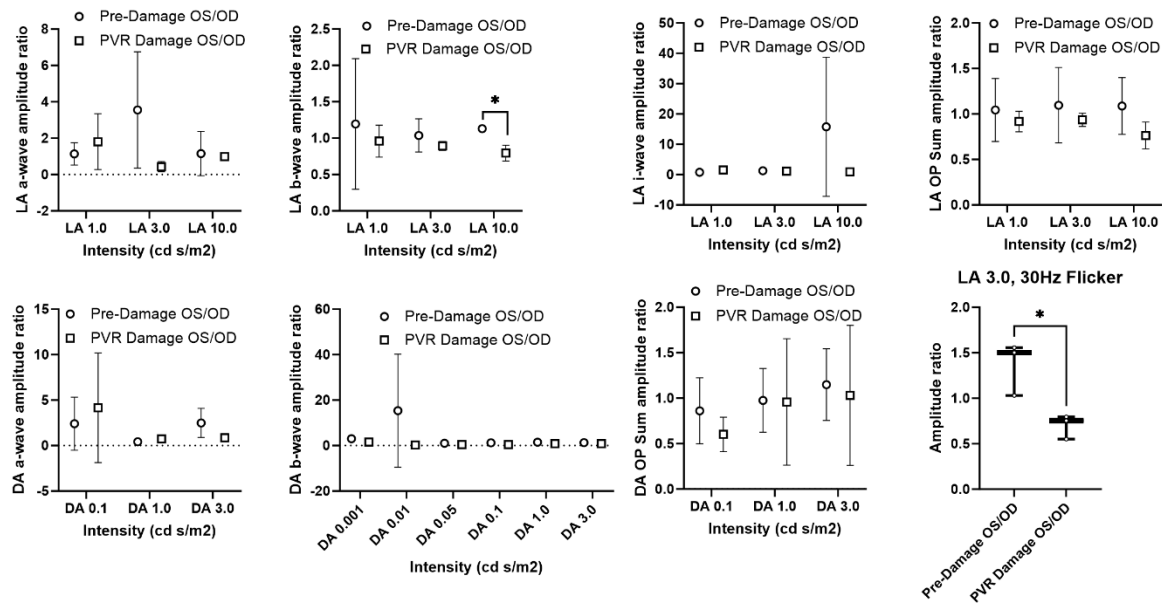


Fig 50. Electrophysiology shows reduction in retinal function in PVR damaged rabbits. Light adapted full field ERGs were performed with intensities of 1.0, 3.0, & 10.0 cd*s/m² where a-wave, b-waves, i-waves, and oscillatory potentials were measured. Light adapted 30Hz flicker tests at 3.0 cd*s/m². Dark adapted ERG were performed at intensities of 0.001, 0.01, 0.05, 0.1, 1.0, and 3.0 cd*s/m² where a-waves, b-waves, and oscillatory potentials were measured. Measured amplitudes were assessed as ratios of the left eye over the right eye, before and after PVR induction. Measurements were acquired on subjects before and after PVR induction. Values above 1.0 represent a greater function in the left eye while values less than 1.0 represent a loss in function. Statistics were calculated with a non-parametric Friedman test with Dunn's multiple comparison adjustment. In the light adapted 10.0 cd*s/m² b-wave measurement, the PVR ratio was significantly lower than the control ratio (p=0.0429). In addition, the flicker test showed a decreased amplitude ratio in the PVR eyes compared to pretreatment eyes (p=0.0429).

Task 3. Determine the ocular pharmacokinetics of clinically relevant MIF inhibitors and their effects on intraocular pressure (IOP) in rabbits.

Subtask 3a: Pharmacokinetic (PK) study. A screening pilot study using chick, performed in parallel to Aim 1, was performed to evaluate drug levels in the retina.

(A) Pharmacokinetic study: The goals of the study are to (1) test that the drug can reach the target tissue (retina) with adequate concentration following delivery via single intravitreal injection and (2) determine drug levels over time in the target tissues, the systemic exposure, and potential toxicity.

(A) A screening pilot study using chick, performed in parallel to Aim 1, will be performed to evaluate retinal drug levels after a single intravitreal treatment in untreated animals (n=1 animal at 1 day).

(n=1/group * 3 drugs and vehicle * 1 endpoint (D1) * ~3 doses * 1 model (untreated) * 1 injection paradigms (1 injection) = 9 chicks)

If needed, the effect of a second injection 12-24h later to assess drug accumulation will be performed.

(n=1/group * 3 drugs and vehicle * 1 endpoint (D1) * ~3 doses * 1 model (untreated) * 1 injection paradigm (2 injections) = 9 chicks)

A. Pharmacokinetic pilot study: We collaborated with the PhASR core to design and perform the PK study in chicks. The pilot PK study was completed as follows: 20ul of 1.0mg/ml ibudilast was injected at time 0 in 9 chicks. The treated eye was harvested at 3min, 10min, 30minutes (min), 1hour (h), 2h, 4h, 8h, 1Day (D), and 2D timepoints. The measured ibudilast level either fell below the LLOQ (lower limit of quantification) or was not found after the 2h time point (**Fig. 51**). Ibudilast was observed to potentially remain in the retina out to the 2D timepoint at a level below the LLOQ. The vitreous half-life was measured to be 26.28 min and the retinal half-life was 31.07 min. These results indicate rapid clearance of ibudilast from the eye.

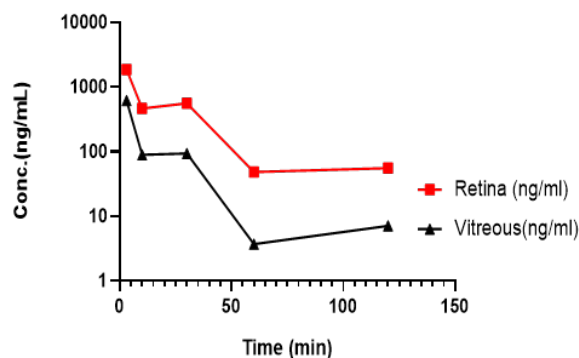


Fig 51: Pilot PK concentration-time profile of ibudilast in the retina and vitreous truncated based on LLOQ (lower limit of quantification). In both the retina and vitreous, ibudilast concentrations either fell below the LLOQ or were not found past the 2 hour timepoint.

Subtask 3b: Pharmacokinetic study. An extended pilot study using chick, performed in parallel to Aim 1, was performed to evaluate retinal drug levels after one (or two) intravitreal treatment of MIF inhibitor in untreated animals using an extended pilot study.

(B) Extended pilot study (n=3/group at day1 and day3):

(n=3/group * 3 drugs and vehicle * 2 endpoints (D1, D3) * 1~2 doses * 1 model (untreated) * 1 injection paradigm (1 or 2 injections) = 18~36 chicks)

A. Pharmacokinetic extended study: An extended PK study in ibudilast treated undamaged chicks (n=4/group) was performed with the following timepoints: 5 min, 10 min, 15 min, 30 min, 60 min, 120 min, 180 min, 480 min, and 1440 min. Retina and vitreous was collected from each subject. Retina and vitreous from the fellow eye were additionally collected from 2 of the chicks at each timepoint. The other two chicks had blood collected and plasma tested. All tests performed on the fellow eyes and

plasma were either not found or below the lower limit of quantification (BLOQ). The injected eye vitreous and retina were averaged and plotted as log ng/ml against time (min) (**Fig 52**). The half-life was determined to be 84.16 minutes in the retina and 82.83 minutes in the vitreous.

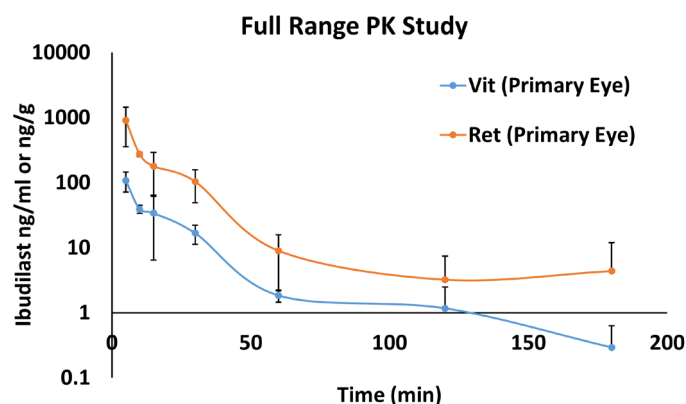


Fig 52: Extended study PK concentration-time profile of ibudilast in the retina and vitreous. In the vitreous, ibudilast concentrations either fell below the LLOQ (1ng/ml) or were not found past the 2-hour timepoint. In the retina, ibudilast concentrations either fell below the LLOQ (25ng/g) or were not found past the 30-minute timepoint.

Subtask 3c: Rabbit pharmacokinetics study: The MIF inhibitor drug showing the most promise in Aims 1/2 will be further evaluated in rabbits for pharmacokinetic properties.

(C) Rabbit pharmacokinetics 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 MIF inhibitor with the dosing scheme established in Aim 1. Fellow eyes will be untreated. Clinical examination will be performed to evaluate inflammation and IOP will be evaluated prior to harvest for drug level evaluation. Clinical examination will be performed to evaluate inflammation and IOP will be evaluated prior to harvest for drug level evaluation. The plasma, vitreous, and retinal drug levels will be analyzed at different timepoints (e.g., hour 0, 1, 3, 12, 24, 72, and 168) with assays developed by the Pharmacodynamic (PhASR) Core Facility at OSU, led by Dr. Mitch Phelps. ERG and SD-OCT 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. (n=3/group * 1~2 drugs * 7 endpoints * 1 dose * 1 model (untreated) * 1 injection paradigm (1 or 2 injections) + 3 vehicle controls = 24 or 45 Rabbits)

- A. Rabbit cadaver samples for PK assay development:** We obtained blood and cadaver eyes from two New Zealand white rabbits obtained through ULAR. Vitreous and retina were isolated. Whole blood, plasma, and platelet rich plasma were obtained for the various assays and rabbit PVR model. A protocol was optimized for the isolation of plasma and platelet rich plasma to standardize the process for upcoming rabbit experiments.
- B. Rabbit training and protocol consultation with ULAR:** ULAR veterinarians regularly consulted with us on the protocol and assisted with planning safety aspects of the rabbit experiments. They optimized processes making the procedures and imaging safer and more efficient. They have provided training to lab members on rabbit handling, blood draws, and USDA-required documentation, and have continued to support the project.
 - i. A total of 12 rabbits were used to perform the PK study. Rabbits received an intraocular injection of Ibudilast and were euthanized at various timepoints to measure drug concentrations in the vitreous, retina, and plasma (**Fig 53**). Timepoints included 5, 10, 30, 60, 120, 240, and 480 minutes post-injection and were guided by the chick PK study.
 - ii. Three 24hr post-injection rabbit necropsies in conjunction with the OSU Veterinary Pathology Core have been performed. One rabbit was injected with a saline vehicle to serve as a control and

two rabbits were injected with ibudilast. ERGs were performed both pre-treatment and post-treatment.

- iii. The half-life of ibudilast in the rabbit retina and vitreous was evaluated and reported by the PhASR core. In the vitreous the half-life for ibudilast is 22 minutes, while in the retina the half-life is 78 minutes.
- iv. We received the results of the necropsies performed by the Veterinary Core Facility. Other than decreased platelet levels in each subject (including the vehicle control), no major differences were noted between the control and treatment groups regarding their blood chemistry analysis.

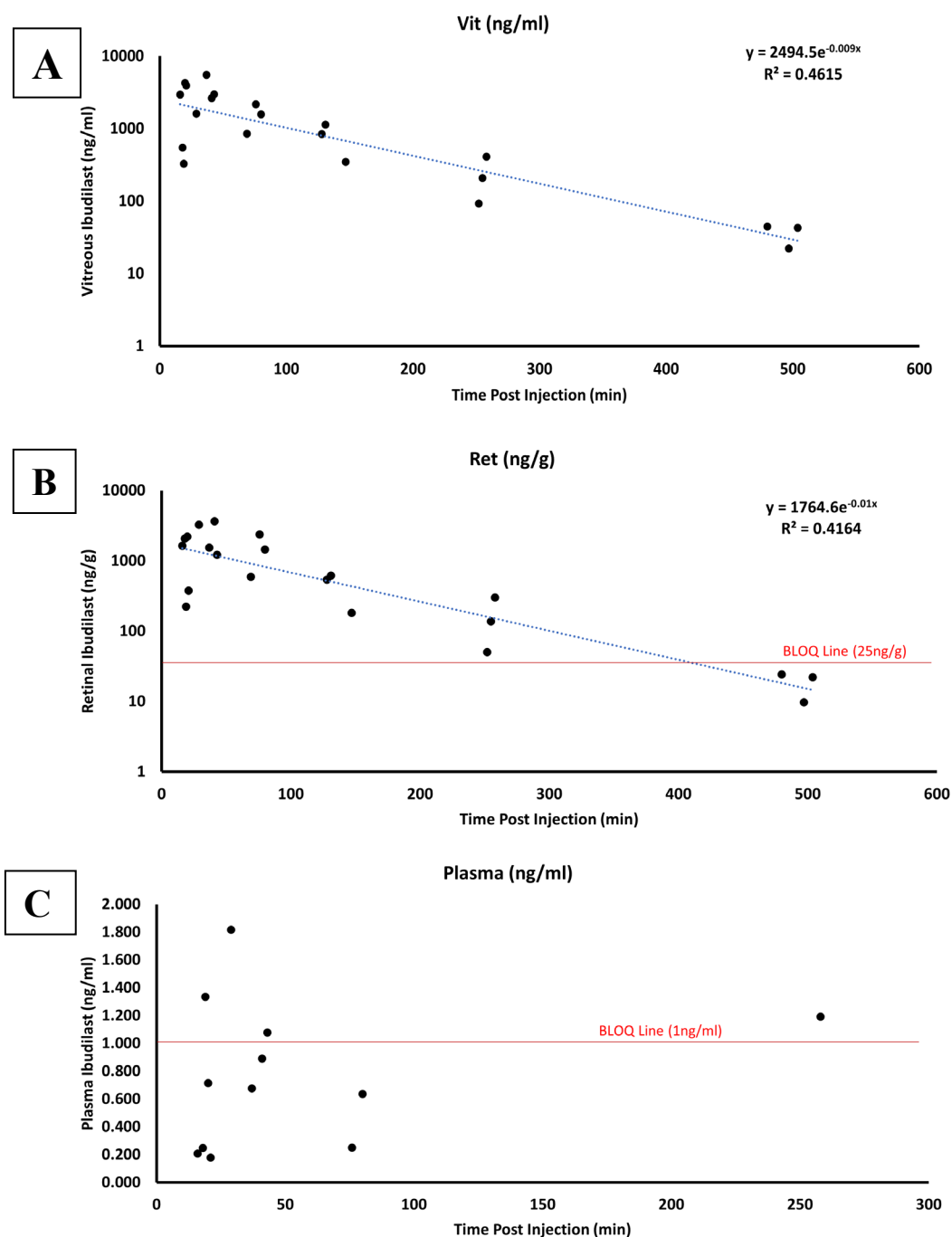


Fig 53 : Rabbit Pharmacokinetic Raw Data. Plots of the concentration of ibudilast in rabbit vitreous (A), retina (B), and plasma (C). Rabbits were planned to be sacrificed at 5, 10, 30, 60, 120, 240, and 480 min after an

intravitreal injection of ibudilast (50ul, 3mg/ml) but plotted by the actual recorded time it took from injection to flash freezing. Data was modeled to find the projected half-life for the vitreous and retina.

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

This project has continued to provide ample opportunity for undergraduates to learn 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. Sumaya Hamadmad is primarily responsible for training and supervising the undergraduates, with assistance from Dr. Cebulla and Dr. Heisler-Taylor.

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 and protocol development. With the expertise gained on this project, Dr. Heisler-Taylor has become the new P30 Vision Sciences Research Core manager for The Ohio State University. Collaborations with other labs that were made possible due to the ERG have continued.

Dr. Heisler-Taylor had three abstracts on the project published in Investigative Ophthalmology and Visual Science that was accepted for presentation at ARVO 2020, 2022, & 2023. These poster presentations were presented in Denver at the 2022 ARVO conference and in New Orleans in the 2023 ARVO conference. The 2020 presentation was presented at the OSU Ophthalmology Graduate Research Day due to the COVID-19 pandemic. This conference brings together many vision researchers and physicians from across the world to share exciting results in basic, translational, and clinical vision research. Additionally, two more abstracts were presented at the 2020 eARVO conference by Dr. Sumaya Hamadmad (research scientist) and Elizabeth Urbanski (research associate). A final abstract was presented at the 2023 ARVO conference by Evan Hawthorn (research assistant). We have trained numerous undergraduate and medical students (see section 7). One summer Optometry masters student, Richard Wan, won the “3 Minute Thesis” award, placing first at the College of Optometry as well as the University level of all graduate students for his presentation

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

1. Manuscripts were submitted for publication.

2. Abstract were submitted and presented at the international ARVO conference (eARVO during the COVID-19 pandemic)
3. Presentations were given at OSU Ophthalmology Graduate Research Days.

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

If this is the final report, state “Nothing to Report.”

Nothing to Report

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Ibudilast was found to be well tolerated with intravitreal delivery. It inhibited retinal neuron death from NMDA excitotoxic damage and reduced PVR grade *in vivo*, at an early time point. Ibudilast had limitations on its pharmacokinetic properties in terms of a short half-life in the retina & vitreous. Therefore, further work to improve the formulation of ibudilast to promote sustained delivery would be beneficial to move this towards clinical ocular development. The phosphodiesterase (PDE) inhibitory properties of ibudilast appear to be critical to its neuroprotective effect since the ibudilast analogue AV1013, which lacks PDE inhibition, did not preserve retinal neurons or their function.

Ibudilast was shown to preserve approximately 40% of dying neurons and additional improvements could be made to further reduce neuronal death, such as by combining ibudilast with other drugs. Steroids, such as dexamethasone, are commonly used in treating damaging retinal conditions in the clinic and their anti-inflammatory properties may produce synergy with ibudilast. Studies to investigate the combination therapy of ibudilast with steroids could lead to a promising future treatment strategy to investigate for patients with retinal conditions.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The neuroprotective effect of ibudilast in the retina potentially implies efficacy towards other neurological diseases. For example, ibudilast is already under clinical trial for ALS and MS in the United States. From our *in vitro* PVR studies, the impact of ibudilast and AV1013 on pathological wound healing (inhibition of proliferation, migration, contraction, and epithelial-mesenchymal transition) implies a potential use in fibrotic and even cancerous disorders.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Ibudilast and potentially AV1013 are worthy of further study in terms of reformulation for the retinal and non-ocular disorders noted above.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions;*
or
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Due to inconsistencies in the chick RD model, we reached out to the scientific officer Dr. Lavinka to consider switching to the mouse RD model. The data in that model provided the basis for the grant preliminary data and could provide more consistent results for analysis of the impact of MIF inhibitors on retinal neuroprotection in RD. We submitted an amendment to the statement of work to include murine RD studies which was approved by the scientific officer. The IACUC has approved the amendment and we submitted all the required documentation to ACURO for approval.

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Delays due to the COVID-19 pandemic added additional no cost extension time.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Addition of murine model for retinal detachment experiments.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

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

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Authors: Tyler Taylor-Heisler, Richard Wan, Elizabeth G Urbanski, Sumaya Hamadmad, Mohd Hussain Shah, Hailey Wilson, Julies Racine, Colleen M Cebulla.

Title: Multimodal imaging and functional analysis of chick NMDA retinal damage model

Journal: PLOS One

Year: 2021

PMC6215716

Status of publication: Published

Authors: Sumaya Hamadmad, Tyler Heisler-Taylor, Sandeep Goswami, Evan Hawthorn, Dena Martini, Sameer Chaurasia, Diana Summitt, Ali Zaatari, Elizabeth G Urbanski, Julie Racine, Abay Satoskar, Heithem M. El-Hodiri, Andy J. Fischer, Colleen M. Cebulla

Title: Ibudilast Protects Retinal Bipolar Cells from Excitotoxic Retinal Damage and activates the mTOR pathway

Pre-Submission

Authors: Sumaya Hamadmad, Diana Summitt, Tyler Heisler-Taylor, Sandeep Goswami, Rahaf Shalash, Ali Zaatari, Dena Martini, Misha Sohail, Julie Racine, Colleen Cebulla

Title: MIF Inhibition Blocks Key PVR Components In Vitro & Protects Against PVR in Rabbits

Pre-Submission

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

- A. Heisler-Taylor T, Hamadmad S, Martini D, Jeng Y, Swindle-Reilly KE, Hill K, Phelps M, Cebulla CM. MIF targeted neuroprotection through human serum albumin nanoparticle drug delivery. ARVO 2023 E-abstract 3885337. Presented at 2023 ARVO conference.
- B. Hawthorn E, Heisler-Taylor T, Goswami S, Cebulla CM. Changes in Morphology and Distribution of Microglia Following MIF Inhibition in Avian Excitotoxic Retinal Damage. ARVO 2023 E-abstract 3886365. Presented at 2023 ARVO conference.
- C. Heisler-Taylor T, Urbanski E, Hamadmad S, Landreth C, Wilson H, Racine J, Cebulla CM. MIF inhibition improves retinal function in NMDA mediated excitotoxic damage. ARVO E-abstract 3714239. Presented at 2022 ARVO conference.
- D. Urbanski E, Heisler-Taylor T, Wan R, Hussain Shah M, Hamadmad S, Fischer AJ, Satoskar A, Cebulla CM. MIF Inhibitor Ibudilast Prevents Retinal Cell Death in Chick Excitotoxic Retinal Damage Model. ARVO 2020 E-abstract 3358663. Presented at 2020 eARVO conference.
- E. Hamadmad S, Reese A, Urbanski E, Heisler-Taylor T, Cebulla CM. Differential effects of MIF inhibitors on an in vitro PVR model. ARVO 2020 E-abstract 3359091. Presented at 2020 eARVO conference.
- F. Heisler-Taylor T, Wan R, Wilson H, Urbanski E, Hussain Shah M, Racine J, Cebulla CM. Effects of age on chick light adapted ERGs with and without inner retinal excitotoxic damage. ARVO E-abstract 3357073. Presented at OSU Ophthalmology Graduate Research Day 2020, winning 2nd place along his peers.
- G. Tyler Heisler-Taylor presented at the OSU Ophthalmology Graduate Research Day 2021 about the work from the PLOS One publication, winning 1st place among his peers for his presentation.
- H. Tyler Heisler-Taylor presented at the Ohio Lions Eye Research Foundation annual meeting 2021 including some of the work from the PLOS One publication.
- I. Richard Wan presented his Master's thesis "Effect of MIF inhibitors Using a N-Methyl-D-Aspartate Damaged Chick Retina Model." in June 2021.
- J. Richard Wan won the "3 Minute Thesis" award, placing first at the College of Optometry as well as the University level of all graduate students for his presentation on the "Effect of MIF inhibitors Using a N-Methyl-D-Aspartate Damaged Chick Retina Model" in February 2021.
- K. Dr. Cebulla presented data in an initial consultation with the Drug and Device Development Services Core at Nationwide Children's Hospital (NCH) on 5/28/21. This group with the Office of Research Regulatory Affairs at NCH has extensive experience in helping investigators develop regulatory strategies to move toward clinical trials. They reviewed preclinical data. Results were not strong enough to proceed with IND application.

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

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report.

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

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

A chick normative database of undamaged and NMDA-damaged chicks was published in PLoS One. This database is publicly available and includes ERG, SD-OCT thicknesses, intraocular pressures, weight, and TUNEL counts. It will allow for other researchers to utilize it for preclinical studies utilizing the chick retinal damage model.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Dr. Colleen M Cebulla

Project Role: Principal Investigator

Researcher Identifier (e.g. ORCID ID): 0000-0002-5636-4968

Nearest person month worked: 4

Contribution to Project: Supervised all studies, equipment installation, protocol and form generation, data analysis, troubleshooting, and all personnel involved in the study. Edited the report.

Name: Dr. Andy J Fischer

Project Role: Co-Investigator

Researcher Identifier (e.g. ORCID ID):

Nearest Person Month Worked: 1

Contribution to Project: Provided advice about chick TUNEL staining and histology and NMDA model.

Recommended new CD45 antibody, provided troubleshooting and feedback on updated immunostaining protocol

Name: Dr. Abhay Satoskar

Project Role: Co-Investigator

Researcher Identifier (e.g. ORCID ID): 0000-0001-5989-1520

Nearest person month worked: 1

Contribution to Project: Advised regarding experimental design for routes of delivery for CPSI-1306 and provided troubleshooting for drug dilution.

Name: Julie Racine

Project Role: Electrophysiologist

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 1

Contribution to Project: Developed ERG protocols. Reviewed ERG data and data analysis, oversaw installation of new ERG machine, trained lab members on use of new ERG machine and software. Consulted on chick ERG data analysis, generated new ERG protocols for rabbit ERGs, and recommended new electrodes for rabbit ERG.

Name: Dr. Mitch Phelps

Project Role: Co-investigator

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: <1

Contribution to Project: Consulted on development of pharmacokinetic study design and assay development to support IND application if positive results were found. Helped perform assay and analyze results. Wrote report and helped produce graphs.

Name: Dr. Kevin Bosse

Project Role: Consultant

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: <1

Contribution to Project: Consulted for regulatory support for IND application. Not enough justification at this time for IND.

Name: Tyler Heisler-Taylor

Project Role: Graduate Fellow/Lab manager

Researcher Identifier (e.g. ORCID ID): 0000-0002-3514-0711

Nearest person month worked: 12

Contribution to Project: Ordered equipment, worked on ULAR and IACUC protocols, communicated with ULAR personnel, used slit lamp, used fundus camera, took IOP measurements, administered isoflurane anesthesia, administered intravitreal injection, collected ocular tissue for histology, provided lab organization, performed laboratory regulatory and safety management, performed clinical exams, sectioned frozen tissue samples, stained, imaged, processed, and analyzed tissue sample images, performed, processed, and analyzed ERG data, operated, processed, and analyzed SD-OCT data, wrote up findings, generated figures, and supervised personnel. Hired as the lab manager for the Department of Ophthalmology and Visual Sciences.

Funding Support: Ohio Lions Eye Research Foundation Norbert Peiker Eye Research Fellowship

Name: Mohd Hussain Shah

Project Role: Post-Doc

Researcher Identifier (e.g. ORCID ID): 0000-0002-3669-1478

Nearest person month worked: 12

Contribution to Project: Used fundus camera, used tonopen, assisted with SD-OCT, administered isoflurane anesthesia, administered intravitreal injection, collected ocular tissue for histology, stained, imaged, processed, and analyzed tissue sample images, used operating microscope, performed clinical exams, performed and analyzed ERG, proofread reports, and supervised personnel.

Name: Elizabeth Urbanski

Project Role: Research Assistant

Researcher Identifier (e.g. ORCID ID): 0000-0003-3576-1275

Nearest person month worked: 6

Contribution to Project: Ordered equipment, used slit lamp, used fundus camera, used tonopen, communicated with ULAR personnel, administered isoflurane anesthesia, administered intravitreal injection, used operating microscope, collected ocular tissue for histology, provided lab organization, performed clinical exams, performed laboratory regulatory and safety management, sectioned frozen tissue samples, stained and imaged tissue samples, analyzed TUNEL data, analyzed INL thickness data, performed ERG, performed SD-OCT, wrote up findings, generated figures, trained new undergraduate volunteer, and supervised personnel.

Name: Sumaya Hamadmad

Project Role: Research Scientist

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 3

Contribution to Project: Stained and imaged tissue samples, performed cell culture experiments, generated figures, wrote up findings.

Funding: Dr. Cebulla's Personal Investigator Fund

Name: Sandeep Goswami

Project Role: Postdoctoral Scholar

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 1

Contribution to Project: Attended meetings with PhASR and assisted with experimental work.

Name: Diana Summitt

Project Role: Research Assistant

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 1

Contribution to Project: Attended meetings with PhASR and assisted with experimental work including tissue collection, fixation, embedding, sectioning, staining and analysis. Performed mouse colony maintenance and IP

injections. Assisted with handling, sedation and anesthesia monitoring of mice and rabbits. Performed rabbit subcutaneous injections and provided rabbit enrichment.

Name: Claire Landreth

Project Role: Electrophysiology Assistant

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: <1

Contribution to Project: Reviewed ERG data and data analysis, assisted with analyzing ERG data.

Name: Richard Wan

Project Role: Optometry Student Volunteer

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 2

Contribution to Project: Wrote up findings, generated figures, analyzed IOP and weight data.

Funding: N/A, Volunteer

Name: Alana Y Reese

Project Role: Undergraduate Researcher

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 2

Contribution to Project: Provided lab organization, performed cell culture experiments, sectioned and stained tissue samples, helped train new undergraduate volunteers, helped with image analysis.

Funding: Dr. Cebulla's Personal Investigator Fund

Name: Hailey Wilson

Project Role: Undergraduate Research Volunteer

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 1

Contribution to Project: Data entry for ERG, helped with sectioning, immunostaining, and imaging of frozen tissue samples.

Funding: N/A, Volunteer

Name: Bayan Shalash

Project Role: Undergraduate Research Volunteer

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 1

Contribution to Project: Stained and imaged tissue samples, helped analyze INL thickness data, and helped train new undergraduate volunteer.

Funding: N/A, Volunteer

Name: Krupa Patel

Project Role: Undergraduate Research Volunteer

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: <1

Contribution to Project: Sectioned frozen tissue samples, stained ocular tissue for histology, and helped analyze INL thickness data.

Funding: N/A, Volunteer

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.

Name: Kayla Bernstein

Project Role: Research Assistant

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 1

Contribution to Project: Aided during chick ocular injections and tissue collection procedures, performed TUNEL staining, imaging, image analysis, ordered lab supplies, communicated with ULAR personnel, and performed laboratory regulatory and safety management.

Name: Evan Hawthorn

Project Role: Research Assistant

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 1

Contribution to Project: Performed immunohistochemistry staining, imaging, and image analysis. Aided during chick ocular injections and tissue collection procedures.

Name: Dena Martini

Project Role: Undergraduate Research Assistant

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: <1

Contribution to Project: Sectioned ocular tissue, recorded data prepared supplies during chick ocular injections, and performed TUNEL and GFAP staining.

Name: Rahaf Shalash

Project Role: Undergraduate Research Volunteer

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: <1

Contribution to Project: Sectioned ocular tissue and performed TUNEL and AQP4 staining.

Name: Ali Zadari

Project Role: Undergraduate Research Assistant

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: <1

Contribution to Project: Sectioned ocular tissue and performed TUNEL staining.

Name: Misha Sohail

Project Role: Undergraduate Research Assistant

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: <1

Contribution to Project: Sectioned ocular tissue and performed TUNEL and PCNA staining.

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

See Appendices A, B, & C

What other organizations were involved as partners?

Nationwide Children's Hospital

700 Children's Drive, Columbus, Ohio 43205

Julie Racine provided crucial support and training in the establishment, experimental process, and analysis of chick and rabbit electrophysiology.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Appendix A: Colleen Cebulla Other Support

Appendix B: Andrew J Fischer Other Support

Appendix C: Abhey Satoskar Other Support

Appendix D: "Multimodal imaging and functional analysis of chick NMDA retinal damage model" Article

Appendix E: Tyler Heisler-Taylor 2020 ARVO Abstract: Effects of age on chick light adapted ERGs with and without inner retinal excitotoxic damage.

Appendix F: Sumaya Hamadmad 2020 ARVO Abstract: Differential effects of MIF inhibitors on an in vitro PVR model.

Appendix G: Elizabeth Urbanski 2020 ARVO Abstract: MIF Inhibitor Ibudilast Prevents Retinal Cell Death in Chick Excitotoxic Retinal Damage Model.

Appendix H: Tyler Heisler-Taylor 2022 ARVO Abstract: MIF inhibition improves retinal function in NMDA mediated excitotoxic damage.

Appendix I: Evan Hawthorn 2023 ARVO Abstract: Changes in Morphology and Distribution of Microglia Following MIF Inhibition in Avian Excitotoxic Retinal Damage.

Appendix J: Tyler Heisler-Taylor 2023 ARVO Abstract: MIF targeted neuroprotection through human serum albumin nanoparticle drug delivery.