Drug-driven identification of inflammatory pathways in retinal microglia

Anne Rombaut [1], Alan Nicol [1], Heela Salrus [2], Pete A Williams 1, Robert A Harris [2], **James R Tribble** [1]

1 Department of Clinical Neuroscience, Division of Eye and Vision, St. Erik Eye Hospital, Karolinska Institutet, Stockholm, Sweden

2 Applied Immunology and Immunotherapy, Department of Clinical Neuroscience, Karolinska Institutet, Center for Molecular Medicine, Karolinska University Hospital, Stockholm Sweden

Introduction

- Inflammation is an important component of glaucomatous damage [1]. Depletion of microglia can be profoundly neuroprotective if prophylactic [2]. However, since glaucoma is diagnosed when symptoms are already present and disease cascades have already initiated, clinically useful treatments for neuroinflammation will require interventional treatment.
- Transcriptional repression of inflammatory pathways offers an attractive option, as non-pathogenic glia will not be deleteriously targeted and can continue to provide homeostatic support to neurons. Importantyl, this would be an interventional treatment.
- Histone deacetylases (HDAC) interact with chromatin structures, thus modulating DNA repair, replication, and transcription [3]. I recently identified the HDAC inhibitor Valproic acid (VPA) as a potential modifier of neuroinflammatory signaling pathways [4] and demonstrated that it limits microglia inflammatory responses, pro-inflammatory cytokine expression, and RGC degeneration following RGC axon injury [5].
- However, VPA is a broad activiting HDAC inhibitor and has an unfovorurable side effect profile. Instead, we will leverage the extensive library of HDAC inhibitors from the cancer research field in a drug-driven approach to identify genes key to neuroinflammatory transcriptional repression and drugs which may target these with greater specificity.

Aims: Using a combined drug and -omics based approach we will identify key neuroinflammatory genes and pathways and identify potential targeted novel drug treatments which can interventionally suppress neuroinflammation in glaucoma.

Design and Methods

Work plans:
1. Screen known HDAC inhibitors for repression of activated microglia
2. Identify key transcriptionally repressed genes and exisitng drugs for repurposing
3. Test new drug candidates for neuroinflammatory suppression

We screened known targeted and broad acting HDAC inhibitors from cancer drug libraries for transcriptional repression of activated microglia

● Whole brains from 3-month old *Cx3cr1*$^{GFP/+}$ mice (for GFP+ microglia) were seeded into T75-cell culture flasks and grown in DMEM/F12 complete medium (inc. 10 % FBS, 1% Pen/Strep, 20 ng/ml m-CSF) for 2 weeks until confluent to yield > 1 million microglia for higher-throughput testing

● Microglia were isolated using MicroBeads attached to anti-mouse CD11b antibodies (Miltenyi Biotec) and cultured at 1,500 cells / well in 96 well plates in serum-free culture conditions (TIC media, [6]). This promotes a more ramified microglial morphology with lower basal inflammatory states.

● Microglia were either:

a) treated with HDAC inhibitors at 50μM, 5μM, and 500nM (*n* = 4 wells / condition) for 24 hours to determine toxicity

or b) exposed to glaucoma relevant pro-inflammatory stimuli (50 ng/ml TNF-α) in the culture media for 24 hrs followed by treatment with HDAC inhibitors at 50μM, 5μM, and 500nM (n = 4 wells / condition) for 24 hrs to determine potential for inflammatory suppression.

● Microglia were fixed with 3.7% PFA for 15 mins and stained with DAPI. DAPI images (5X) were aquired for cell counting and GFP images (20X) were aquired for analysis of microglial morphology using a Leica DMi8 microscope. Individual cell morphology was anlyzed using Imaris software (Bitplane).

Best candidates were sent for RNA-sequencing to identify key transcriptionally repressed genes for microglial neuroinflammatory suppression

● We repeated the above protocol in 24-well plates with 50,000 cells / well for VPA (5μM), ACY-957 (500nM), SW-100 (500nM) following inflammatory activation

● Samples have been sent for bulk RNA-sequencing (Illumina TruSeq)

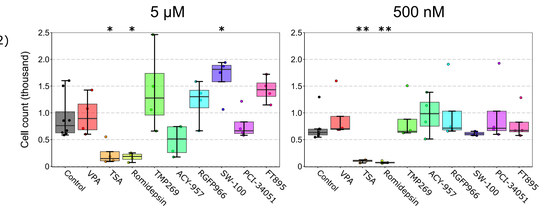
We tested best candidate HDAC inhibitors for microglial inflammatory suppression and neuroprotection

● Adult C57BL/6J mice were euthanized and retinas dissected and maintained as retinal explants for 1 day *ex vivo* on cell culture inserts (Millicell 0.4 μm pore) in Neurobasal-A media (2 mM L-glutamate, 1% Pen/Strep) at (37 °C, 5% CO2).

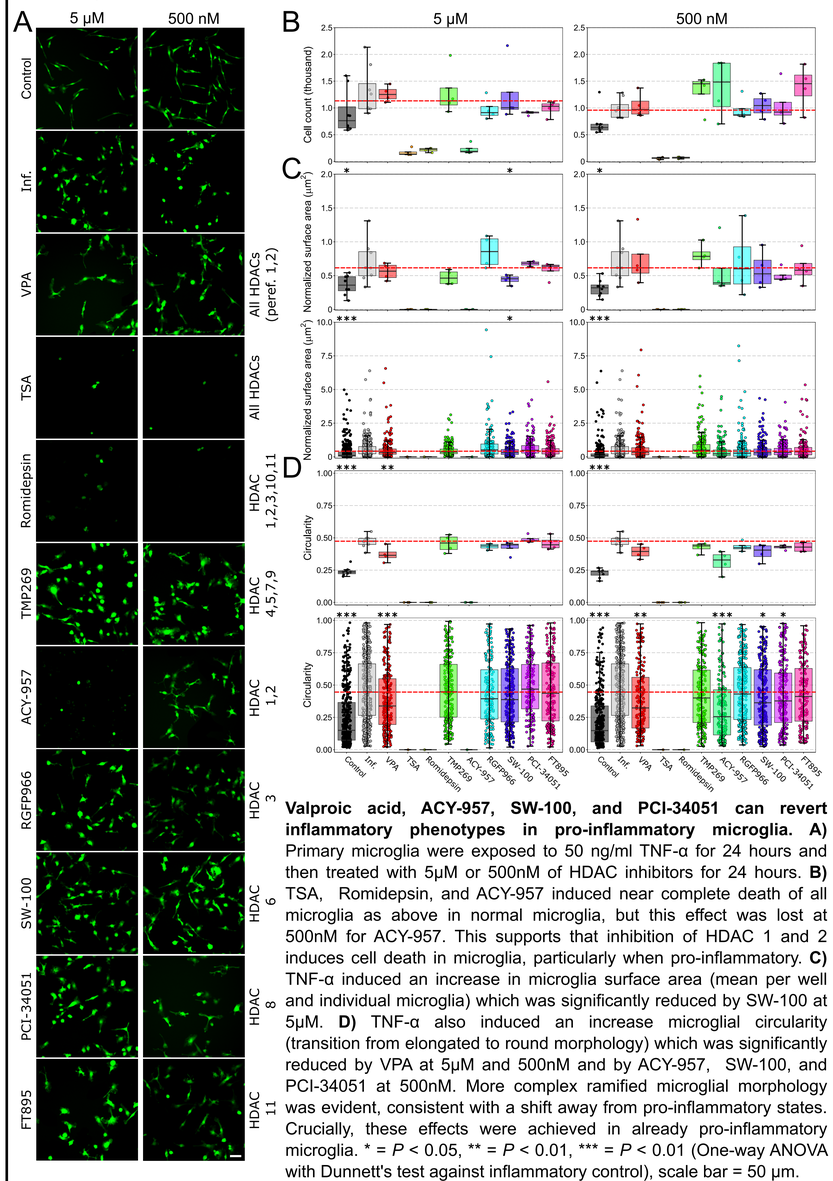
● Retinas were fixed with 3.7% PFA for 1 hour and labelled with anit-Iba1 (microiglia) and stained with DAPI. Confocal images were acquired on an LSM-800 (Zeiss) to assess micorglia morphology as above.

Results

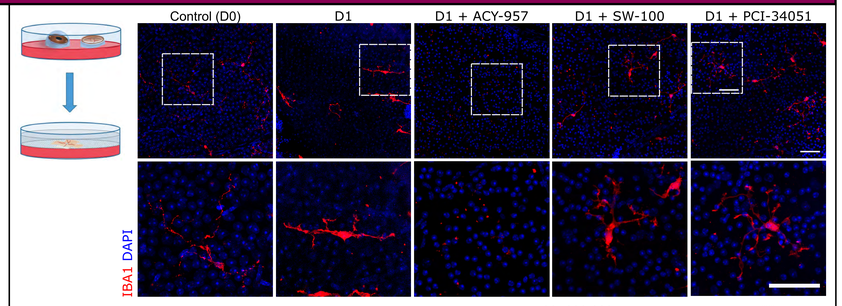
Broad	VPA	All HDACs (peref. 1,2)
	TSA	All HDACs
	Romidepsin	HDAC 1,2,3,10,11
	TMP269	HDAC 4,5,7,9
Specific	ACY-957	HDAC 1,2
	RGFP966	HDAC 3
	SW-100	HDAC 6
	FT895	HDAC 8
	PCI-34051	HDAC 11



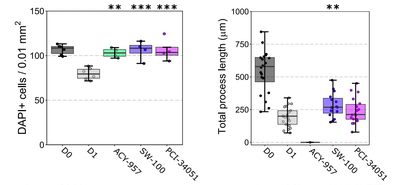
Inhibition of HDAC 1 and 2 induces cell death in microglia. Primary microglia were exposed to 50μM, 5μM, and 500nM of HDAC inhibitors for 24 hours. Compared to untreated controls, all HDAC inhibitors induced cell death or had solubility issues at 50μM, except for VPA (data not shown). TSA and Romidepsin also induced significant cell death at 5μM, and 500nM. SW-100 induced an increase in cell numbers at 5μM but not 500nM. (*n* = 4 wells / condition, *n* = 8 wells for control). * = $P < 0.05$, ** = $P < 0.01$ (One-way ANOVA with Dunnett's test against control). NB VPA is a much less potent HDAC inhibitor and has other potential mechanisms of action.



Valproic acid, ACY-957, SW-100, and PCI-34051 can revert inflammatory phenotypes in pro-inflammatory microglia. A) Primary microglia were exposed to 50 ng/ml TNF-α for 24 hours and then treated with 5μM or 500nM of HDAC inhibitors for 24 hours. **B)** TSA, Romidepsin, and ACY-957 induced near complete death of all microglia as above in normal microglia, but this effect was lost at 500nM for ACY-957. This supports that inhibition of HDAC 1 and 2 induces cell death in microglia, particularly when pro-inflammatory. **C)** TNF-α induced an increase in microglia surface area (mean per well and individual microglia) which was significantly reduced by SW-100 at 5μM. **D)** TNF-α also induced an increase microglial circularity (transition from elongated to round morphology) which was significantly reduced by VPA at 5μM and 500nM and by ACY-957, SW-100, and PCI-34051 at 500nM. More complex ramified microglial morphology was evident, consistent with a shift away from pro-inflammatory states. Crucially, these effects were achieved in already pro-inflammatory microglia. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.01$ (One-way ANOVA with Dunnett's test against inflammatory control), scale bar = 50 μm.



SW-100 is a novel drugs that could be re-purposed for glaucoma treatment. HDACs were tested in a retinal explant model in which I have previously demonstarted that VPA protects against RGC loss and neuroinflammation [4,5]. Retinas were maintained for 1 day ex vivo (D1) or fixed immediately as naïve controls (D0). Retinas were treated with either ACY-957, SW-100, or PCI-34051(100μM). At D1, significant nuclear loss in the GCL occurrs compared to D0. ACY-957, SW-100, and PCI-34051 provided significant neuroportection compared to untreated D1 with no significant loss of neurons relative to D0. ACY-957 killed all microglia, recapitulating effects *in vitro*. PCI-34051 had no significant effect on microglia morphology but SW-100 induced a significantly improved complexity to microglial morphology with greater total process length. This supports that SW-100 can support the supression of pro-inflammatory microglia and provide neuroprotection. *n* = 4-6 retinas for all conditions, scale bars = 50 μm, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. (One-way ANOVA with Tukeys HSD test for DAPI; One-way ANOVA with Dunnett's test against D1 for microiglia).



Conclusion

- From a screen of HDAC inhibitors *in vitro* we identify SW-100 as a novel drug that can revert microglial pro-inflammatory phenotypes and provide neuroprotection *ex vivo*.
- We identify that ACY-957 can induce dose dependent cell death of microglia without neuronal death supporting its utility as a tool compound for future research.
- We identify that the previous neuroprotective and anti-inflammatory effects of VPA can be explained by direct suppression of pro-inflammatory microglia.
- We can maintain microglia in serum free conditions following amplification in serum containing media, without serum deprivation effects. This method provides high yields of microglia with more ramified morphology for high throughput testing of inflammatory mechanisms and inflammatory modifying drugs.

Next Steps

- We will test VPA and SW-100 *in vivo* in a rat bead model of glaucoma, where the drugs will be administered as intravitreal injections at 3 days post IOP (once inflammatory responses have begun). We will determine the effects on inflammation and neuroprotection.
- We will continue with RNA-sequencing analysis to identify which gene changes are important for inflammatory suppression.
- From these gene list we will perform *in silico* drug screening using the Broad Institute CMAP database. This will identify existing drugs from a library of ~5,000 small-molecule compounds and ~3,000 genetic reagents (>1000 FDA approved) that can induce similar gene profile changes. We will test identified drugs *in vitro* in microglia and expand to glaucoma models.

References

[1] Tribble JR, Hui F, Quintero H, et al. Neuroprotection in glaucoma: Mechanisms beyond intraocular pressure lowering. Mol Aspects Med. 2023;92:101193. doi: 10.1016/j.mam.2023.101193

[2] Rombaut A, Brautaset R, Williams PA, Tribble JR. Glial metabolic alterations during glaucoma pathogenesis. Front. Ophthalmol. 2023,3. doi: 10.3389/fopht.2023.1290465

[3] Ho TCS, Chan AHY, Ganesan A. Thirty Years of HDAC Inhibitors: 2020 Insight and Hindsight. J Med Chem. 2020;63(21):12460-12484. doi: 10.1021/acs.jmedchem.0c00830

[4] Enz TJ, Tribble JR, Williams PA. Comparison of Glaucoma-Relevant Transcriptomic Datasets Identifies Novel Drug Targets for Retinal Ganglion Cell Neuroprotection. J Clin Med. 2021;10(17):3938. doi:10.3390/jcm10173938

[5] Tribble JR, Kastanaki E, Uslular AB, Rutigliani C, Enz TJ, Williams PA. Valproic Acid Reduces Neuroinflammation to Provide Retinal Ganglion Cell Neuroprotection in the Retina Axotomy Model. Front Cell Dev Biol. 2022;10:903436. doi:10.3389/fcell.2022.903436

[6] Bohlen CJ, Bennett FC, Tucker AF, Collins HY, Mulinyawe SB, Barres BA. Diverse Requirements for Microglial Survival, Specification, and Function Revealed by Defined-Medium Cultures. Neuron. 2017;94(4):759-773.e8. doi:10.1016/j.neuron.2017.04.043

James Tribble | Assistant Professor | james.tribble@ki.se | ki.se | Twitter: @James_R_Tribble