Glaucoma is commonly described as a heterogeneous family of optic neuropathies that lead to irreversible blindness. Major risk factors for glaucoma include age and race; however, the most attributable risk factor for the development of glaucoma is an increase in intraocular pressure (IOP). To date, glaucoma therapies have focused solely on lowering IOP, either surgically or pharmacologically. Although these treatments have proven effective, progression of glaucomatous degeneration persists in some cases [1], and nearly 13.5% of glaucoma patients lose vision in one eye and 4% lose vision in both eyes [2]. Glaucoma results in degeneration of the retinal ganglion cells (RGCs), which are terminally differentiated neurons that lack the ability to regenerate. Therefore, it is imperative to develop approaches that can promote robust neuroprotection of these cells.

The current study focuses on the ocular endothelin (ET) system, which includes the vasoactive peptides ET-1, ET-2, and ET-3 and their G protein coupled receptors, the
ET$_A$ and ET$_B$ receptors. ET-1 and its receptors are expressed in multiple ocular tissues, including the iris, choroid, retina, RPE, and cornea [3,4]. Although the precise role of ET-1 in ocular tissues is not completely understood, studies have shown increased levels of endothelin-1 (ET-1) in both the aqueous humor and plasma of patients and in animal models of glaucoma [5-8]. In animal studies, ocular administration of ET-1 has been shown to produce ischemic damage, leading to RGC axon injury and optic nerve degeneration [9-11]. Intravitreal administration of 2 n mole of ET-1 produced a decline in fast anterograde axonal transport associated with the transportation of mitochondrial subcomponents in RGCs [12]. Another study, using a lower concentration of ET-1 (1 nM), showed a reversible disruption of the optic nerve fast axonal transport [13]. The relevance of these findings stems from studies that have reported mitochondrial dysfunction as a risk factor for RGC neurodegeneration and development of glaucoma [14-16]. One study found that mitochondrial activity and integrity were preserved by oral administration of nicotinamide (vitamin B$_3$), which protected RGCs from glaucomatous damage [17].

Some debate still persists regarding the initial site of damage; however, clinical examinations and experimental findings have provided evidence for the optic nerve head as the first site of damage in glaucomatous degeneration [18-20] and that activation of optic nerve head astrocytes also contributes to axonal degeneration [21-23]. ET-1 has been shown to induce the proliferation of optic nerve head astrocytes, and this could be inhibited by blocking either the ET$_A$ receptor or the ET$_B$ receptor [24]. In addition, both the ET$_A$ and ET$_B$ receptors have been shown to contribute to the ET-1 mediated production and release of collagen I and collagen VI from cultured human lamina cribrosa cells [25]. Multiple studies have shown a significant contribution of the ET$_B$ receptor to glaucomatous degeneration [26-29]. An increase in the ET$_A$ receptor leads to increased expression of the ET$_B$ receptor, which has been shown to contribute to glaucomatous degeneration following IOP elevation [30].

ET-1 has the ability to generate an array of degenerative effects observed in primary open angle glaucoma (POAG), and studies have found in an inheritable (DBA/2J) mouse model of glaucoma that the endothelin system is activated early in the disease and occurs before any noticeable morphological damage [31]. These findings highlight the likelihood of the endothelin system being a potential target for neuroprotective intervention. Further evidence for this was demonstrated by the prevention of glaucomatous damage through the administration of dual endothelin receptor antagonists: bosentan and macitentan [31,32]. While these previous studies with dual endothelin receptor antagonists showed significant protection, administration of the drugs had been started before the clinical manifestation (IOP elevation) of the disease. The aim of the present study was to determine whether initiation of macitentan treatment after the induction of IOP elevation can still promote the neuroprotection of RGCs.

**METHODS**

**Animals:** Adult male and female retired breeder Brown Norway rats (8–11 months old; Charles River Laboratories, Wilmington, MA) were used for all experiments in this study. Rats were housed individually in each cage under constant dim lighting (90 lux) to minimize diurnal variations in IOP. All procedures involving animals were performed in accordance with the ARVO resolution for the Use of Animals in Ophthalmic and Vision Research and approved by the University of North Texas Health Science Center (UNTHSC) Institutional Animal Care and Use Committee (IACUC).

**IOP measurements:** IOP measurements were taken on conscious rats using a TonoLab tonometer (iCare, Finland), 2–3 times per week, between 8 and 11 a.m., for the duration of the study. Rats were handled gently but firmly while IOP measurements were performed. For each eye, ten IOP readings were recorded and averaged to give each IOP measurement. Total IOP exposure was calculated by computing the integral product of IOP elevation and the number of days for which it was maintained (expressed as mmHg-days). Naïve rats were used as controls.

**Induction of ocular hypertension using Morrison’s method:** The Morrison model of ocular hypertension was performed as previously described [30,33]. Rats were anesthetized by intraperitoneal injection (100 µL/100 g body wt) of a ketamine (VEDCO, Saint Joseph, MO) / xylazine (VEDCO, Saint Joseph, MO) / acepromazine (Lloyd Laboratories, Walnut, CA) cocktail with final concentrations of 55.6 mg/ml / 5.6 mg/ml / 11.1 mg/ml, respectively. A small incision was made in the conjunctiva to expose the episcleral veins. Approximately 50 µl of hypertonic saline (1.8 M NaCl) solution was then injected via a glass micropipette (TIP01TW1F, WPI, Sarasota, FL) at a flow rate of 309 µL/min for 10 s into one eye (left eye) of each rat. Triple antibiotic ointment was applied to the surgical area to prevent any infections. Induction of ocular hypertension typically occurs 7–10 days following Morrison surgery.

Macitentan treatment (5 mg/kg body wt) was started following the induction of IOP elevation and performed 3 days per week for 4 weeks. Since macitentan has higher affinity, longer receptor occupancy time, and longer half-life than
other endothelin receptor antagonists, including bosentan and ambrisentan, we decided to use a dose of 5 mg/kg body wt. To ensure proper consumption, macitentan was administered orally by mixing the drug (in powder form) into DietGel® Recovery (Clear H2O, Westbrook, ME). Rats were individually housed for the experiments and carefully observed on a daily basis. Gels containing macitentan as well as gels without macitentan (untreated controls) were administered to the rats and monitored for complete consumption of the gels.

**Pattern electroretinography (PERG):** PERG was performed following IOP elevation in rats treated with either macitentan (5mg/kg body wt in dietary gels) or dietary gels alone (untreated). Rats were anesthetized by intraperitoneal injection (100 μl/100 g body wt) of a ketamine (VEDCO, Saint Joseph, MO) / xylazine (VEDCO, Saint Joseph, MO) / acepromazine (Lloyd Laboratories, Walnut, CA) cocktail with final concentrations of 55.6 mg/ml / 5.6 mg/ml / 11.1 mg/ml, respectively. Pattern ERG analysis was performed using the Jörvec instrument (Intelligent Hearing Systems, Miami, FL). Rats were placed onto a heated platform that was adapted for rats and allowed unobstructed views of the visual stimulus monitors, which were kept 10 cm apart. Rats were maintained at 37 °C for the duration of the procedure. Reference and ground electrodes were placed subcutaneously in the scalp and base of the tail, respectively. Saline eye drops were applied to both eyes to prevent drying, and corneal electrodes were positioned at the lower fornix, in contact with the eye globe. LED monitors were used to display contrast-reversing horizontal black and white bars at a spatial frequency of 0.095 cycles/degree and luminance of 500 cd/m². Pattern ERG waveforms from both eyes were simultaneously recorded, with each run consisting of 372 sweeps (on-off). Subsequently, three waveforms were averaged and processed using PERG software to identify and calculate the peak amplitude and latency. The latency was determined from the starting time of recording, with no pre-stimulus recording time.

The PERG signal is an aggregate of the activity of several RGCs and represents RGC function, as well as connectivity in the inner retinal circuitry that feeds to the RGCs. The integration of this signal requires time that is reflected by the latency following the onset of the stimulus [34]. Thus, the PERG machine computes the latency based upon the time required to integrate the signal from the inner retinal circuitry, which is reflected in the elapsed time leading to peak P1 of the PERG waveform [34].

**Retinal flat mounts and immunostaining:** Rats were euthanized by intraperitoneal injection of pentobarbital (120 mg/kg body wt.). Following enucleation, the eyes were briefly washed in ice-cold 1X PBS (0.135 M Sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate dibasic, 1.4 mM potassium phosphate monobasic) prepared by diluting 10X PBS (P0496, Teknova, Hollister, CA). An ophthalmic microsurgical knife (MVR 20G, 160,710, Cambrian-Medical, Cedar, UT) was used to create a circumferential incision just posterior to the limbus, and the eyes were then incubated in 4% paraformaldehyde for 30 min at room temperature. Small vannas scissors were then used to cut circumferentially around the globe until the anterior segment, including the lens, was completely removed. The posterior segments were then placed in 4% paraformaldehyde overnight at 4 °C, followed by three 10 min washes in 1 ml PBS. The posterior segments were then immersed in permeabilization buffer (0.1% sodium citrate and 0.2% Triton-X-100 in PBS) for 10 min, followed by three 10 min washes in 1 ml PBS. The posterior segments were then immersed in blocking buffer (5% normal donkey serum and 5% bovine serum albumin [BSA] in PBS) and incubated overnight at 4 °C. The blocking buffer was removed, and the posterior segments were given three 10 min washes in 1 ml PBS, followed by immersion and incubation in primary antibody goat anti-Brn3a (sc-31894, Santa Cruz; diluted 1:500 in PBS containing 1% BSA) for 72 h at 4 °C. The posterior segments were given three 10 min washes in 1 ml PBS and then placed in the secondary antibody solution of donkey anti-alexa 488 (Invitrogen; diluted 1:1000 dilution in PBS containing 1% BSA). The posterior segments were incubated overnight at 4 °C, followed by three 10 min washes in 1 ml PBS. The retinas were then carefully separated from the retinal pigment epithelium and completely removed from the posterior segment. Small surgical scissors were used to make 4 cuts around the retina to allow it to be flattened. The retinas were then placed onto glass slides and mounted using Prolong® Gold anti-fade reagent (P36935, Invitrogen).

**Flat mount imaging and RGC counts:** All images were taken using either a Zeiss LSM 510 META confocal microscope or a Cytation5 cell imaging multi-mode reader (Agilent Technologies). For this study, images were taken at two different eccentricities, located at one-third and two-thirds of the distance from the optic nerve head to periphery of the retina. For each retina, two pictures were taken at each eccentricity in the superior, inferior, nasal, and temporal quadrants. Images were randomized, and the RGC counts were performed manually by a masked observer using ImageJ software and calculated as cells/mm².

**Paraphenylenediamine (PPD) staining of the optic nerve for the assessment of axonal damage:** Degeneration of axons was evaluated using PPD staining, which stains the myelin around the axons. Briefly, rats with IOP elevation were maintained...
for four weeks with or without oral feeding of macitentan in dietary gel. After euthanasia, the rat eyes were enucleated and the optic nerves were excised posterior to the globe. The optic nerves were then immediately fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Before dehydration, the optic nerves were transferred to 2% osmium tetroxide in PBS for 1 h and embedded in Epon. Optic nerve cross sections were obtained with a ultramicrotome and stained with 1% PPD. Images of the stained sections were taken at a magnification of 100× with a Zeiss LSM 510 META confocal microscope equipped with an oil immersion lens. Images were taken at a few points in the center, as well as in the peripheral region, of each quadrant of every optic nerve section. The analysis of the axon counts was performed using ImageJ software (National Institutes of Health). Briefly, after adjusting the brightness/contrast, the sharpest image of the optic nerve sections from the Z stack images was selected using the stack option in the software. The image was then converted into 8 bit format, and the threshold was adjusted. The counts were then analyzed based on the particle size parameters, including size and circularity. The total axonal counts were the average of the axonal counts from the central and peripheral regions of each optic nerve. Based on the statistical analysis of the axon counts, the neuroprotective/neurodegenerative effects were analyzed further.

**Statistical analysis:** Statistical analysis was performed using Sigmaplot 12.5 (Systat Software Inc.) and GraphPad Prism 7 (GraphPad Software, La Jolla, CA). The data between multiple groups were compared using one-way ANOVA followed by Tukey’s multiple comparison test, and comparisons between two groups were made using an unpaired Student t test. Values of p<0.05 were considered statistically significant.

**RESULTS**

**Effect of macitentan on IOP elevation:** To determine the effectiveness of macitentan in preventing RGC death during IOP elevation, Brown Norway rats were subjected to IOP elevation in one eye, and dietary administration of macitentan was started following the induction of IOP elevation. An additional group of naïve rats that did not undergo any surgical manipulation or treatment was also included in the experiment. One week following the surgery, at every studied time point, a significant elevation of IOP was noted in the untreated rats compared to naïve rats. Following IOP elevation the rats were fed 5 mg/kg body wt macitentan (in Diet gel recovery packs) three times per week for a total of 4 weeks. No significant difference was found in the average IOP exposures between untreated rats (130 mmHg-days, n=7) and macitentan-treated rats (132 mmHg-days, n=8; Figure 1), indicating no effect on IOP by the macitentan treatment. At day 7 following IOP elevation, a decrease in IOP became apparent in the macitentan-treated IOP-elevated rats compared to the untreated IOP-elevated rats; however, the difference was not statistically significant.

**Effect of Macitentan on RGC loss following IOP elevation:** Since we did not find any effect of macitentan on IOP, we determined whether macitentan could protect RGC function during IOP elevation. We assessed RGC function by performing PERG on adult retired breeder Brown Norway rats that were either untreated or were treated with macitentan during IOP elevation. IOP was elevated in rats using Morrison’s method and maintained for 2 to 4 weeks. After the elevation of IOP, macitentan (5 mg/kg body wt/day) was administered three days per week for 4 weeks. After 2 weeks of IOP elevation, PERGs were recorded. No difference was found in the recorded latency times between untreated and macitentan-treated rats (IOP-elevated untreated: 89.13±3.92 ms; IOP-elevated macitentan-treated: 89.82±6.4 ms; naïve 91.1±1.76 ms). The PERG recordings showed a significant reduction in peak amplitude in the IOP-elevated untreated rats compared to naïve rats (IOP-elevated untreated: 5.08±0.62μV, * p=0.012 (n=6), naïve 8.9±0.71 μV (n=10), one-way ANOVA), whereas the macitentan-treated rats showed a protective trend (not statistically significant) against a decline in peak amplitude when compared to the IOP-elevated untreated rats (IOP-elevated macitentan: 7.81±1.08μV, p=0.11, n=7 one-way ANOVA) (Figure 2A). After 4 weeks of IOP elevation, the PERGs were again recorded. No difference was found in the recorded latency times between untreated and macitentan-treated rats (IOP-elevated untreated: 92.39±7.19 ms; IOP-elevated macitentan-treated: 94.33±6.35 ms; naïve 91.26±1.9 ms). By contrast, the PERGs of macitentan-fed rats (5 mg/kg body wt) continued to show a trend toward preservation (not statistically significant) in the peak amplitude when compared to untreated rats (IOP-elevated untreated: 5.12±0.55 μV; IOP-elevated macitentan-treated: 7.07±0.71 μV; naïve 8.1±0.54 μV), indicating a potential of macitentan to have protective effects against a decline in RGC function (Figure 2B,C).

**Effect of macitentan on RGC loss following IOP elevation:** The extent of RGC loss was determined by preparing retinal flat mounts and immunostaining them with the RGC-selective marker Brn3a. Fluorescence images were taken at the peripheral and mid-peripheral eccentricities located at a distance of two-thirds and one-third of the width of the retina from the optic nerve head, respectively. The RGC counts in naïve, IOP-elevated untreated, and IOP-elevated
macitentan-treated rats (Figure 3A) were performed by a masked observer. The naïve animals had an RGC count of 1004±102 cells/mm$^2$ (n=6), whereas rats with IOP elevation for 4 weeks showed a significant reduction in RGC counts of 716±19 cells/mm$^2$ at the peripheral eccentricity (p=0.049, n=7 one-way ANOVA). By contrast, the macitentan-treated rats showed an RGC count of 998±84 cells/mm$^2$ in the peripheral eccentricity, indicating a significant protection against RGC loss compared with the IOP-elevated untreated rats (p=0.036, n=8, one-way ANOVA; Figure 3B).

In the mid-peripheral eccentricity, comparison of the naïve animals, which had an RGC count of 1861±51.87 cells/mm$^2$ (n=6), indicated a significant loss in the IOP-elevated untreated rats, which showed an RGC count of 1218±106 cells/mm$^2$ (p=0.0022, n=7, one-way ANOVA). This RGC loss was significantly attenuated in IOP-elevated macitentan-treated rats, which had an RGC count of 1724±128 cells/mm$^2$ (p=0.0086, n=8, one-way ANOVA) (Figure 3C). Comparison of the average counts of peripheral eccentricity plus mid-peripheral eccentricity to those of the naïve controls, which had an RGC count of 1432±71 cells/mm$^2$, indicated a significant loss of RGCs in the IOP-elevated untreated rats, which had an RGC count of 967±56 cells/mm$^2$ (p=0.0024, n=7, one-way ANOVA). By contrast, the IOP-elevated macitentan-treated rats had an RGC count of 1361±95 cells/mm$^2$, suggesting significant protection against IOP-mediated damage to the RGCs (p=0.0053, n=8, one-way ANOVA) (Figure 3D). No significant difference was detected between the naïve animal RGC counts and the macitentan-treated RGC counts (p=0.99, peripheral eccentricity; p=0.65, mid-peripheral eccentricity; p=0.81, total RGC counts).

Effect of macitentan on axonal degeneration following IOP elevation: We also assessed the axonal integrity of the animals treated with or without macitentan following IOP elevation. After four weeks of IOP elevation, the rats were euthanized and optic nerve sections stained with PPD were analyzed (Figure 4A). When compared with naïve animals, IOP-elevated untreated rats showed significant disruption of the optic nerve axonal bundles (p=0.02, n=7; Figure 4B), intense staining of myelin, and glial scar formation (indicated by pink arrows; Figure 4A). However, rats treated with macitentan following IOP elevation showed significantly higher axon counts (p=0.04, n=7; Figure 4B), compared to the untreated IOP-elevated rats. Comparison of the optic nerves of macitentan-treated animals with those of naïve animals did not reveal any significant difference in axon counts.
Glial scarring was less prominent in macitentan-treated rats than in the untreated rats following IOP elevation (Figure 4A). Several collapsed axons (yellow arrow heads in Figure 4A) were detected in the IOP-elevated untreated rats but were minimally seen in the IOP-elevated macitentan-treated rats. These findings indicate that oral consumption of macitentan could have protective effects on the axons of the optic nerve, in addition to preventing RGC loss.

DISCUSSION

Although current IOP-lowering treatments have proven effective for both ocular hypertensive and normotensive patients, the extent to which a patient’s IOP can be lowered is limited. In the clinical setting by adding another IOP lowering medication, the IOP in most patients cannot be lowered beyond 20% of what is obtained using a combination of therapies targeting aqueous humor formation and outflow [35]. Thus, the development of additional IOP-independent strategies...
that could prevent neurodegeneration of RGCs and promote neuroprotection would be advantageous.

The endothelin system is composed of three distinct 21-amino acid peptides, endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3), which are encoded by three separate genes in the human genome [36]. ET-1, which was first isolated from porcine aortic endothelial cells and described as a potent vasoactive peptide [37], has been shown to play a significant role in the maintenance of vascular tone and homeostasis [38]. Endothelin peptides bind to either of two distinct G-protein coupled receptors (GPCRs), endothelin A (ET\textsubscript{A}) receptor and endothelin B (ET\textsubscript{B}) receptor [39]. Studies have revealed that all three peptides have approximately equal affinities for the ET\textsubscript{B} receptor; however, ET-3 has much lower affinity for the ET\textsubscript{A} receptor and could be considered a selective agonist of the ET\textsubscript{B} receptor [40,41]. Since its original discovery, the endothelin system has been found to be expressed in several tissues in the body, including the renal system [42-44], brain [45,46], and eye [47].

Figure 3. Macitentan (5 mg/kg body wt) enhances retinal ganglion cells (RGCs) survival in rats during IOP elevation for 4 weeks. Representative images of Brn3a staining of RGCs in retinal flat mounts from rats that were either untreated or treated with macitentan, 3 days per week for 4 weeks following IOP elevation (A). Quantitation of Brn3a-positive RGCs in naïve eyes, untreated IOP-elevated eyes, and macitentan-treated IOP-elevated eyes of Brown Norway rats in the peripheral (B) and mid-peripheral (C) eccentricities, located at two-thirds and one-third of the width of the retina from the optic nerve head, respectively, and total RGC counts (D). Bars represent mean RGC count ± SEM * p<0.05, **p<0.005 indicates statistical significance, one-way ANOVA followed by Tukey’s multiple comparison test. Scale bar: 100 μm, n=7 (3 male rats and 4 female rats) for untreated rats, n=8 (4 male rats and 4 female rats) for macitentan-treated rats, and n=6 (3 male rats and 3 female rats) for naïve rats.
Several studies point to the endothelin system as an important contributor to glaucomatous neurodegeneration [47-52]. Both intravitreal and peribulbar administrations of ET-1 have been shown to produce RGC loss through apoptotic mechanisms and damage to optic nerve axons [11,27,53,54]. Several prominent cellular events that occur in the optic nerve head, as well as in the retina, could contribute to the neurodegenerative effects occurring during the progression of glaucoma prior to RGC death [20,55,56]. These events include the disruption of RGC axonal transport [57-62], activation and redistribution of optic nerve head astrocytes [63-66], and changes in the optic nerve head extracellular matrix milieu [67-69]. Many studies have shown that the endothelin system, specifically ET-1, contributes to key glaucomatous events, including a decrease in axonal transport [12,13,70,71] and proliferation of optic nerve head astrocytes [24,72,73]. In addition, Rao et al. (2008) demonstrated that cultured lamina cribrosa cells treated with ET-1 showed a concentration-dependent increase in the production and secretion of both collagen I and collagen VI, indicating a possible ET-1 mediated extracellular deposition of collagens by lamina cribrosa cells [25]. However, how these endothelin-mediated changes in collagen expression and release could affect the anatomy and ultrastructure of the lamina cribrosa in vivo is unclear. Nevertheless, since endothelins act through either the $\text{ET}_A$ and $\text{ET}_B$ receptor (or both) to mediate these damaging effects, the use an $\text{ET}_A$/ET$_B$ dual receptor antagonist would be prudent to block the neurodegenerative effects in glaucoma.

Macitentan was approved by the FDA in 2013 for the treatment of pulmonary arterial hypertension, and its safety in humans has already been established. In the current study, we have demonstrate the ability of macitentan (5 mg/kg bodyweight) to promote neuroprotection of RGCs following IOP elevation, without lowering IOP, in the Morrison’s rat model of glaucoma. Moreover, in this study, we started the macitentan treatment after the onset of IOP elevation, thereby simulating the clinical scenario where human patients start their treatment after their diagnosis of glaucoma. Previous

Figure 4. Integrity of optic nerve axons following intraocular pressure (IOP) elevation with or without macitentan treatment compared to naive animals. Following 4 weeks of IOP elevation, rats were euthanized, and optic nerve sections obtained were subjected to PPD staining to assess optic nerve degeneration. Axonal degeneration accompanied by gliosis and glial scar were observed in IOP-elevated untreated rats compared to naive eyes. IOP-elevated macitentan-treated rats show significant protection of their axons, compared to those of untreated rats. Pink arrows point to glial scarring, which was found mainly in the retinas from IOP-elevated untreated rats. Dark spots (yellow arrowheads) indicate the collapsed axons in the untreated IOP-elevated rats (A). The mean counts of healthy axons were significantly reduced in untreated IOP-elevated animals compared to naive animals (*p=0.022, n=7) and a significant protection was seen in macitentan-treated rats with IOP elevation (*p=0.013, n=7; B) compared to untreated IOP-elevated rats (one-way ANOVA followed by Tukey’s multiple comparisons test). Scale bar: 20 μm. n=7 (3 male rats and 4 female rats) for untreated rats, n=7 (3 male rats and 4 female rats) for macitentan-treated rats and n=10 (5 male rats and 5 female rats) for naive rats.
work by Howell et al. (2011) demonstrated that bosentan treatment (100 mg/kg) had neuroprotective effects in the DBA/2J mouse model of glaucoma [31]. In a subsequent publication, Howell et al. (2014) treated DBA/2J mice with dietary macitentan (30 mg/kg) and found significant neuroprotective effects in the RGC axons [32]. Although the dose of macitentan used in our study is lower than that used by Howell et al. (2014), it is still much higher than that used in patients with pulmonary hypertension (Osumit, a brand name of macitentan, is administered at a dose of 10 mg in patients). Macitentan has greater affinity, better efficacy, higher receptor occupancy time, and longer duration of action than several endothelin receptor antagonists, including bosentan and ambrisentan [74,75]. The half-life of its active metabolite ranges from 40 to 65 h [75], and this was the reason for administering macitentan 3 days per week in this rat study.

All current clinical therapies only aim to lower IOP; therefore, an IOP-independent therapy could be a viable option for primary open-angle glaucoma patients, especially those who continue to show glaucomatous progression. In addition to finding that macitentan promoted RGC survival, we also found a trend toward maintenance of the PERG amplitude by macitentan, indicative of maintenance of RGC function. Chou et al. (2013) demonstrated that retrograde signaling is required for the PERG response [76], further suggesting that signal transduction between the retina and the brain was preserved in macitentan-fed rats in the current study. Nonetheless, the present study has some limitations, since Sandalon and Ofri (2012) have shown a significant decline in PERG in aged Lewis rats [77]. This may account for the variability in PERG amplitude in our retired breeder Brown Norway rats and the lack of statistical significance in the protection seen with macitentan against the decline in PERG amplitude during IOP elevation.

Increased IOP could produce direct damage to the axons through mechanical effects. In addition, IOP elevation could indirectly damage axons of RGCs by inducing astrocytes to release ET-1, which could act upon endothelin receptors in RGCs. Studies have shown an increased mRNA expression of ET-2, as well as of the ET<sub>B</sub> receptor, in the retina as early as 1 day following IOP elevation in rats [78], suggesting that elevated IOP could alter expression of endothelins and their receptors at an early stage when damage to axons has not yet commenced. Macitentan treatment does not lower IOP, so the mechanical effects would persist, but the treatment with macitentan, as an endothelin antagonist, would prevent endothelin-mediated damage. This indicates that while mechanical effects are contributors to the damage, IOP-mediated increases in endothelins could be a significant cause of neurodegeneration. This hypothesis is supported by our previous publication that demonstrated significant protection of RGCs and their axons in ET<sub>B</sub> receptor-deficient rats [28]. Macitentan treatment could possibly increase endothelin levels [79]; however, due to the high affinity and efficacy of the receptor blockade, this would shield against continued stimulation of the endothelin receptors. While the mechanical effects of IOP elevation would continue to occur, endothelin antagonists would significantly protect against both axonal injury (Figure 4) and RGC loss (Figure 3). This suggests that IOP-mediated damage is manifested to a large extent through an activation of the endothelin signaling pathway.

We have assessed the effect of macitentan in the posterior segment of the eye; however, this drug could also possibly have some beneficial effects on aqueous humor dynamics in the anterior segment of the eye (this would not be obvious in the Morrison’s model due to irreversible sclerosis of the trabecular meshwork). For instance, IOP elevation is understood to result from cellular alterations within the trabecular meshwork (TM), as well as from changes in the extracellular matrix. The alterations in the TM reduce the outflow of aqueous humor from the anterior segment of the eye, thereby resulting in an increase in IOP [80]. The aqueous humor of patients with glaucoma shows increased levels of TGF-β [81-86], which has been shown to contribute to the pathogenesis of POAG [87]. Increased levels of ET-1 have been shown following 24 h treatment with TGF-β in the TM cells [88]. Interestingly, ET-1 has been found to contribute to TGF-β-induced fibrosis in skin and lung tissues, but this effect was ameliorated by bosentan [89,90]. Similar to our findings, macitentan was unable to prevent IOP elevation in the DBA/2J mouse model, however, the point to be noted is that the IOP increase in this model is due to dispersion of iris pigment, which mechanically impedes aqueous humor outflow from the TM [32]. While studies that focus on the relationship between TGF-β and ET-1 in the eye are limited, a worthwhile pursuit would be to investigate the potential of a dual endothelin receptor antagonist, like macitentan, to prevent increased ECM deposition within the TM and possibly the LC region of the optic nerve head.

In summary, oral administration of the macitentan, a dual ET<sub>A</sub>/ET<sub>B</sub> receptor antagonist, had neuroprotective effects on RGC survival when administered orally at a dose of 5 mg/kg body wt in ocular hypertensive rats. Macitentan also had some beneficial effects in maintaining RGC function, as determined by PERG analysis. The study findings raise exciting possibilities for the use of macitentan as an oral formulation to promote neuroprotection in glaucoma patients. This would be particularly helpful for elderly patients and
those with motor deficits leading to non-compliance issues stemming from difficulties in instilling eye drops. Future studies will examine the cellular mechanisms and signaling pathways that contribute to macitentan-mediated neuroprotection in glaucoma.

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