Screening of a Randomised Compound Library to Uncover Novel Drugs Capable of Improving Vision in a Zebrafish Model of Inherited Blindness

Student: Jodie Kearney, Supervisors: Dr. Patrizia Colucci, Dr. Tess McCann

Background & Aims:

Worldwide, over two million people suffer from a variety of debilitating diseases known as inherited retinal diseases (IRDs), caused by mutations in over 300 genes. Individuals suffering from these conditions, which result in partial or complete blindness, often face a poorer quality of life and loss of independence. Currently there is only 1 available FDA-approved gene therapy capable of slowing down disease progression in IRDs: LUXTURNA™, but it has only been shown to be beneficial for a small subset of patients[1].Thus, there is an urgent need to identify therapeutic options against retinal degeneration.

Drug discovery can be a lengthy and time-consuming process. However, combining an orthogonal drug pooling approach with an *in vivo* phenotypic-based compound screen can allow for a faster strategy to drug discovery and a reduction in sample numbers[2]. Zebrafish are a promising *in vivo* model to study vision loss due to their high genome homology with humans and their conserved anatomical and functional retinal structure. The use of an *in vivo* model is key not only for understanding the underlying mechanisms of these diseases, but also for advancing pharmacological interventions.

The *dying on edge* (*dye*) zebrafish mutants are a result of a 180 bp deletion in the *atp6v0e1* gene. From 3 days post fertilisation (dpf), *dye* larvae are morphologically distinguishable from their siblings. *dye* mutants have hypopigmentation (HP), pericardial oedema (PO), a deflated swim bladder (DSB) and decreased body length at 5 dpf (Figure 1). Microscopically, their photoreceptor outer segments are also shown to be shorter with increased cell death of retinal stem cells[3]. Due to this mutation, *dye* mutants exhibit impaired visual function, making it a promising *in vivo* model to identify novel compounds that can improve/restore vision.

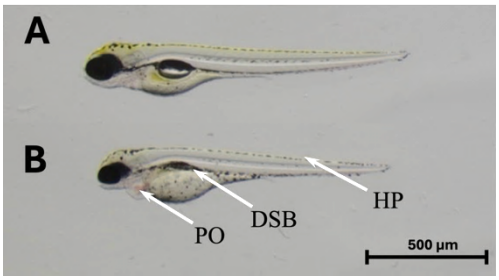

Figure 1: A - Image of a sibling larva, **B –** image of a *dye* mutant.

The aim of this project is to uncover novel compounds capable of improving vision in the *dye* zebrafish model of inherited blindness. By validating known compounds[4] as positive controls of visual rescue and using an orthogonal drug pooling protocol in conjunction with a behavioural assay known as the optokinetic response (OKR), we assessed the ability of novel small molecules to improve vision in the *dye* mutants.

Methods:

Zebrafish Husbandry, Embryo Collection and Screening:

atp6v0e1 heterozygous adults were kept under a standard 14 hr light/10 hr dark cycle in a recirculating water system at 28 °C. Embryos were collected from crosses of heterozygous *dye* carrier adults and raised in petri dishes containing embryo medium. At 3 dpf, the larvae were screened for the *atp6v0e1*$^{-/-}$ mutation, this will be approximately 1/4th of the clutch and can be easily identified by their difference in pigmentation. The identified *dye* mutants were then placed in a separate dish.

Pharmacological Treatment of Zebrafish Larvae:

To determine robust and reproducible positive controls for the library screening, *dye* larvae were placed in a 48 well plate (5 per well). At 3 dpf, the larvae were treated with selected compounds between a range of concentrations or a 1% DMSO vehicle control for 48 hours. Prior to performing the OKR assay, at 5 dpf, the treatments were removed and replaced with embryo medium.

Randomised Compound Library Screening:

The Chembridge DIVERSet-Exp MF6 was chosen as the compound library. Compounds were stored in a 96 well plate and placed on ice during solution preparation. Drug pools were created by combining the compounds found in the same row and column. Each pool contained 10 µM of each compound found in that row/column up to 1% DMSO. Drug solutions were also prepared for our negative (1% DMSO) and positive (100 µM repistat) controls. Each solution was added to the respective well in a 48 well plate and incubated for 48 hours before performing the OKR assay.

OKR Assay:

The OKR is a behavioural assay used to determine the visual function of the larvae, from this we can deduce the ability of the compounds to improve vision in our *dye* model. At 5 dpf, each larva was placed in a dish at the centre of a black and white striped rotating drum. The larva's eye movements (saccades) were tracked as it followed the stripes for 1 minute[5].

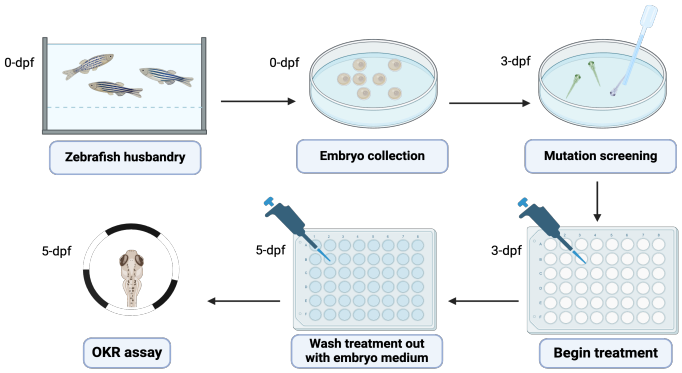

Figure 2: Experimental workflow of pharmacological treatment of zebrafish larvae.

Results:

A variety of compounds were tested to validate positive controls of visual rescue. The drugs tested, including HDAC6 inhibitors (HDAC6i, such as ACY-1215, tubastatin A and repistat) and an antioxidant (NACA), were ranked according to the best OKR response (Figure 3A). Although treatment with 100 µM repistat did not result in a high rank, probably due to the small sample size tested, we decided to retest it again based on promising results published in the literature[4]. Treatment with 100 µM repistat, 250 and 150 µM tubastatin A showed a 3.8-, 3.39- and 3.24-fold improvement in visual function compared to 1% DMSO, respectively (Figure 3B & 3C). Thus, they were chosen as our positive controls moving forwards with the library screen. However, due to the limited amount of wells available and sample numbers, repistat was the compound chosen as the positive control for the library screen.

A

Rank	Compound	Concentration	Mean saccade/min	N of larvae
1	tubastatin A	250 µM	9.714	14
2	tubastatin A	150 µM	8.765	17
3	tubastatin A	100 µM	6.5	18
4	NACA	200 µM	4.214	14
5	ACY-1215	20 µM	4.2	10
6	NACA	300 µM	4	12
7	repistat	100 µM	3.75	4
8	ACY-1215	30 µM	3.556	10
9	NACA	400 µM	3.364	11
10	ACY-1215	5 µM	2.444	9
11	DMSO	0.1-0.6%	2.209	48
12	tubastatin A	200 µM	2	13
13	NACA	100 µM	1.6	20
14	ACY-1215	10 µM	0.8	10

B

Rank	Compound	Concentration	Mean saccades/min	N of larvae
1	repistat	100 µM	7.533	15
2	tubastatin A	250 µM	6.7	10
3	tubastatin A	150 µM	6.414	29
4	DMSO	1%	1.979	48

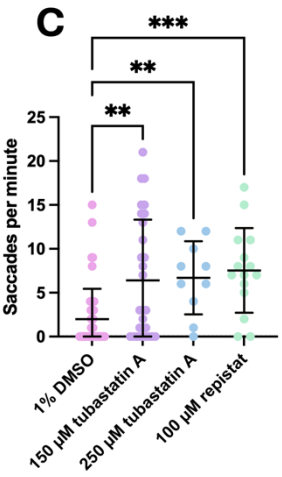

Figure 3:
Determination of a Robust Positive Control
A – Ranking of compounds tested with 0.1-0.6% DMSO concentration in control and test groups based on mean saccades/minute. **B –** Ranking of compounds tested with 1% DMSO concentration in control and test groups based on mean saccades/minute. **C –** Combined data of compounds eliciting an higher OKR response. 1% DMSO concentration in control and test groups. n ≥ 10 larvae for each group from 4 biological replicates. Statistical analysis carried out by Kruskal-Wallis test followed by Dunn's multiple comparisons test. **p < 0.01, ***p < 0.001.

160 small molecules grouped in 36 orthogonal pools of the Chembridge DIVERSet-Exp MF6 compound library were tested in total. In the individual library plate screens, ~60-80% of the pools were shown to be toxic to the larvae. None of the viable pools from either plate screen was shown to have a promising effect on the *dye* mutant's visual function (Figure 4A). We decided to run intermediate experiments to determine the cause of the toxicity. Individual compounds from plate 20 were screened at 100 µM to determine whether it was the pooling of the compounds. The levels of toxicity shown were similar to that of the library plate (data not shown). Thus we tested individual compounds from plate 21 at a lower concentration of 50 µM (Figure 4B). This resulted in a lower toxicity rate, and potential 'hits' with compounds A11, E6, G2, H2, C7 & D4 as their mean OKR response was higher than our positive control.

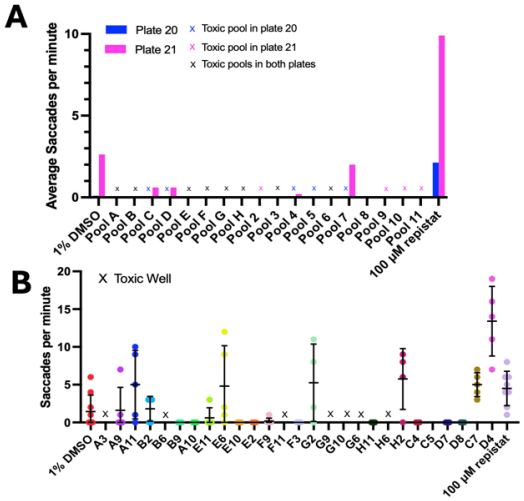

Figure 4:
Randomised Compound Library Screen & Protocol Optimisation
A – Combined data from compound library plate 21 & 20. Each test pool at a concentration of 100 µM. Many test groups showed high levels of toxicity where ≥ 3 larvae are dead at 5 dpf. n ≥ 3 larvae for each biological replicate. **B –** Individual compound testing from plate 20. Each test well at 50 µM. Lower levels of toxicity observed. Statistical analysis carried out by Kruskal-Wallis test followed by Dunn's multiple comparisons test, p > 0.05, no significance.

Discussion:

Overall, two compounds were found to be effective in improving the larvae's visual function. The confirmation of repistat and tubastatin A as a robust positive control was to be expected with the data found in previous literature[4]. Many of the other compounds tested as candidates for our positive control did not have any consistent effect on the larvae's OKR response, such as ACY-1215, another HDAC6i. NACA has previously been shown to protect RPE cells against oxidative stress induced cell death in mice[6]. However, NACA displayed no evidence for improving vision in our *dye* zebrafish model.

The orthogonal drug pooling protocol at an overall concentration of 100 µM per pool resulted in unexpected high levels of toxicity. The individual compound screening experiments allowed us to say with a high level of certainty that the toxicity was induced by the overall concentration of the drug pools rather than the pooling protocol itself. Further protocol optimisation revealed potential compound 'hits' when library plate compounds were individually tested at 50 µM. This finding not only validates this approach as a viable drug discovery strategy but also paves the way for further research to identify potential drug candidates for IRDs.

Future Directions

More extensive testing of the hit compounds is required to confirm they actually have a positive effect on the larvae's vision as is shown in this preliminary data. If this result can be replicated with a larger sample number, further studies into the mechanism of action and therapeutic target of the compounds will be investigated.

Value to Patients, Student & Lab:

Due to the limited number of FDA-approved drugs to combat IRDs uncovering compounds which can not only prevent disease progression but improve vision in these patients is vital. The screening of a randomised compound library acts as an unbiased method of drug discovery, allowing for the rapid testing of compounds for their ability to improve vision.

Through the Biochemical society summer studentship, I was given the opportunity to receive the necessary training to plan, set-up and execute experiments independently. This allowed me to gain first-hand experience conducting independent practical lab work which will benefit my future career in which I wish to apply to do post graduate research. This funding also allowed me to gain experience working with an *in vivo* model, which is something I would like to continue with in the future. This opportunity gave me the chance to learn from fellow lab groups through attending weekly lab meetings. As I conducted my research project in a different university, I was introduced to new PIs, allowing me to expand my professional network.

The work presented will act as a baseline for future researchers that decide to continue with the compound library screen in terms of protocol optimisation with the identification of a positive control.

Figure 5: Group photo. Left to right – Dr. Patrizia Colucci, Marzia Pendino, Grace Ruddin, Dr. Tess McCann, Jodie Kearney, Ellie Swords, Dr. Elin Strachan & John Fehilly

References:
1. Maguire, A. M *et al.* (2017). Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. Lancet (London, England), 390(10097), 849–860. https://doi.org/10.1016/S0140-6736(17)31868-8
2. Kennedy, B.N *et al.*, 2019. Orthogonal Drug Pooling Enhances Phenotype-Based Discovery of Ocular Antiangiogenic Drugs in Zebrafish Larvae. Frontiers in Pharmacology 10. https://doi.org/10.3389/fphar.2019.00508
3. Daly, C., Shine, L., Heffernan, T. et al. A Brain-Derived Neurotrophic Factor Mimetic Is Sufficient to Restore Cone Photoreceptor Visual Function in an Inherited Blindness Model. Sci Rep 7, 11320 (2017).
4. Campiani, G *et al.*, 2024. Targeting Relevant HDACs to Support the Survival of Cone Photoreceptors in Inherited Retinal Diseases: Identification of a Potent Pharmacological Tool with In Vitro and In Vivo Efficacy. J. Med. Chem. https://doi.org/10.1021/acs.jmedchem.4c00477
5. Gómez Sánchez A, Álvarez Y, Colligris B and Kennedy BN. Affordable and effective optokinetic response methods to assess visual acuity and contrast sensitivity in larval to juvenile zebrafish]. Open Res Europe 2022, 1:92 (https://doi.org/10.12688/openreseurope.13923.2)
6. Apte, R. S *et al.* (2011). N-acetylcysteine amide (NACA) prevents retinal degeneration by up-regulating reduced glutathione production and reversing lipid peroxidation. The American journal of pathology, 178(5), 2032–2043. https://doi.org/10.1016/j.ajpath.2011.01.036