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Activating the Melanocortin System Resolves Inflammation, Reduces VEGF Signaling in Diabetic Retinopathy (DR), and Provides Retinal Ganglion Cell (RGC) Protection

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Purpose

- Diabetic retinopathy (DR) is a consequence of chronic, inadequately controlled diabetes that often results in impaired vision and in severe cases leads to blindness[1]
- Management of DR often involves the use of anti-VEGF agents; however, there are many attributes of anti-VEGF therapy that warrant the need for additional treatment options[2]
- The melanocortin system (MS) offers a new approach to treating inflammatory diseases by harnessing an endogenous pathway to reestablish tissue homeostasis and resolve inflammation[3]
  - Agonism of the MS has been shown to be efficacious in a range of ocular diseases[4]
- Melanocortin receptor (MCR) agonists reestablish the inflammatory resolution response, reduce stress signaling, and promote healthy homeostasis to defend against future insults[5-7]
- We hypothesize that the MS could provide a much wider response to retinopathy through modulating cell populations, multiple pathways, barrier function, and neuronal protection
- The objective was to assess the efficacy of the MCR agonists PL9654 and PL9655 in preclinical models

Methods

- MC1R/3R/4R/5R pan-agonists, PL9654 and PL9655, were investigated in a streptozotocin (STZ)-induced rat model of diabetic retinopathy (DR), a choroidal neovascularization (CNV) mouse model, and a retinal ischemia/reperfusion (I/R) mouse model to evaluate their effects on retinopathy by modulating inflammation, retinal cell populations, VEGF signaling, angiogenesis and barrier function, and neuroprotection

STZ Model of Induced Hyperglycemia in Rats

- Hyperglycemia was induced with STZ, and glucose levels were checked at Day 4 to exclude animals without high glucose levels
- Animals received PL9654 (0.05-0.5 mg/kg, subcutaneously [SC]), PL9655 (0.1 mg/mL, topical), or vehicle BID on days 4-113
- Retinas were analyzed with snRNA-seq or data-independent acquisition liquid chromatography-mass spectroscopy (DIA LC-MS) for differential expression, transcript clustering, and gene set enrichment and quantified for Iba1+ cells
- Blood-retinal barrier (BRB) breakdown was measured by intravenous administration of fluorescein isothiocyanate-tagged (FITC)-albumin tracer

Choroidal Neovascularization Model in Mice

- In the CNV mouse model, intravitreal treatment (IVT) with vehicle, anti-VEGF (0.2 mg/mL), or PL9654 (10 µM or 100 µM) occurred on Day 0 and Day 15
- Analysis by Fundus Fluorescein Angiography (FFA) for lesion leakage and immunohistochemistry for isolectin B4 (angiogenesis) and collagen 1 (fibrosis) was conducted on Day 35 (end of study)

Ischemia/Reperfusion Model in Mice

- In the I/R mouse model, anterior chamber injections with 1 µL of α-MSH or PL9654 (1 µg/mL) were given on Days 1 and 5; eyes were collected on Day 7
- Neuroprotection was evaluated on Day 7 after I/R
- Cell nuclei of the retinal-ganglion-cell (RGC) layer were stained with Brn3a, and viable RGCs were measured by Tuj1 staining

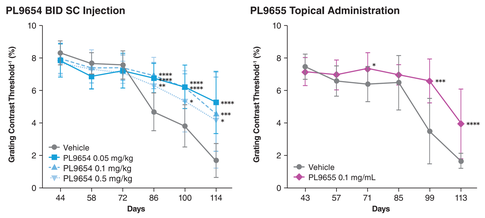
Results

STZ Model of Induced Hyperglycemia in Rats

Change in Visual Contrast Threshold

- PL9654 and PL9655 showed significant efficacy at maintaining contrast vision compared with vehicle (**Figure 1**)

Figure 1. Visual Contrast Threshold Maintained With PL9654 AND PL9655

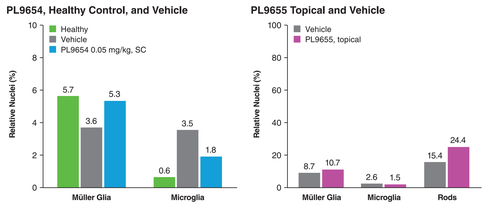


Values are mean +/- SD; vehicle for PL9654, n=8; PL9654, n=7/dose; vehicle for PL9655, n=6; PL9655, n=6. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs vehicle (Mann-Whitney test).

Changes in Percentage of Nuclei in Microglia and Müller Glial Cells

- snRNA-seq analysis showed that PL9654/PL9655 decreased the relative percentage of nuclei in microglia cells and increased the relative percentage of nuclei in Müller glial cells compared with vehicle (**Figure 2**)
  - The relative percentage of microglia and Müller glial cells in PL9654 treated animals was comparable to healthy control
  - PL9655 topical treatment also showed an increase in rods compared with vehicle

Figure 2. Improvement in Percentage of Nuclei in Microglia and Müller Glial Cells

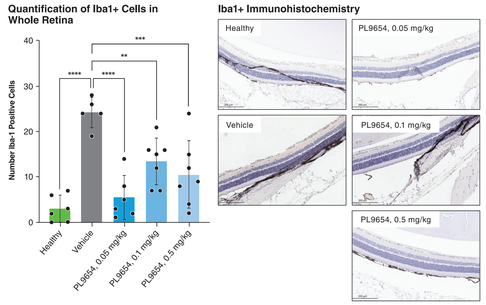


Healthy for PL9654, n=6; vehicle for PL9654, n=6; PL9654, n=7; vehicle for PL9655, n=4; PL9655, n=7.

Changes in Number of Iba1+ Cells in STZ-induced Retinas

- PL9654 significantly (P<0.01) reduced Iba1+ cells compared with vehicle (**Figure 3**)

Figure 3. PL9654 Reduces Iba1+ Cells in the Retina

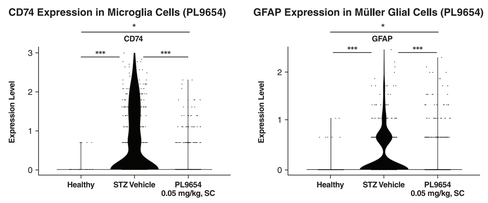


Dots represent number of positive cells in each sample; healthy, n=6; vehicle, n=5; PL9654, n=6-7/dose.
One-way ANOVA, Dunnett's post hoc test to compare each treatment to vehicle.
**P<0.01, ***P<0.001, ****P<0.0001.

Changes in Expression of Inflammatory Pathway Genes

- Differential gene expression analysis of microglia and Müller glial cells showed down-regulation of multiple immune marker genes
  - CD74, a known marker for microglial activation in the retina, was significantly down-regulated in PL9654 and PL9655 treated microglial cells compared with vehicle
  - Glial fibrillary acidic protein (GFAP), a molecular marker for reactive retinal gliosis and a molecular marker of inflammation in DR, showed significantly reduced expression in Müller glial cells with PL9654 and PL9655 compared to vehicle
- PL9654/PL9655 treatment significantly reduced CD74 and GFAP expression (P<0.001; **Figure 4**)

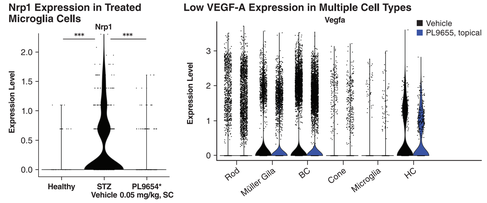
Figure 4. CD74 and GFAP Are Reduced by PL9654



STZ, streptozotocin.
Healthy, n=6; vehicle for PL9654, n=6; PL9654, n=7; vehicle for PL9655, n=4; PL9655, n=7.
*P<0.05, **P<0.01, ***P<0.001.

- Nrp1, a transmembrane glycoprotein that acts as a co-receptor of VEGF, facilitates VEGF interaction with the primary VEGF receptor (VEGFR2) to play a critical role in angiogenesis
- Significantly reduced expression of Nrp1 (P<0.0001) was observed in PL9654-treated and healthy rats compared with vehicle, and a significantly lower expression of VEGF-A (P<0.0001) was observed in multiple cell types of PL9655 treated rats (**Figure 5**)

Figure 5. Nrp1 and VEGF-A Are Reduced by PL9654/PL9655

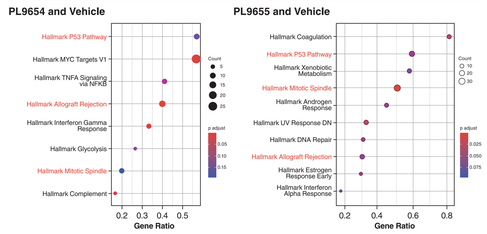


BC, bipolar cells; HC, horizontal cells.
Healthy, n=6; vehicle for PL9654, n=6; *PL9654 (subset of PL9654 treated samples), n=5; vehicle for PL9655, n=4; PL9655, n=7.
***P<0.001.

Changes in Pathways Enriched in Microglia Cells

- Gene set enrichment analysis identified negative enrichment of diverse immune-related pathways in microglia cells for PL9654 and PL9655 vs vehicle (**Figure 6**)

Figure 6. Negative Enrichment of Inflammatory Pathways in Microglia Cells With PL9654/PL9655 Treatment

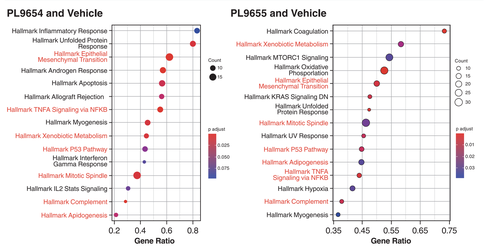


*Shared pathways are highlighted.

Changes in Pathways Enriched in Müller Glial Cells

- Gene set analysis identified negative enrichment of diverse immune-related pathways in Müller glial cells for PL9654 and PL9655 vs vehicle (**Figure 7**)

Figure 7. Negative Enrichment of Inflammatory Pathways in Müller Glial Cells With PL9654/PL9655 Treatment

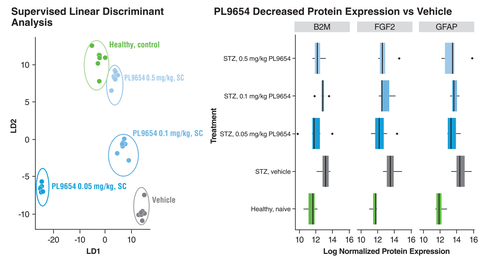


*Shared pathways are highlighted.

Proteomic Analysis

- Proteome profiling using LC-MS was performed on 43 rat retina samples; specific rat retina spectral libraries were created consisting of 2,683 proteins and 31,682 peptides (**Figure 8**)
- Supervised linear discriminant analysis of proteomic (DIA LC-MS) data results in perfect separation of treatment groups using 2 latent dimensions
- Beta-2 microglobulin (B2M; a potential indicator of DR), fibroblast growth factor (FGF)-2, and GFAP proteins were reduced by PL9654, showing a pro-resolution protein expression pattern

Figure 8. Effects of PL9654 on Protein Expression

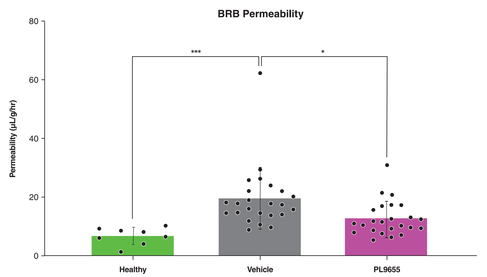


Healthy, n=7; vehicle, n=8; PL9654, n=7/dose.

Changes in BRB Permeability

- PL9655 reduced BRB permeability (P<0.01; **Figure 9**)

Figure 9. BRB Permeability Reduced with PL9655 Topical vs Vehicle

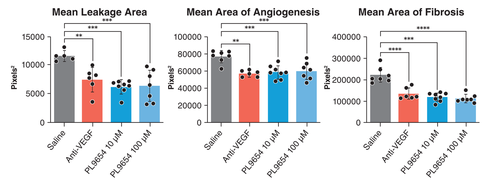


Healthy, n=8; vehicle, n=24; PL9655, n=24.
Each dot represents an individual animal.
Statistics: One-Way ANOVA with Dunnett's post hoc test to compare differences with vehicle.
*P<0.05, ***P<0.001.

Choroidal Neovascularization Model in Mice

- PL9654 significantly reduced CNV-driven angiogenesis and fibrosis (P<0.01; **Figure 10**)

Figure 10. PL9654 Significantly Reduces Leakage Area, Angiogenesis, and Fibrosis



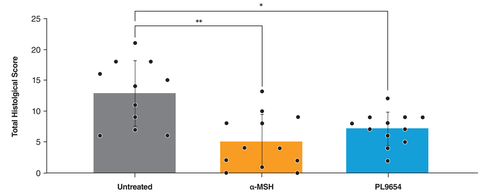
Saline, n=5-7; anti-VEGF, n=6; PL9654, n=7-8/dose.
One-Way ANOVA with Dunnett's post hoc test comparing to saline.
Each dot represents an individual animal.
**P<0.01, ***P<0.001, ****P<0.0001.

Ischemia/Reperfusion Model in Mice

Changes in Retinal Damage from I/R

- As assessed by total histological score, a rubric for damage within the retina after I/R, PL9654 significantly reduced retinal damage driven by I/R, similar to α-MSH (**Figure 11**)

Figure 11. PL9654 Significantly Reduces Retinal Damage Driven by I/R

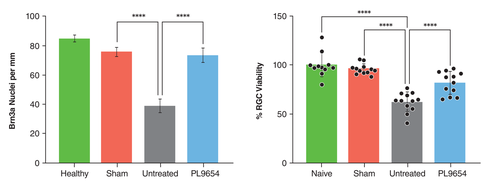


Values are mean +/- SD of total histological score; n=12 animals/group.
One-way ANOVA with Kruskal-Wallis post hoc test comparing treatment to untreated.
*P<0.05, **P<0.01.

Changes in RGC Health After I/R

- PL9654 significantly maintained Brn3a- and Tuj1-positive cells, indicating protection of RGC viability after I/R-induced damage (P<0.0001; **Figure 12**)

Figure 12. PL9654 Protects RGC Health in the I/R Mouse Eye



Mean +/- SD; n=12 animals/group.
Brn3a positive cell number per mm OR %RGC viability measured by number of Tuj1 positive cells/total cell number per mm.
***P<0.001, ****P<0.0001.

Conclusions

- Melanocortin agonists PL9654 and PL9655 show promise in treating retinopathy by down-regulating multiple inflammatory pathways, suppressing angiogenesis and BRB breakdown, and providing neuroprotection
- With multiple routes of administration, including topical, these agents could be used earlier than the current standard of care
- Together, these results support the continued development of PL9654 and PL9655 for the treatment of DR

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Disclosures Paul Kayne, Alison Obr, Priyanka Dhingra, John Dodd, and Carl Spana are employees of and own stock in Palatin Technologies. Hongkwan Cho, Zhenhua Xu, Lijuan Wu, Shirley Wu, and Haining Lu have nothing to declare. Andrew W. Taylor received SRA and consulting fees from Palatin Technologies. Elia J. Duh received a sponsored research agreement with Palatin Technologies.

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