Topical Treatment With Bromfenac Reduces Retinal Gliosis and Inflammation After Optic Nerve Crush

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Submitted: July 30, 2016
Accepted: October 6, 2016


PURPOSE. To study the effect of topical administration of bromfenac, a nonsteroidal anti-inflammatory drug (NSAID), on retinal gliosis and levels of prostaglandin E$_2$ (PGE$_2$) after complete optic nerve crush (ONC).

METHODS. Adult albino rats were divided into the following groups ($n = 8$ retinas/group): (1) intact, (2) intact and bromfenac treatment (twice a day during 7 days), (3) ONC (7 days), and (4) ONC (7 days) + bromfenac treatment (twice a day during 7 days). Animals from groups 3 and 4 were imaged in vivo with spectral-domain optical coherence tomography (SD-OCT) before the procedure and 15 minutes, 3, 5, or 7 days later. Retinas from all groups were analyzed by immunodetection, Western blotting, or enzyme-linked immunosorbent assay (ELISA).

RESULTS. Quantification of Brn3a (brain-specific homeobox/POU domain protein 3A) + RGCs (retinal ganglion cells) in cross sections showed that bromfenac treatment does not accelerate ONC-induced degeneration. Cellular retinaldehyde binding protein 1 regulation indicated that bromfenac improves retinal homeostasis in injured retinas. Spectral-domain OCT showed that the thickness of the retina and the retinal nerve fiber layer at 7 days post ONC was significantly reduced in bromfenac-treated animals when compared to untreated animals. In agreement with these data, hypertrophy of astrocytes and Müller cells and expression of glial fibrillary acidic protein and vimentin were greatly diminished by bromfenac treatment. While no changes in cyclooxygenase (COX) enzyme COX1 and COX2 expression were observed, there was a significant increase of PGE$_2$ after ONC that was controlled by bromfenac treatment.

CONCLUSIONS. Topical administration of bromfenac is an efficient and noninvasive treatment to control the retinal gliosis and release of proinflammatory mediators that follow a massive insult to the RGC population.

Keywords: nonsteroidal anti-inflammatory drug, NSAID, astrocyte, Müller cell, prostaglandin, cyclooxygenase, optical coherence tomography

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most common drugs used to treat ophthalmic pain, photophobia, inflammation, and edema (reviewed in Refs. 1, 2). Nonsteroidal anti-inflammatory drugs inhibit cyclooxygenases, and this inhibition in turns results in a reduced level of proinflammatory prostaglandins. Although corticosteroids have a stronger anti-inflammatory effect than NSAIDs, they also have various side effects and may trigger neuronal apoptosis and axonal loss.1-6 Therefore, NSAID treatment is preferred, and these compounds are being extensively studied in patients and animal models to assess their toxicity, pharmacokinetics, neuroprotective properties, and anti-inflammatory and anti-edema effects, among others.7-16

One such NSAID, bromfenac, is an inhibitor of the cyclooxygenase (COX) enzymes COX1 and COX2, and is more selective for COX2 than for COX1.17 In rabbits, topical bromfenac reaches the vitreous and retinochoroidal tissues, and after a single topical administration is detectable in the retinochoroid up to 24 hours.8,17,18 Kida et al.19 showed that compared to nepafenac and diclofenac, bromfenac concentration after topical application on rabbit eyes was continuously higher in the retinochoroidal tissues. Thus, bromfenac may have a better therapeutic effect than these two other NSAIDs in retinochoroidal inflammatory diseases.

In patients, bromfenac treatment has been shown to be more efficient and safer than dexamethasone and fluorometholone to control postoperative inflammation.16 Moreover, it has been recently demonstrated that bromfenac as well as two other NSAIDs, indomethacin and nepafenac, significantly reduced, in the same fashion, the levels of prostaglandin E$_2$ (PGE$_2$) in the vitreous of patients undergoing vitrectomy for macular pucker.14 Finally, in patients, as in rabbits, bromfenac shows good penetration, and its concentration remains stable in the aqueous humor up to approximately 12 hours after instillation.20

Regarding the therapeutic effect of bromfenac eye drops in animal models, it has been shown that they reduce retinal edema triggered by lipopolysaccharide systemic injection in...
Topical Bromfenac and Retinal Inflammation

Inflammation and edema are, as well, common responses to tissue damage or disease, as has been shown for the retina in animal models of glaucoma or optic nerve injury.34–38,40,41 Complete intraorbital optic nerve crush (ONC) is an acute insult that kills the vast majority of retinal ganglion cells (RGCs) within the first 2 weeks after injury.34,38,40,41 Moreover, this injury also triggers an important inflammatory response within the injured and contralateral fellow eye.27,28,42 Optic nerve crush is a model that has been widely used to test neuroprotective therapies36,37,41,43–47 and to investigate the molecular events underlying central nervous system neuronal death upon axonal trauma.25,38,46 Optic nerve crush is as well often used as a simplified model of glaucomatous injury. To the best of our knowledge there are no previous studies investigating the effects of topical administration of a NSAID on the retinal inflammation after complete optic nerve axotomy. Thus, in the present study we investigated the effect of topical bromfenac on retinal degeneration, gliosis, and release of PGE2 in the rat retina after ONC.

Materials and Methods

Animal Handling

This study was approved by the Committee of Animal Care of the University of Murcia (Murcia, Spain). All experimental procedures were performed in accordance with the European Union Directive 2010/63/EU for animal experiments and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult female Sprague-Dawley rats (200–250 g body weight) were fed ad libitum and kept in an environmentally controlled room with an alternating 12-hour/12-hour light/dark cycle. Surgery and spectral-domain optical coherence tomography (SD-OCT) analysis were carried out under general anesthesia induced by intraperitoneal injection of a mixture of xylazine (10 mg/kg, Rompun; Bayer, Kiel, Germany) and ketamine (70 mg/kg, Imalgene; Merial laboratories S.A., Barcelona, Spain). Analgesia was provided by subcutaneous administration of buprenorphine (0.1 mg/kg). All efforts were made to minimize animal suffering. For euthanasia, rats were killed with an IP overdose of sodium pentobarbitol diluted 1:1 in saline (Dolethal, Vetoquinol; Especialidades Veterinarias, S.A., Madrid, Spain).

Several groups were formed (n=8 retinas/group): (1) intact, (2) intact + bromfenac treatment (7 days), (3) ONC (7 days), and (4) ONC + bromfenac treatment (7 days). Animals from groups 3 and 4 were imaged in vivo with SD-OCT. Retinas from all groups were analyzed by immunodetection, Western blotting, or ELISA (see below).

Surgery

The left optic nerve was intraorbitally crushed at approximately 2 mm from the optic disc following previously reported methods.34–36,50

Topical Administration of Bromfenac

On the left eye of experimental animals and both eyes of intact animals, two drops of bromfenac (0.9 mg/mL 0.09% Yellox; Bausch & Lomb (S.A., Alcobendas, Madrid, Spain) were instilled topically right after the injury and then every 12 hours for 7 days. This dosage was selected following the prescribed treatment for human patients.51 When the administration of the drug did not coincide with the SD-OCT analysis, animals were anesthetized with inhalational anesthesia (2% isoflurane, IsoFlo; Laboratorios Veterinarios Esteve, City, Spain), and they were kept anesthetized for 15 further minutes after applying the drops.

Spectral-Domain Optical Coherence Tomography

The left retinas from the ONC and ONC + bromfenac groups were analyzed using SD-OCT before the lesion (baseline), after the injury (15 minutes) and 3, 5 and 7 days after the injury. At the end of the study (7 days) animals were euthanized and the retinas dissected for further analysis.

Briefly, from the second measurement onward, animals were anesthetized with intraperitoneal anesthesia, and a drop of tropicamide (Tropicamida 1%; Alcon-Cusi, S.A, Barcelona, Spain) was instilled in both eyes to induce mydriasis. Eyes were kept hydrated with artificial tears, and a custom-made contact permeable lens (3.5-mm posterior radius of curvature, 5.0-mm optical zone diameter, +5.0-diopter [D] back vertex power) was placed on the cornea to maintain corneal hydration and clarity. Then, the retinas were imaged according to manufacturer instructions (Spectralis; Heidelberg Engineering, Heidelberg, Germany). To adapt for the rat’s eye, a commercially available 78-D double aspheric fundus lens (Volk Optical, Inc., Mentor, OH, USA) was mounted in front of the camera unit. Imaging was performed with a proprietary software package (Eye Explorer, version 3.2.1.0; Heidelberg Engineering) as described.22

Retinas were scanned with a raster pattern of 31 equally spaced horizontal B-scans spanning the central retina (3000-μm length). In four central sections/animal, the retinal nerve fiber layer (RNFL) and retinal thickness were manually measured at 600 (center) and 1200 (periphery) μm from the optic nerve.

Retinal Cross Sections and Immunodetection

Animals were perfused transcardially with 4% paraformaldehyde (PFA) in phosphate buffer 0.1 M after a saline rinse. Eyes were dissected and cryoprotected in increasing gradients of sucrose (15% to 30%; Sigma-Aldrich, Madrid, Spain). Tissue was embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA), frozen in liquid nitrogen, and kept at −80°C. Retinas (n=4/group) were sectioned at 15 μm in a cryostat. For immunodetection, sections were blocked in 2% donkey serum in phosphate-buffered saline (PBS) with 0.1% Triton (PBST) and incubated overnight at 4°C with a mixture of rabbit anti-galalid fibrillary acidic protein (GFAP; 1:500 Sigma-Aldrich) and goat anti-vimentin (1:500 sc-7557; Santa Cruz Biotechnologies, Heidelberg, Germany) or a mixture of mouse anticalcular retinaldehyde binding protein 1 (CRALBP1 [B2], 1:200; ab15051; Abcam, Cambridge, UK) and goat anti-brain-specific homeobox/POU domain protein 3A (Brn3a, 1:750, sc-31984; Santa Cruz Biotechnologies). Secondary detection was done with donkey anti-goat Alexa Fluor 488 or 594, donkey antichick Alexa 488, and donkey anti-rabbit Alexa 594 (1:500; Molecular Probes, ThermoFisher, Madrid, Spain). Finally, sections were counterstained with DAPI (4',6-diamidino-2-phenylindole) (Vectorshield mounting medium with DAPI; Vector Laboratories, Palex Medical, Barcelona, Spain). Images were acquired with a ×20 objective under an epifluorescence microscope (Axioskop 2 Plus; Zeiss Mikroskopie, Jena, Germany).

Brn3a+RGCs were manually counted, in a masked fashion, in sections spanning the optic nerve (3 sections/animal).
Numbers are given as the mean number ± standard difference of RGCs per section.

**Western Blotting**

Retinas were fresh dissected and immediately frozen in dry ice (n = 4/group). Then, retinas were homogenized in lysis buffer (Pro-prep protein extraction solution; Intron Biotechnologies, Sevilla, Spain). Lysates were incubated 1 hour on ice and centrifuged to remove particulate matter. Protein concentration was determined using SimpliNano spectrophotometer (Biochrom Ltd, Cambridge, UK). A total of 20 to 60 μg protein was resolved in 4% to 20% SDS-PAGE gels (Bio-Rad laboratories, SA, Madrid, Spain) and transferred to nitrocellulose membranes (GE Healthcare, Barcelona, Spain) by electroblotting. Blots were blocked for 1 hour with 5% skim milk in PBS containing 0.5% Tween-20 (PBS-T; pH 7.4) and then were incubated overnight at 4°C with rabbit anti-cyclooxygenase 2 (COX2, 1:1000), mouse anti-cyclooxygenase 1 (COX1, 1:2000), rabbit anti-vimentin (1:1000), mouse anti-CRALBP1 (1:1000), all from

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**FIGURE 1.** Bromfenac treatment diminishes retinal swelling after ONC. (A) OCT sections spanning the optic disc acquired before (baseline) and 15 minutes or 7 days after ONC or ONC + bromfenac. (a, b) Magnifications from the squared areas. (B) Graphs showing the course of the retinal (top) and RNFL (bottom) thickness after ONC and ONC + bromfenac. These data were measured in the OCT images at 1200 and 600 μm from the optic disc. §‡Significant compared to baseline values (§ONC, ‡ONC + bromfenac; Kruskal-Wallis, Dunn’s post hoc, §P < 0.05, ‡P < 0.05, ‡‡P < 0.01). †Significant compared to 15 minutes for both groups (Kruskal-Wallis, Dunn’s post hoc, P < 0.05) *Significant difference between both groups (Kruskal-Wallis, Dunn’s post hoc, *P < 0.05, **P < 0.01). B, baseline; Imm, immediately after the lesion; d, days; ON, optic nerve; ONC, optic nerve crush.

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Abcam, or rabbit anti-GFAP (1:500, Sigma-Aldrich). Detection was carried out with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit or anti-mouse, Santa Cruz Biotechnologies) at 1:5000 dilution. Membranes were developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare). As loading control, total protein/lane in the membranes was visualized using reversible protein staining (R-PROB, Sigma-Aldrich) followed by β-actin identification using anti-β-actin–HRP mouse monoclonal antibody (Sigma-Aldrich). β-actin signal was equivalent to total protein staining in all lanes, and thus its intensity was used for normalization. The density of the protein bands was quantified using image analyzer Chemidoc XRS+ (Bio-Rad, Hercules, CA, USA) and the software (ImageLab 5.2.1).

Prostaglandin E₂ Detection

Prostaglandin E₂ levels in retinal homogenates from the different experimental groups (n = 4/group) were assayed by PARAMETER PGE₂ enzyme-linked immunoabsorbent assay.
FIGURE 3. Bromfenac treatment decreases reactive gliosis and PGE₂ production in axotomized retinas. (A) GFAP (astrocytes and Müller cells) and vimentin (Müller cells) immunodetection in retinal sections. ONC causes hypertrophy of astrocytes and Müller cells (gliosis). This gliotic response is greatly reduced by bromfenac treatment. (B) Western blotting showing the expression levels of COX2, COX1, vimentin, and GFAP in extracts from intact retinas (I), intact retinas treated during 7 days with bromfenac (I+B 7 d), retinas dissected 7 days after axotomy (ONC 7 d), or 7 days after ONC and bromfenac treatment (ONC7d+B7d). a.u., arbitrary units taking intact retinas as 100% and normalized versus β-actin signal and total protein.
staining (see Methods). *Significant compared to intact retinas (t-test, $P < 0.05$ for I-B, $P < 0.01$ ONC+B, $P < 0.0001$ ONC). †Significant compared to ONC (t-test, $P < 0.001$). ‡Significant compared to intact + bromfenac (t-test, $P < 0.001$ for ONC and $P < 0.05$ for ONC+B). (C) PGE$_2$ concentration (pg/mL) in extracts from the same groups as above. ONC causes a significant increase of PGE$_2$ that is abolished by bromfenac treatment (Kruskal-Wallis, Dunn’s post hoc, $P < 0.001$).

(ELISA) (R&D Systems, Minneapolis, MN, USA). Tests were performed according to the manufacturer’s instructions.

Graphs and Statistical Analysis
Graphs and pairwise multiple comparisons (Kruskal-Wallis ANOVA, Dunn’s post hoc) were done with GraphPad Prism v. 6 software (GraphPad, San Diego, CA, USA). Data are presented as mean ± standard deviation (SD), and differences were considered significant when $P < 0.05$.

RESULTS
Retinal Thickness
In both groups, ONC and ONC + bromfenac, 15 minutes after the lesion the retina had swollen compared to baseline values, although this swelling was transient and at day 3 the retina was back to normal (Fig. 1). Thereafter, the retinal thickness decreased gradually, reaching statistical significance for both groups at day 5 in the periphery, and closer to the optic disc in the bromfenac-treated group. Seven days after the injury, the retina was significantly thinner in both groups and at both distances. Interestingly, comparing both groups, the retina was thinner at day 7 in the bromfenac-treated animals, reaching this difference statistical significance at 600 μm from the optic disc (for detailed values see Supplementary Table S1).

At 1200 μm from the optic disc, the thickness of RNFL significantly decreased by day 5 in the bromfenac-treated group and at day 7 in both groups. At this latter time point, the RNFL was significantly thinner in the treated versus untreated group. At 600 μm from the optic disc, there were not significant changes in either group compared to baseline. However, 7 days after the lesion, the RNFL thickness was significantly reduced in the bromfenac-treated retinas compared to untreated ones.

Bromfenac Treatment Does Not Accelerate Retinal Degeneration
In view of the above data, the question arises: Does the reduced RNFL thickness in treated animals reflect a reduction in edema/gliosis, or does it reflect increased neurodegeneration? To answer it, we quantified Brn3a expression in retinal cross sections (Figs. 2A, 2B) and analyzed the expression of CRALBP1, a protein expressed by Müller cells that is a marker of retinal homeostasis. 52 Bromfenac treatment did not alter the number of Brn3a+RGCs, either in intact or in ONC-injured retinas. Regarding CRALBP1, compared to intact retinas, its expression declined after ONC but was maintained in ONC-treated samples. In intact but treated retinas, CRALBP levels declined as well, although to a lesser extent than after ONC.

Retinal Gliosis
Next, we assessed astrocyte and Müller cell hypertrophy using the classical gliosis markers GFAP and vimentin. Optic nerve crush triggers a strong glial activation, which, at 7 days, almost reverted with bromfenac treatment, as observed by immunodetection and Western blotting (Figs. 3A-B). In retinal extracts from intact animals treated during 7 days with bromfenac, Western blotting analysis showed that GFAP and vimentin are upregulated compared to intact retinas, but their levels are still lower than those found in ONC + bromfenac retinas.

Cyclooxygenases and Prostaglandin E$_2$
Bromfenac mechanism of action is due to its ability to block prostaglandin synthesis by inhibiting COX1 and COX2. Thus, in retinal extracts we studied the regulation of both enzymes and measured the levels of PGE$_2$ (Figs. 3B, 3C). Neither COX1 nor COX2 showed a significant regulation at 7 days post lesion and/or treatment (Fig. 1B). Regarding PGE$_2$, its concentration in intact or intact and treated retinas was similar (9.6 ± 4 or 8.7 ± 2 pg/mL, respectively), and significantly increased ~4 fold after ONC (33.4 ± 7 pg/mL). Interestingly, in the ONC + bromfenac group, PGE$_2$ levels were reduced almost to the values found in intact retinas (13 ± 2 pg/mL) (Fig. 3C).

DISCUSSION
The main findings of this work are that topical instillation of bromfenac (0.09%) reduces retinal thickness, retinal gliosis, and the release of PGE$_2$ after a complete intraorbital ONC, a drastic injury to the retina that results in acute and massive RGC death. Importantly, bromfenac treatment does not affect the number of RGCs in intact retinas or accelerate ONC-induced degeneration. In fact, current experiments in our lab indicate that bromfenac treatment increases RGC survival after ONC, although this protection is not immediate but is delayed to 9 days onward (Rovere G, Nadal-Nicolás FM, Vidal-Sanz M, Agudo-Barriuso, M. unpublished data, 2016). Macrogial cells respond quickly to retinal stress or injury. 33 Indeed, it has been recently shown that macroglial cells hypertrophy across the whole retina after an apparently innocuous manipulation such as intravitreal injection of phosphate-buffered saline. 29 It was interesting to find that they also responded to topical administration of bromfenac, as evidenced by the significant increase of GFAP and vimentin and the decrease of CRALBP1 in extracts from intact retinas treated during 7 days with the NSAID. However, these changes reverted in ONC + bromfenac retinas when compared to ONC alone, indicating, firstly, that the gliosis triggered by ONC is very strong and almost but not completely abolished by bromfenac, and, secondly, that treatment with this NSAID maintains retinal homeostasis after ONC. In line with this, it is worth highlighting that gliotic and homeostatic markers seem to be regulated in opposite ways here, after ONC, and in isolated retinal explants. 52

Retinal gliosis is reported to have both neuroprotective as well as neurodegenerative effects (reviewed in Ref. 55). Data in this work do not support neuroprotection, but they indicate that, at least in this model and at this time point, the reduced gliosis is neutral in terms of RGC degeneration. But, because as mentioned above we have preliminary data indicating that indeed bromfenac treatment protects RGCs from 9 days onward, it would seem that a reduction in gliosis is neuroprotective. However, this might well occur because the reduced gliosis is accompanied by maintenance of the retinal homeostasis or by other mechanism(s) not related to the giotic response.
Bromfenac mechanism of action goes through the nonselective inhibition of COX1 and COX2, although recent work indicates that bromfenac is more selective for COX2.17 Expression of COX2 is inducible in response to injury and the release of cytokines and proinflammatory molecules,54 both of which occur in the retina after ONC.55 In mild brain injuries, COX2 expression elevates shortly after the lesion, but this upregulation is extended (>3 days) if the injury is severe,55 and this may explain why we did not find upregulation of COX2 in our experiments, since we analyzed the retinas 7 days after ONC. Nevertheless, we found that at this time point there was approximately a 4-fold increase of PGE2 concentration in ONC retinas compared to intact ones, indicating that even though COX2 is not upregulated, it is activated. Furthermore, bromfenac instillation almost suppressed completely the increment of PGE2, suggesting that bromfenac inhibits COX2 increased activity. Interestingly, in spite of the increase of macroglial markers in intact + bromfenac retinas compared to intact ones, the concentration of PGE2 was similar in both groups, suggesting that perhaps the cytokines/inflammatory mediators that activate COX are released by microglial cells.56,57

Finally, we found that bromfenac treatment significantly reduced in injured retinas the retinal and RNFL thickness 7 days after the insult, without this thinning being due to an increased RGC death. This is of special importance in the central retina because in clinical practice, RNFL measurement is employed as an index of RGC loss. Our group and others22,58–61 have shown in animal models that following axotomy of the optic nerve or ocular hypertension there is a 9-day time lapse mismatch between the onset of RGC disappearance and the slower, more protracted, degeneration of the intraretinal axons, which form the RNFL.58 It is tempting to suggest that such a time delay could be influenced by a RNFL swollen by the astrocyte and Müller cell hypertrophy that occurs upon lesion.

Besides reducing gliosis, bromfenac abolishes the release of PGE2, which in turn may effectively reduce the retinal edema, since prostaglandins in the eye disrupt the blood-ocular barrier, increase vasodilation, and facilitate leukocyte migration causing edema and inflammation.2 If this hypothesis is sustained, it would be of interest to instill NSAIDs in patients with retinal edema when diagnosed by in vivo measurement of the retina.

**Acknowledgments**

The authors thank José María Díaz Llopis (W.M. Bloss, S.A. Barcelona, Spain) for providing the Spectralis OCT system (Heidelberg Engineering) for these studies and Antonio Parrado (Immunología, IMIB-Arrixaca, Murcia, Spain) for providing the Chemidoc Image analyzer.


Disclosure: G. Rovere, None; F.M. Nadal-Nicolás, None; P. Sobrado-Calvo, None; D. García-Bernal, None; M.P. Villegas-Pérez, None; M. Vidal-Sanz, None; M. Agudo-Barruso, None

**References**

Topical Bromfenac and Retinal Inflammation


