

## Letter to the Editor

# Dramatic Effect of Oral CSF-1R Kinase Inhibitor on Retinal Microglia Revealed by In Vivo Scanning Laser Ophthalmoscopy

Andreas Ebner, Despina Kokona, Joël Jovanovic, and Martin S. Zinkernagel

Department of Ophthalmology and Department of Clinical Research, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland

**Correspondence:** Andreas Ebner; Universitätsklinik für Augenheilkunde, Inselspital, CH - 3010 Bern, Switzerland; e-mail ebner.a.andreas@gmail.com

This report provides sound evidence that the small molecule pharmaceutical PLX5622, a highly selective CSF-1R kinase inhibitor, crosses the blood-retina barrier and suppresses microglia activity. Members of this class of drug are in advanced clinical development stages and may represent a novel approach to modulate ocular inflammatory processes.

**Received:** 2 July 2016

**Accepted:** 1 March 2017

**Published:** 19 April 2017

**Keywords:** retina; microglia; in vivo imaging; modulation; inflammation

**Citation:** Ebner A, Kokona D, Jovanovic J, Zinkernagel MS. Dramatic effect of oral CSF-1R kinase inhibitor on retinal microglia revealed by in vivo scanning laser ophthalmoscopy. *Trans Vis Sci Tech.* 2017;6(2):10, doi:10.1167/tvst.6.2.10  
Copyright 2017 The Authors

## Introduction

Colony-stimulating factor 1 receptor (CSF-1R) inhibition results in effective elimination of brain microglia.<sup>1</sup> However, the effect of such treatment on the retinal microglia has not been reported to our knowledge. To this end, we performed in vivo imaging of the retina in transgenic microglia reporter mice (CX3CR1-GFP<sup>+/+</sup>) treated orally with PLX5622 for 7 days, a highly selective CSF-1R inhibitor.<sup>2</sup> Using scanning laser ophthalmoscopy, we found dramatically reduced microglia counts in the mouse retina.

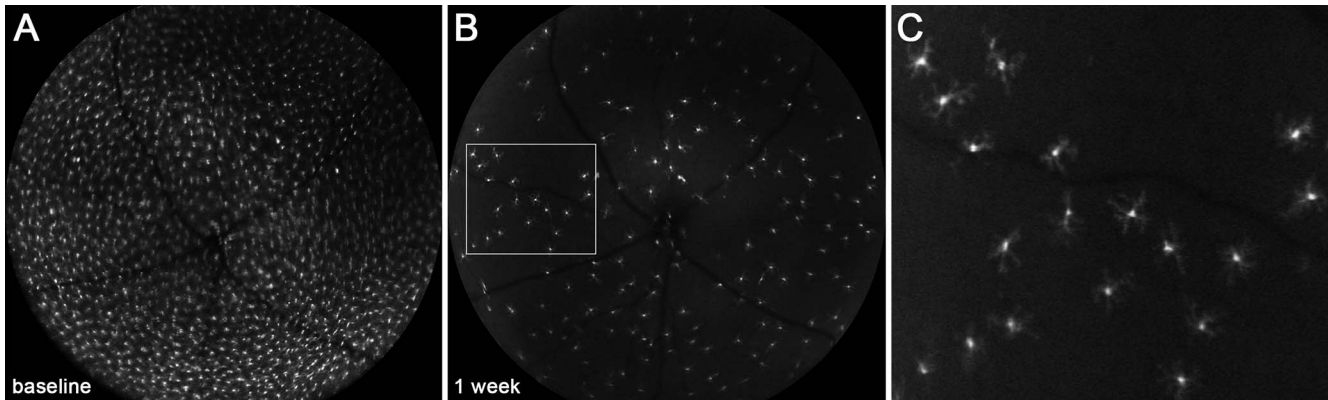
## Methods

Heterozygous fractalkine receptor reporter mice were bred by crossing homozygous CX3CR1-GFP<sup>+/+</sup> male mice<sup>3</sup> with wildtype Balb/c females. Two-month-old heterozygous mice then were fed with chow containing PLX5622 (1200 parts per million [ppm] formulated in AIN-76A standard rodent diet; Research Diets, Inc., New Brunswick, NJ) or identical

chow without this compound. Animals were housed under temperature and humidity-controlled conditions in individually ventilated cages with a 12-hour light/12-hour dark cycle. This study was approved by the local Animal Ethics Committee (Veterinärdienst des Kantons Bern: BE 14/16) and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

At baseline and 1 week after commencement of the PLX5622 diet, the retinas of the mice were imaged using confocal laser scanning ophthalmoscopy (Heidelberg Spectralis HRA+OCT; Heidelberg Engineering GmbH, Heidelberg, Germany) after intraperitoneal anesthesia with 1 mg/kg medetomidine (Dormitor 1 mg/mL; Provet AG, Lyssach, Switzerland) and 80 mg/kg ketamine (Ketalar 50mg/mL; Parke-Davis, Zurich, Switzerland). Some mice were killed to confirm findings on whole-mounts and by fluorescence-activated cell sorting (FACS) of retinas.

Retinal whole-mounts were prepared as described previously,<sup>4,5</sup> and double-stained for fluorescence immunohistochemistry, labeling microglia (rabbit anti-iba1 diluted 1:200, Cat #019-19741; Wako Pure



**Figure 1.** Ultra-widefield blue light fundus autofluorescence images at baseline (A) and after 1 week of CSF-1R kinase inhibitor treatment (B) from a representative animal. The area outlined in (B) is magnified in (C) to highlight the ramified processes typical for quiescent retinal microglia.

Chemical Industries Ltd., Osaka, Japan), and retinal ganglion cells (goat anti-brn3a [C-20] diluted 1:500; sc-31984; Santa Cruz Biotechnologies, Heidelberg, Germany).

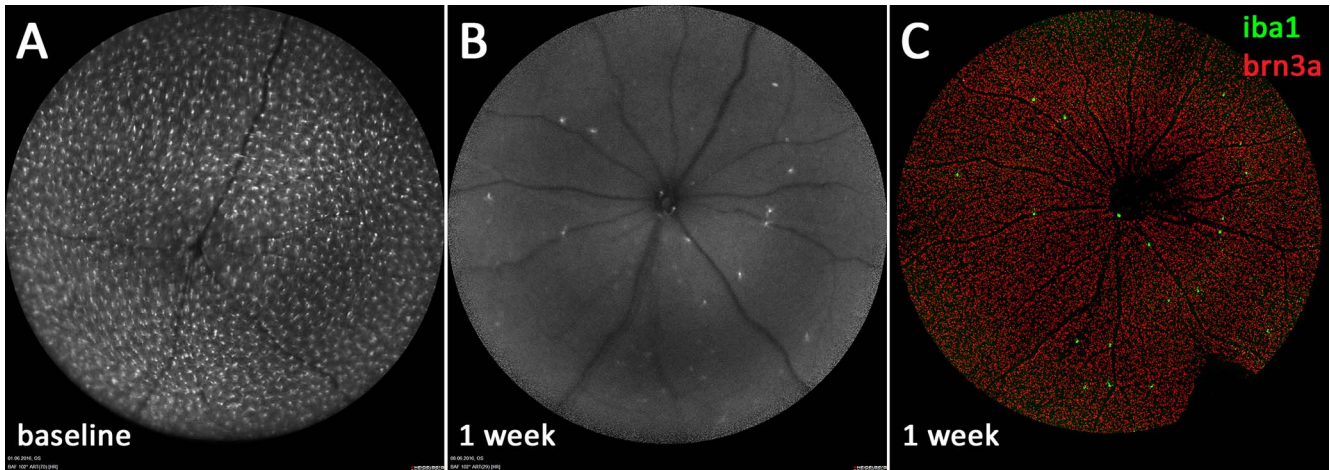
For FACS analysis, the two retinas of individual mice were combined and analyzed as one sample to ascertain a sufficient number of cells in the specimen for cell sorting.<sup>6</sup> Briefly, the retinas were dissociated in 0.4 Wunsch units/mL Liberase TM Grade (Roche, Basel, Switzerland) in DPBS at 37°C for 30 minutes. Then, the samples were washed in DPBS and resuspended in FACS buffer (100mM EDTA, 20% FBS, 0.5% Na-Azide, in DPBS) for antibody staining. DNase I (0.01%; Roche) was present in all solutions. Retinal microglia were identified using fluorescent-labelled antibodies against CD45 (30-F11, #103116, 1:100) and CD11b (M1/70, #101212, 1:100) from Biolegend (San Diego, CA). Samples were incubated for 20 minutes with antibodies and then analyzed with a LSR II Cytometer System using the BD FACSDiva software (BD Biosciences, Allschwil, Switzerland). The FACS data were analyzed with the Flowjo Single Cell Analysis Software V10 (TreeStar, Ashland, OR).

For retina in vivo imaging, the pupils were dilated with tropicamide 0.5%/phenylephrine 2.5% eyedrops (Hospital Pharmacy, Inselspital, Bern, Switzerland). Hypromellose 20 mg/mL (Methocel 2%; OmniVision AG, Neuhausen, Switzerland) was applied on the eyes to avoid dry corneas. Images of the retina then were obtained in the blue light (488 nm) fundus autofluorescence mode (BAF) using a noncontact ultra-widefield 102° lens (Heidelberg Engineering GmbH).

For statistical analysis, the number of microglia in the widefield BAF images was counted using ImageJ (version 1.48v; provided in the public domain at [\[imagej.nih.gov\]\(http://imagej.nih.gov\) by the National Institutes of Health, Bethesda, MD\). Briefly, images were exported as jpegs and background subtraction was performed \(rolling ball radius 4.0\). The files then were converted into 8-bit images and a threshold was manually set before counting the cells using the particle analyzer \(particle size 25 to  \$\infty\$ \).<sup>7</sup> The mean of the two eyes of each animal was used for further calculations, and results are given as mean  \$\pm\$  standard deviation. The paired or unpaired Student's \*t\*-test was used as appropriate. Confidence intervals were 2-sided, and a \*P\* value of  \$< 0.05\$  was considered statistically significant.](http://</a></p>
</div>
<div data-bbox=)

## Results

The number of retinal GFP-expressing cells per widefield BAF image decreased dramatically by 92.2% (confidence interval [CI], 83.8–100;  $P = 0.0001$ , paired *t*-test;  $n = 5$ ) from  $881 \pm 116$  cells at baseline to  $70 \pm 28$  cells after 1 week of PLX5622 treatment. The cells that still were present displayed the characteristic morphology of quiescent microglia with ramified processes (Fig. 1). Immunohistochemistry staining of retinal whole-mounts for ionized calcium-binding adapter molecule 1 confirmed these findings (Fig. 2). The effect of PLX5622 on retinal microglia was further investigated in FACS analysis. The retinal microglia phenotype is characterized by low expression levels of CD45 and high CD11b ( $CD45^{\text{low}}CD11b^+$ ).<sup>8</sup> Here too, a significant decrease of the cell population exhibiting these characteristics was seen (Fig. 3). On cursory evaluation of optical coherence tomography scans in treated animals, no changes of total retinal thickness or layer architecture were found. Based on histologic studies, it has been



**Figure 2.** Comparison between scanning laser ophthalmoscopy and retinal whole-mount. Blue light autofluorescence scanning laser ophthalmoscopy (BAF) was performed in vivo at baseline (A) and 1 week after CSF-1R kinase inhibitor treatment (B). The retinal whole-mount (C) was collected immediately after in vivo imaging and double-stained using fluorescence immunohistochemistry. Individual cells visible in the BAF image (B) can be identified in the whole-mount (C) stained for calcium-binding adapter molecule 1 (iba1, corresponding cells highlighted for better visibility). Only the area of the whole-mount captured in the BAF image (B) is shown (the [Supplementary Figure](#) shows the unmodified whole-mount), and ganglion cells have been stained (brn3a) to highlight retinal vessels for spatial orientation.

reported previously that PLX5622 does not cause overt loss of other retinal cells (Dharmarajan S, et al. *IOVS* 2016;57:ARVO E-Abstract 2223), at least in the short-term.

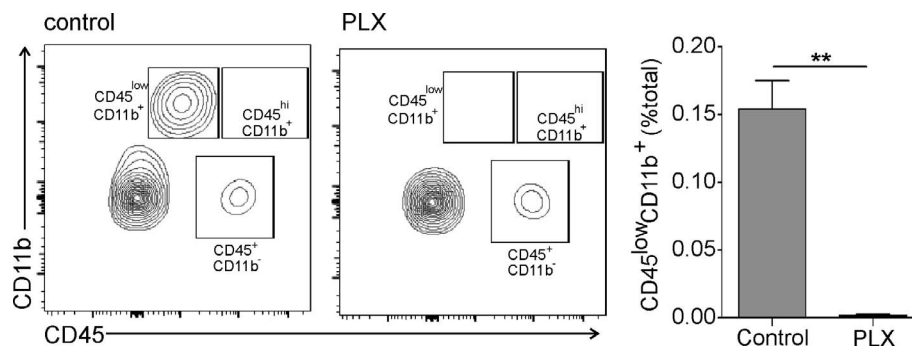
## Discussion

In the retina, we found drastic changes of GFP expression in microglia reporter mice during treatment with PLX5622, an orally administered selective CSF-1R kinase inhibitor. We have not specifically investigated cell death, but previous work in the central nervous system suggests that microglia are subject to apoptosis.<sup>1,9</sup> Alternatively, the observed

phenotypic changes might be caused solely by altered protein expression. Recently, Guan et al.<sup>10</sup> reported upregulation of the fractalkine receptor gene in microglia of the spinal cord dorsal horn after intrathecal administration of CSF-1. Further investigations are warranted, whereby noninvasive imaging of the retina might be a helpful tool with high spatial resolution, affording identification of individual cells in vivo.

In conclusion, selective CSF-1R kinase inhibition may be a promising new approach for targeted modulation of ocular microglia and inflammatory processes in the retina.

**Citation:** Ebnetter A, Kokona D, Jovanovic J, Zinkernagel MS. Dramatic effect of oral CSF-1R



**Figure 3.** Evaluation of microglia depletion in the retinas of PLX5622 fed mice. Representative FACS contour plots and corresponding bar graph of the cell populations with typical microglia phenotype (CD45<sup>low</sup>CD11b<sup>+</sup>) in controls and PLX5622 fed animals (PLX) 7 days after the initiation of PLX5622 chow ( $n = 2$  per group;  $**P < 0.01$ , unpaired  $t$ -test).



kinase inhibitor on retinal microglia revealed by in vivo scanning laser ophthalmoscopy. *Trans Vis Sci Tech.* 2017;6(2):10, doi:10.1167/tvst.6.2.10

## Acknowledgments

Homozygous fractalkine receptor reporter mice (CX3CR1-GFP<sup>+/+</sup>) were kindly donated by Steffen Jung (Weizmann Institute of Science, Rehovot, Israel). PLX5622 chow was provided by Plexxikon, Inc. under a Materials Transfer Agreement. The sponsor or funding organizations had no role in the design or conduct of the experiments. The authors alone are responsible for the content and writing of this letter.

This project is supported by CTU Research-Grant from Inselspital (84800858), Bern, Switzerland; Dr. Streuli-Fonds, Bern, Switzerland; Foundation OPOS, Stiftung zugunsten von Wahrnehmungsbehinderten, St. Gallen, Switzerland; Swiss National Science Foundation (Grant 320030\_156019). PLX5622 rodent diet was provided without financial support under a Materials Transfer Agreement with Plexxikon Inc., Berkeley, CA, USA.

A.E.: received honoraria from Bayer for lectures and non-financial support from Allergan and Novartis. D.K.: receives grant support from Bayer. M.S.Z.: received financial support from Allergan, Bayer, Heidelberg Engineering, Novartis, and equity Novartis.

Disclosure: **A. Ebnetter**, Bayer (R), Allergan (C, R), Novartis (F, R); **D. Kokona**, Bayer (F); **J. Jovanovic**, None; **M. S. Zinkernagel**, Allergan (F), Bayer (F, C), Heidelberg Engineering (F), Novartis (C, I)

## References

1. Elmore MRP, Najafi AR, Koike MA, et al. Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. *Neuron.* 2014;82:380–397.
2. Dagher NN, Najafi AR, Kayala KM, et al. Colony-stimulating factor 1 receptor inhibition prevents microglial plaque association and improves cognition in 3xTg-AD mice. *J Neuro-inflamm.* 2015;12:139.
3. Jung S, Aliberti J, Graemmel P, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol.* 2000;20:4106–4114.
4. Giannakaki-Zimmermann H, Kokona D, Wolf S, Ebnetter A, Zinkernagel MS. Optical coherence tomography angiography in mice: comparison with confocal scanning laser microscopy and fluorescein angiography. *Transl Vis Sci Technol.* 2016;5:11.
5. Rovere G, Nadal-Nicolas FM, Agudo-Barriuso M, et al. Comparison of retinal nerve fiber layer thinning and retinal ganglion cell loss after optic nerve transection in adult albino rats. *Invest Ophthalmol Vis Sci.* 2015;56:4487–4498.
6. Zinkernagel MS, Petitjean C, Wikstrom ME, Degli-Esposti MA. Kinetics of ocular and systemic antigen-specific T-cell responses elicited during murine cytomegalovirus retinitis. *Immunol Cell Biol.* 2012;90:330–336.
7. Ebnetter A, Casson RJ, Wood JP, Chidlow G. Estimation of axon counts in a rat model of glaucoma: comparison of fixed-pattern sampling with targeted sampling. *Clin Exp Ophthalmol.* 2012;40:626–633.
8. Eter N, Engel DR, Meyer L, et al. In vivo visualization of dendritic cells, macrophages, and microglial cells responding to laser-induced damage in the fundus of the eye. *Invest Ophthalmol Vis Sci.* 2008;49:3649–3658.
9. De I, Nikodemova M, Steffen MD, et al. CSF1 overexpression has pleiotropic effects on microglia in vivo. *Glia.* 2014;62:1955–1967.
10. Guan Z, Kuhn JA, Wang X, et al. Injured sensory neuron-derived CSF1 induces microglial proliferation and DAP12-dependent pain. *Nat Neurosci.* 2016;19:94–101.