# Evaluation of the neuroprotective mechanism of 7,8-dihydroxyflavone and generation of corresponding sustained release nano/microparticles for improved ocular drug delivery

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## Introduction

Age-related macular degeneration and inherited retinal diseases are pathologies of the back of the eyes leading to visual impairment and often total blindness. Although much progress has been made in understanding ocular anatomy and disease pathophysiology, there is still a need for effective therapeutic interventions[1,2]. Recently, we demonstrated that 7,8-dihydroxyflavone (7,8-DHF), restores vision in a zebrafish model of inherited blindness[3]. 7,8-DHF is brain derived neurotrophic factor (BDNF) mimetic, able to positively stimulate the TrkB pathway with consequent cell survival and proliferation[4]. However, recent reports question the direct agonism of the TrkB receptor *in vitro*[5]. Thus, the molecular mechanism by which 7,8-DHF restores vision remain unclear. Furthermore, we generated data suggesting systemic delivery of 7,8-DHF in mice did not result in high levels in the retina warranting alternative delivery system for ocular drug availability (unpublished).

Indeed, the eye is a particularly challenging tissue in terms of drug delivery: various barriers from tear dilutions to blood-retinal barrier make the retina a difficult tissue to reach. Several routes (Fig.2) can be used to reach the retina, such as topical, systemic and intraocular injections [6]. Here we investigate the mechanism by which 7,8-DHF restores vision and use Poly D-L(lactide-co-glycolide), PLGA, an FDA approved polymer, for the formulation of 7,8-DHF nano/microparticles[7].

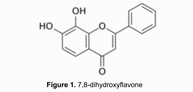


Figure 1. 7,8-dihydroxyflavone

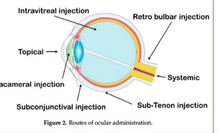
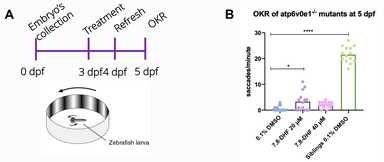


Figure 2. Routes of ocular administration.

## Methods

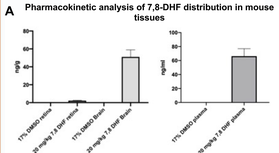
- Visual behavior was assessed using the optokinetic response assay (OKR)
- The analysis of disease pathomechanism and the therapeutic mechanism of 7,8-DHF was investigated via RNA sequencing. Differentially expressed genes were analyzed using Cytoscape and gProfiler
- PLGA nano and microparticles were produced by single emulsion solvent evaporation method
- Nanoparticles size, charge and zeta potential were assessed by DLS
- Encapsulation efficiency (E.E%) of both nano and microparticles was analyzed using the HPLC
- The SEM was used to assess microparticles size and shape

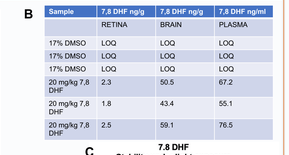

## Aim 1: How does 7,8-DHF rescue vision?




**Behavioural analysis shows that 7,8-DHF rescues vision in *atp6v0e1*$^{-/-}$ larvae. (A):** Schematic of experimental design. Treatment is performed at 3 dpf, refreshed daily and optokinetic response assay performed at 5 dpf after immobilizing the larva into methylcellulose. Saccades per minute are recorded manually. **(B)** 20 µM of 7,8-DHF rescues vision in *atp6v0e1*$^{-/-}$ mutant larvae compared to the DMSO vehicle control *atp6v0e1*$^{-/-}$ (p=0.04). 40 µM 7,8-DHF and DMSO vehicle control *atp6v0e1*$^{-/-}$ have similar OKR score (p=0.58).

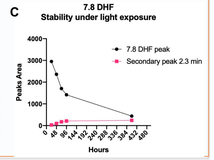
## Aim 2: How to deliver 7,8-DHF to the eye



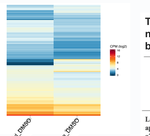
B

| Sample | 7,8 DHF ng/g | 7,8 DHF ng/g | 7,8 DHF ng/ml |
|---|---|---|---|
| | RETINA | BRAIN | PLASMA |
| 17% DMSO | LOQ | LOQ | LOQ |
| 17% DMSO | LOQ | LOQ | LOQ |
| 17% DMSO | LOQ | LOQ | LOQ |
| 20 mg/kg 7,8 DHF | 2.3 | 50.5 | 67.2 |
| 20 mg/kg 7,8 DHF | 1.8 | 43.4 | 55.1 |
| 20 mg/kg 7,8 DHF | 2.5 | 59.1 | 76.5 |




**Pharmacokinetics analysis shows that systemic 7,8-DHF has poor bioavailability in the retina. (A-C):** As systemically adminstered 7,8-DHF appeare not to reach the retina and beacuse 7,8-DHF is unstable in light, challenges in delivery to the eye need to be overcome. In particular, HPLC-MS/MS shows that 20 mg/g of interperitoneal 7,8 DHF for 16 days, twice a day, resulted in only 2.2 ng/g detected in the retina compared to 51 ng/g and 66.3 ng/ml in brain and plasma, respectively (unpublished).

## 3. Identification of genes altered in IRD and by 7,8-DHF

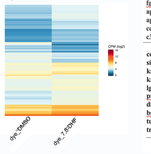




**Table 1. Top 20 differentially expressed gene between normal phenotype siblings and *atp6v0e1*$^{-/-}$ mutants selected by Log2(FC)**

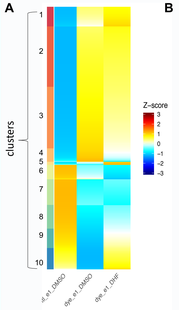
| Gene name | Description | Log2 (FC) | Padjus |
|---|---|---|---|
| LOC100536946 | uncharacterized | 9.09 | 7E-43 |
| apobb.1 | apolipoprotein Bb, tandem duplicate 1 | 8.76 | 8E-18 |
| si2ml | alpha-2-macroglobulin-like | 8.55 | 6E-17 |
| jac9 | jacalin 9 | 7.56 | 6E-121 |
| apoba | apolipoprotein Ba | 7.41 | 4E-12 |
| fgg | fibrinogen gamma chain | 7.17 | 6E-16 |
| apoa1a | apolipoprotein A-Ia | 6.72 | 1E-15 |
| apoa4b.1 | apolipoprotein A-IVb, tandem duplicate 1 | 6.59 | 3E-10 |
| cxl34a.4 | chemokine (C-C motif) ligand 34a, duplicate 4 | 6.36 | 4E-44 |
| c3a.1 | complement component c3a, duplicate 1 | 6.36 | 4E-18 |
| ... | ... | ... | ... |
| cxl34b.1 | chemokine (C-C motif) ligand 34b, duplicate 1 | -3.05 | 3E-21 |
| si:ch211-103a14.5 | si:ch211-103a14.5 | -2.59 | 3E-23 |
| krt97 | keratin 97 | -2.47 | 1E-27 |
| krt96 | keratin 96 | -2.35 | 5E-14 |
| lgals3bpb | lectin, galactoside-binding, soluble, 3 binding protein b | -2.24 | 2E-10 |
| pfkmb | phosphofructokinase, muscle b | -2.17 | 1E-17 |
| dio3b | iodothyronine deiodinase 3b | -2.17 | 8E-10 |
| bglap | bone gamma-carboxyglutamate (gla) protein, like | -2.13 | 2E-13 |
| tubb2 | tubulin, beta 2A class IIa | -2.09 | 4E-09 |
| tmprss5 | transmembrane serine protease 5 | -2.07 | 2E-08 |

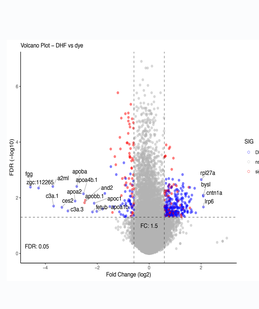
**Table 2. Top 20 differentially expressed gene between *atp6v0e1*$^{-/-}$ mutants and 7,8-DHF treated *atp6v0e1*$^{-/-}$ selected by Log2(FC)**

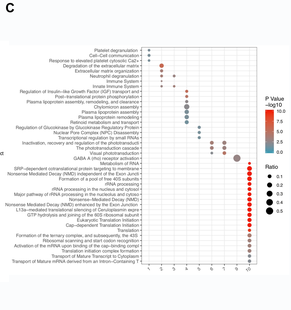
| Gene name | Description | Log2 (FC) | Padjus |
|---|---|---|---|
| kdm6al | lysine (K)-specific demethylase 6A, like | 2.98 | 7E-43 |
| ccdc149b | coiled-coil domain containing 149b | 2.57 | 8E-18 |
| si:dkey-74.21 | si:dkey-74.21 | 2.20 | 6E-17 |
| ddx39aa | DEAD (Asp-Glu-Ala-Asp) box polypeptide 39Aa | 2.08 | 6E-121 |
| ctcf | CCCTC-binding factor (zinc finger protein) | 2.02 | 4E-12 |
| mccc1 | methylcrotonoyl-CoA carboxylase 1 (alpha) | 2.01 | 6E-16 |
| Cct2 | chaperonin containing TCP1, subunit 2 (beta) | 1.96 | 1E-15 |
| CR381638.1 | MAM domain containing glycosylphosphatidylinositol anchor 2a | 1.93 | 3E-10 |
| zc3h12b | zinc finger CCCH-type containing 12B | 1.86 | 4E-44 |
| reck | reversion-inducing-cysteine-rich protein with kazal motifs | 1.84 | 4E-18 |
| ... | ... | ... | ... |
| rpl10a | ribosomal protein L10a | -6.93 | 7E-43 |
| c3a.1 | complement component c3a, duplicate 1 | -4.08 | 8E-18 |
| si:ch73 | si:ch73 | -3.86 | 6E-17 |
| c3a.3 | complement component c3a, duplicate 3 | -3.81 | 6E-121 |
| ces2 | carboxylesterase 2 (intestine, liver) | -3.43 | 4E-12 |
| apoa2 | apolipoprotein A-II | -3.12 | 6E-16 |
| apoba | apolipoprotein Ba | -3.09 | 1E-15 |
| apobb.1 | apolipoprotein Bb, tandem duplicate 1 | -2.82 | 3E-10 |
| si:dkey-13e23.3 | si:dkey-13e23.3 | -2.71 | 4E-44 |
| apoa1b | apolipoprotein A-Ib | -2.51 | 4E-18 |

**Multiple pathways are deregulated in IRD and altered by 7,8-DHF. (A):** Experimental design of RNA sequencing. **(B):** Among 2565 genes significantly altered in 5 dpf eyes of *atp6v0e1*$^{-/-}$, the top 10 upregulated genes encode apolipoproteins and complement system-related proteins, whilst the top 10 downregulated genes are related with development, visual perception and translation, as shown by the heatmap and **table 1. (C)** RNAseq analysis identified 1633 genes upregulated and 932 downregulated in *atp6v0e1*$^{-/-}$ after the restoring vision treatment 7,8-DHF, as shown by the heatmap and **table 2**.

## 4. 7,8-DHF reduces inflammation in the eye

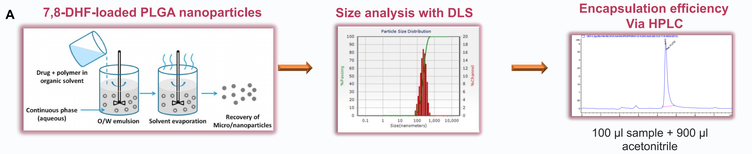


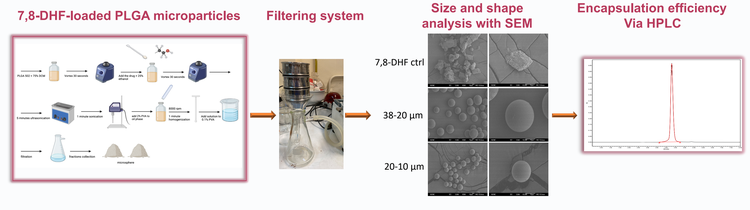




**7,8-DHF normalizes genes previously altered in the mutant: (A-B):** Heatmap and volcano plot showing a triple analysis between the vehicle control-treated siblings, vehicle control treated *atp6v0e1*$^{-/-}$ and 7,8-DHF treated *atp6v0e1*$^{-/-}$. The statistically altered genes were clustered into 10 cluster using apcluster v1.4.8 in R. Using a cut-off of 1.5 and FDR < 0.05, cluster 4 and 10 were identified to contain genes normalized by 7,8-DHF, e.g genes involved in lipid transport and inflammation (*e.g apoba, apoa4b.1, apoa2, c3a.1, c3a.3*) and genes involved in translation (*e.g rpl27a, bysl*) previously altered in the mutant. **(C)** biological processes altered by 7,8-DHF were analyzed. Cluster 4 and 10 involve the highest number of biological processes normalized after the treatment, as shown by the functional gene enrichment analysis performed via gProfiler. Among these, lipid transport, remodeling and assembly are the processes altered in cluster 4; whilst processes involved in translation are the major constituent of cluster 10.

## 5. Biodegradable PLGA particles for 7,8-DHF ocular drug delivery




| Table 1 | Diameter | Polarity | Zeta Potential | Encapsulation Efficiency (E.E%) |
|---|---|---|---|---|
| Nanoparticles | 222.6 nm (DLS) | Negative | 8.6 mv | 59.14 ± 4.97 % |
| Microparticles | 38-20 µm (SEM) | - | - | 58.8±3.1%. |
| Microparticles | 20-10 µm (SEM) | - | - | 32.2±12.9%. |

**PLGA nano and microparticle for 7,8-DHF encapsulation. (A)** 7,8-DHF nanoparticles were generated using a single emulsion solvent evaporation method, in which 1 mg of 7,8-DHF and 3.5 mg/ml PLGA (38000-54000 RG504H) were dissolved into 2 ml of dichloromethane (DCM) to form the oil phase. The oil phase was added, drop by drop, into 10 ml of aqueous phase 1% Poloxamer (surfactant). The solution was kept stirring until complete evaporation of DCM at room temperature. Nanoparticles were collected after a 15 minutes centrifuge at 3000 rpm, 4 °C, and analysed by DLS to measure particle size, charge, zeta potential and E.E% by HPLC, as shown in figure **A** and **table 1**. **(B)** Single emulsion solvent evaporation method was also used to generate microparticles. 10 mg of 7,8-DHF and 100 mg of PLGA 502 were dissolved into 75% DCM and 25% ethanol as shown by the schematic in figure **B**. The oil phase was added into 100 ml of 2% PVA 72 kDa. After the evaporation of the organic phase, microparticles were filtered while washing with $dH_2O$. Particle shape was analysed by SEM and the E.E via HPLC, as shown by figure **B** and **Table 1.**

## 6. Conclusions and future directions

- This research provides an insight on the 7,8-DHF mechanism of restoring vision in a zebrafish model of inherited blindness (*atp6v0e1*$^{-/-}$)
- The disease pathomechanism of *atp6v0e1*$^{-/-}$ is characterized by a significant upregulation of apolipoproteins and downregulation of development and translation related genes
- 7,8-DHF revert the expression of those genes in a disease model normalizing their level
- RNA seq validation via qPCR is currently ongoing
- Future directions include release study during secondment at UCM, Madrid, and efficacy and safety analysis with preclinical models of blindness at Experimentica, Finland

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## References

1. Gheorghe et Al. Rom J Ophthalmol. 59(2):74–7. (2015)
2. Vázquez-Domínguez et Al. Genes-basel 10, 654 (2019)
3. Daily et Al. Sci Rep-uk 7, 11320 (2017)
4. Liu et Al. Transl Neurodegener 5, 2 (2016)
5. Boltaev et Al. Sci Signal 10, eaal1670 (2017)
6. Chen at A.l Tzu Chi Medical J 20, 25–34 (2008)
7. Herrero-Vanrell et Al. Prog Retin Eye Res 42, 27–43 (2014)

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