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NCX 470 Exerts Retinal Cell Protection and Enhances Ophthalmic Artery Blood Flow After Ischemia/Reperfusion Injury of Optic Nerve Head and Retina

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Purpose: The purpose of this study was to assess the retinal protective activity and ocular hemodynamics after NCX 470 (0.1%) compared to bimatoprost administered as the US Food and Drug Administration (FDA)-approved drug (Lumigan — 0.01% ophthalmic solution, LUM) and at an equimolar dose (0.072%, BIM) to that released by NCX 470.

Methods: Endothelin-1 (ET-1) induced ischemia/reperfusion injury model in rabbits was used. ET-1 was injected nearby the optic nerve head (ONH) twice/week for 6 weeks. Starting on week 3, the animals received vehicle (VEH), NCX 470, LUM, or BIM (30 µL/eye, twice daily, 6 days/week) until the end of ET-1 treatment. Intraocular pressure (IOP), ophthalmic artery resistive index (OA-RI), and electroretinogram (ERG) data were collected prior to dosing and at different time points postdosing. Reduced glutathione, 8-Hydroxy 2-deoxyguanosine, and Caspase-3 were determined in the retina of treated eyes. DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining.

Results: ET-1 increased IOP ($VEH_{IOP\_Baseline} = 20.5 \pm 0.8$ and $VEH_{IOP\_Week6} = 24.8 \pm 0.3$ mmHg) and OA-RI ($VEH_{OA\text{-}RI\_Baseline} = 0.36 \pm 0.02$ and $VEH_{OA\text{-}RI\_Week6} = 0.55 \pm 0.01$) and reduced rod/cone responses over time. Oxidative stress, inflammation, and apoptotic markers increased in ET-1-treated eyes. NCX 470 prevented IOP ($NCX\ 470_{IOP\_Week6} = 18.1 \pm 0.6$ mmHg) and OA-RI changes ($NCX\ 470_{OA\text{-}RI\_Week6} = 0.33 \pm 0.01$) and restored ERG amplitude leaving unaltered the respective latency; these effects were only partially demonstrated by LUM or BIM. Additionally, NCX 470 reduced oxidative stress, inflammation, and apoptosis in the retinas of treated eyes. BIM and LUM were numerically less effective on these parameters.

Conclusions: NCX 470 repeated ocular dosing ameliorates ocular hemodynamics and retinal cell dysfunction caused by ischemia/reperfusion *via* nitric oxide- and bimatoprost-mediated mechanisms.

Translational Relevance: If confirmed in clinical setting our data may open new therapeutic opportunities to reduce visual field loss in glaucoma.

Introduction

Glaucoma comprises a group of eye diseases which can lead to irreversible blindness.[1] Elevated intraocular pressure (IOP) is the primary risk factor for glaucoma and lowering IOP is the only therapy available to slow disease progression.[2] The reduction of IOP, albeit important, is not sufficient to effectively and completely counteract the retinal and optic nerve

progressive degeneration observed in these patients.[1] Among others, ischemia due to vasospastic and/or occlusive ocular circulatory disturbances followed by tissue reperfusion are regarded as significant events in the progression of the glaucomatous neuropathy.[3] Ischemia/reperfusion leads to the production of reactive oxygen species (ROS) and several studies suggest that ROS play a major role in the genesis of post-ischemic retinopathy as well as of glaucoma.[3,4] Indeed, increased levels of oxidative stress markers, such as superoxide dismutase, were recently shown to positively correlate with the onset and progression of glaucoma.[5]

Cytokines and chemokines are proteins secreted by immune cells and exert a central role in the progression of glaucoma and ischemic insult.[6,7] Among others, interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNFα), and interleukin-6 (IL-6) act as primary initiators of inflammation following infection or tissue damage in the eyes as in other organs.

Prostaglandin F2 alpha receptor (FP) agonists, including latanoprost, bimatoprost, travoprost, tafluprost, and unoprostone isopropyl, are potent and effective US Food and Drug Administration (FDA)-approved IOP-lowering prodrugs for the treatment of ocular hypertension and glaucoma.[8] These drugs mostly act *via* an increase of aqueous humor (AH) drainage from the uveoscleral pathway upon the release of the respective free acids. As with other commonly used IOP-lowering agents, despite their satisfactory IOP control and safety profile, these compounds seem to only partially affect ocular blood flow with tafluprost being the most effective.[9] Furthermore, FP agonists seem to lack any direct meaningful activity on the inflammation sometimes observed in glaucomatous eyes and are not reported to have an effect on retina cell survival, as has been reported for other IOP-lowering agents, such as the alpha–2 adrenergic agonist, brimonidine.[10]

Nitric oxide (NO) and its intracellular second messenger cyclic guanosine monophosphate (cGMP) lowers IOP mainly *via* an increase of AH drainage through the Trabecular meshwork/Schlemm's canal (TM/SC) pathway, known as the conventional outflow facility.[11] Moreover, it has been shown that, independently from its effect on IOP, physiologic concentrations of NO may protect retinal cells from irreversible functional damage.[12] In a rat model of retinal ischemia, endothelial NO synthase (eNOS) messenger RNA (mRNA) decreased and treatment with the NO precursor, L-arginine, increased the recovery of retinal function[13] thereby demonstrating that NO plays an important role in protecting the retina from ischemic damage. Likewise, low nanomolar concentration of NO has also been shown to inhibit pro-inflammatory cytokines,[7] whereas NO depletion increases ROS generation and pro-inflammatory biomarkers.[14]

Recently, an entirely new class of drugs has been proposed for the treatment of ocular hypertension and glaucoma, namely: NO-donating FP agonists that include Vyzulta (latanoprostene bunod 0.024% ophthalmic solution), which is marketed in many countries including the United States,[8] and NCX 470 (a novel dual acting NO-donating bimatoprost – 0.1% ophthalmic solution) which recently demonstrated non-inferiority for IOP lowering as compared with latanoprost 0.005% ophthalmic solution in a pivotal phase III clinical trial (https://clinicaltrials.gov/ct2/show/NCT04445519). These latter compounds act as IOP-lowering agents by simultaneously activating two independent mechanisms: (1) FP receptor-mediated increase in uveoscleral outflow, and (2) cGMP-dependent activation of TM/SC conventional outflow facility.[11] Interestingly, these dual acting drugs have also been shown to affect ocular blood flow in patients[15,16] and optic nerve head oxygen saturation in healthy volunteers[17] as well as in a model of endothelin-1 (ET-1)-induced ischemia/reperfusion in rabbits where repeated ocular dosing of NCX 470 reversed the ocular hemodynamic changes as well as the impairments in retinal function observed after ET-1 administration.[18]

Here, we used the same ET-1-induced ischemia/reperfusion model to compare the effect of NCX 470 to that of bimatoprost administered as the FDA-approved drug (Lumigan, 0.01% bimatoprost ophthalmic solution) or at a dose (0.072%) equimolar to that released by 0.1% NCX 470. Furthermore, in an attempt to understand the cellular and molecular mechanism/s involved, we investigated changes in various biochemical markers potentially affecting retinal cell physiology.

Methods

Animals

Twenty-four (24) adult New Zealand White (NZW) male rabbits weighting 2.0 to 2.5 kg were used for this study. The experimental procedures were conforming to those of the Association for Research in Vision and Ophthalmology Resolution on the use of the animals and were conducted in accordance with the Italian regulation on the protection of animals used for experimental and other scientific purpose (Italian Legislative Decree 26, March 13, 2014) as well as with the EU Regulations (Council Directive of the

European Community 2010/63/EU). The study was approved by the local animal care committee of the University of Florence (Italy) and followed Ministry of Health recommendations (authorization n. 110/2021-PR). Every effort was made to minimize animal suffering and reduce the number of animals used.

The animals were kept in individual cages, food and water were provided ad libitum. The animals were identified with a tattoo on the ear, numbered consecutively, and housed on a 12 to 12 hour light/dark cycle in a temperature-controlled room (22–23°C).

Experimental Animal Model and Test Item Dosing

The ET-1-induced ischemia/reperfusion model previously described[18] was used.

Vehicle (Kolliphor HS15, benzalkonium chloride, boric acid, EDTA, sorbitol, dibasic sodium phosphate, pH = 6.0, 30 µL/eye, twice daily [BID]), NCX 470 (0.1% dissolved in vehicle, 30 µL/eye, BID), Lumigan (bimatoprost 0.01% ophthalmic solution, 30 µL/eye, BID-LUM) or bimatoprost (0.072% dissolved in vehicle, 30 µL/eye, BID-BIM) were administered as eye drops with the following schedule: on Monday to Friday at approximately 10:00 AM and 4:00 PM, on Saturday at approximately 10:00 AM only, and on Sunday the animals are kept treatment-free. All treatments were masked to the investigator starting on week 3 until the end of the experiment (week 6).

Functional Measurements

Out of the 24 animals used in this study, 12 animals (left and right eyes) were randomly assigned to vehicle (VEH, $n = 7$ eyes), NCX 470 (0.1%, $n = 7$ eyes), (LUM, $n = 4$ eyes or BIM, $n = 6$ eyes) and tested for IOP and Ophthalmic Artery Resistive Index (OA-RI). The remaining 12 animals received the same treatment as those in the previous set and assigned for electroretinogram (ERG) determinations.

Intraocular Pressure

The IOP was measured at the indicated time points using a pneumatonometer (Model 30 Classic; Reichert, Depew, NY, USA) on Monday (after 36 hours free of treatment) before the AM dosing of test items. One drop of oxybuprocaine hydrochloride (4 mg/mL) was instilled immediately before each set of pressure measurements. Values reported for each time point are the mean of 2 consecutive measurements taken 1 minute apart. IOP changes from baseline were calculated as follows: IOP_{Tx}-IOP_{T0} where IOP_{Tx} and IOP_{T0} are the IOPs at the time of interest and at baseline, respectively.[18]

Ophthalmic Artery Resistive Index

Measurements were taken using an Echo Color Doppler (Philips Ultrasound HD7XE; Philips, Milan, Italy) with the S12–4 ultrasound transducer and a frequency of 6.0 MHz, before ET-1 treatment (baseline, time 0), and weekly thereafter on Mondays until the end of the study. Pourcelot resistive index for ophthalmic artery (OA-RI) was calculated using the following formula: (OA-PSV–OA-EDV)/OA-PSV where OA-PSV and OA-EDV are the ophthalmic artery peak systolic velocity and ophthalmic artery end diastolic velocity, respectively.[18]

Electroretinogram

ERG signals were recorded using Retimax (CSO, Florence, Italy) and according to the current International Society for Clinical Electrophysiology of Vision (ISCEV) indications, as previously reported.[18] Measurements were taken before ET-1 first dose (baseline, time 0), then at the end of week 2 (before VEH, NCX 470, LUM or BIM first day-first dose), and at the end of week 6 (36 hours after VEH, NCX 470, LUM, or BIM last day-last dose).

Biochemical Measurements

The retinas were collected from all animals at the end of the study as well as from naïve animals for comparative assessments. Animals were euthanized with an overdose of anesthetic (pentothal sodium 0.15 g/kg, intravenous bolus) and tissues dissected and frozen until further processing. Reduced glutathione (GSH) and 8-Hydroxy-2-deoxyguanosine (8-OH2dG) were used as representative markers of oxidative stress. Caspase-3 activity and protein expression were used as representative markers of cell apoptosis.

GSH Levels

The retinas were homogenized in 2 mL of 10 mM phosphate buffer, pH 7.4 and then centrifuged at 10,000 g for 30 minutes at 4°C, then a fixed volume of supernatant was diluted to 2 mL with phosphate buffer containing ethylene diamine tetra-acetic acid (EDTA). The resulting solution was then processed to determine the levels of GSH, as previously reported.[18]

8-OH2dG Levels

The isolation of DNA was performed as described previously by our group[19] with minor modifications. Samples were homogenized in 1 mL of 10 mM phosphate-buffered saline (PBS), pH 7.4, sonicated on ice for 1 minute, added with 1 mL of 10 mM Tris-HCl

buffer, pH 8, containing 10 mM EDTA, 10 mM NaCl, and 0.5% sodium dodecyl sulfate (SDS), incubated for 1 hour at 37°C with 20 µg/mL RNase 1 (Sigma-Aldrich, St. Louis, MO, USA) and kept overnight at 37°C in the presence of 100 µg/mL proteinase K (Sigma-Aldrich). The mixture was extracted with chloroform/isoamyl alcohol (10/2 v/v) and DNA precipitated from the aqueous phase with 0.2 volumes of 10 mM ammonium acetate, solubilized in 200 µL of 20 mM acetate buffer, pH 5.3, and denatured at 90°C for 3 minutes. The extract was then supplemented with 10 IU of P1 nuclease (Sigma-Aldrich) in 10 µL and incubated for 1 hour at 37°C with 5 IU of alkaline phosphatase (Sigma-Aldrich) in 0.4 M phosphate buffer, pH 8.8. The mixture was filtered by an Amicon Micropure-EZ filter (Merck-Millipore) and 50 µL of each sample was used for 8-OH2dG determination using an ELISA kit (JalCA, Shizuoka, Japan), following the instructions provided by the manufacturer. The absorbance of the chromogenic product was measured at 450 nm and expressed as ng/mg of DNA. The results were calculated from a standard curve based on 8-OH2dG known concentrations.

Caspase-3 Activity

The activity of Caspase-3 was determined using a fluorescent substrate following the methods described by Stennicke and Salvesen.[20] Frozen tissue samples were homogenized with 10 mM N-2-hydroxymethylpiperazine-N-2-ethansulfone acid (HEPES, pH 7.4) containing 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), 42 mM KCl, 5 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/mL leupeptin, and 1 µg/mL pepstatin A, and then centrifuged at 10,000 g for 10 minutes. The resulting supernatant (containing about 70–80 µg total protein) was incubated with 40 µM of the caspase-3 substrate AC-DEVD-AMC for 60 min at 37°C. At the end of incubation, substrate cleavage was monitored fluorometrically with a λ excitation at 380 nm and λ emission at 460 nm. Data are expressed as mU/μg protein where one unit of enzyme activity is defined as the amount of enzyme required to liberate 40 µmol of AC-DEVD-AMC for 60 minutes at 37°C.

Caspase-3 Protein Expression

Retina tissues were homogenized with radioimmunoprecipitation assay (RIPA) buffer enriched in protease inhibitors and PMSF (1 mM). Homogenates were then centrifuged at 15,000 g for 10 minutes and total protein levels were determined in the collected supernatants using BCA Protein Assay (ThermoFisher Scientific, Waltham, MA, USA). Forty (40) µg of proteins were separated by SDS-PAGE, electro-transferred on PVDF membranes, and incubated with Caspase-3 Monoclonal Antibody 2 µg/mL (74T2; Invitrogen, Waltham, MA, USA) followed by incubation with appropriated horseradish peroxidase (HRP)-conjugated secondary antibodies. Total protein loading was checked by reblotting the membrane with a tubulin antibody (1:5000; Sigma-Aldrich). Bands were visualized by enhanced chemiluminescence (ECL; ThermoFisher Scientific) and quantified by densitometric analysis with the ImageJ 1.33 software.

Apoptotic Index (%) was calculated as the % ratio between cleaved Caspase-3 protein expression (17-19 kDa) normalized to tubulin and total Caspase-3 (17-19kDa plus 35kDa) normalized to tubulin.

IL-1β, IL-6, and TNFα Levels

The levels of inflammatory cytokines, IL-1β, IL-6, and TNFα, were quantitatively determined on aliquots (100 µL) of retina homogenate supernatants, using the RayBio Rabbit ELISA kit for IL-1β, IL-6, and TNFα (RayBiotech, Norcross, GA, USA), following the protocol provided by the manufacturer. The antibody specific for each cytokine was coated on a 96-well plate and exposed to tissue extracts. The wells were then reacted with biotinylated anti-rabbit cytokine antibody and reacted with HRP conjugated streptavidin and finally exposed to 3,3′,5,5′-tetramethylbenzidine (TMB). Cytokine quantification was performed using standard curves obtained with known cytokine concentrations exposed to an identical processing as the samples. Values are indicated as mean ± SEM and expressed as pg/mL.

In Situ Staining

Some eyes from each treatment group were dissected, paraffin-embedded, and later processed for the presence of apoptotic nuclei.

TUNEL DNA Fragmentation Staining

To determine DNA fragmentation the paraffin-embedded eyes were cut into 5 µm thick sections, deparaffinized, and rehydrated. Tissues were then incubated with proteinase K solution at room temperature and refixed with formaldehyde 4%. Sections were incubated with DNA labeling solution, containing Br-dUTP, for 60 minutes at 37°C and then with the antibody solution for 30 minutes at room temperature. Finally, sections were counterstained with 7-amino-actinomycin D (7-AAD)/RNase A, a fluorescent intercalant that binds selectively GC regions of DNA and can be excited at 488 nm. Sections were analyzed with a confocal Leica TCS SP5 microscope. All sections

were stained in a single session to minimize artifactual inconsistencies during the staining process.

Results

Intraocular Pressure Following Repeated NCX 470, LUM or BIM Dosing in Endothelin-1-Treated Rabbits

Baseline IOP before ET-1 injection did not differ significantly between eyes subsequently randomized for the different groups (20.5 ± 0.8, 20.7 ± 0.7, 21.5 ± 0.5, and 19.5 ± 1.1 mmHg for eyes later randomized for VEH, NCX 470, LUM and BIM, respectively (Table 1). Twice weekly treatments with ET-1 for 6 weeks increased average IOP to 24.8 ± 0.3 mmHg. Conversely, in eyes treated with ET-1 and NCX 470 (30 µL/eye, BID, starting from week 3), IOP did not change significantly over the course of treatment (18.1 ± 0.6 mmHg at week 6; see Table 1). LUM and BIM only provided partial reversal of ET-1-induced IOP changes (see Table 1).

Ophthalmic Artery Resistive Index Following Repeated NCX 470, LUM or BIM Dosing in Endothelin-1-Treated Rabbits

Ophthalmic artery resistive index (OA-RI) was calculated as previously reported.[18] OA-RI did not differ significantly under baseline conditions for eyes subsequently randomized for VEH, NCX 470, LUM,

Table 1. Intraocular Pressure (IOP) After Repeated (30 µL/eye, BID) VEH, NCX 470, LUM, or BIM Treatments in ET-1-Treated Eyes

	Intraocular Pressure (IOP, mmHg)					
Time After ET-1 First Dose	Week 0 Baseline	Week 2 ET-1	Week 3 ET-1 + VEH or Test Items	Week 4 ET-1 + VEH or Test Items	Week 5 ET-1 + VEH or Test Items	Week 6 ET-1 + VEH or Test Items
VEH	20.5 ± 0.8	23.9 ± 0.4	24.0 ± 0.6	24.7 ± 0.4	24.8 ± 0.3	24.8 ± 0.3
NCX 470	20.7 ± 0.7	22.4 ± 0.9[b]	18.7 ± 0.9[a,b]	18.4 ± 0.5[a,b]	18.1 ± 0.5[a,b]	18.1 ± 0.6[a,b]
LUM	21.5 ± 0.5	22.1 ± 0.9	20.5 ± 1.3	20.6 ± 0.9[a]	21.1 ± 0.4[a]	20.7 ± 0.7[a]
BIM	19.5 ± 1.1	25.4 ± 0.3[a]	23.1 ± 0.8	22.8 ± 1.1	22.3 ± 0.7[a]	21.6 ± 0.5[a]

Week 0 refers to baseline IOP (mmHg); week 2 refers to IOP values registered in eyes treated for 2 weeks with ET-1 (twice/week, 200 µL of 250 nM); weeks 3, 4, 5, and 6 refer to IOP values registered in eyes treated with ET-1 + VEH, ($n = 7$) or test items (NCX 470, $n = 7$; LUM, $n = 4$; BIM, $n = 6$). Data are reported as mean ± SEM, multiple t-test with Welch correction.

[a] $p < 0.05$ versus VEH.

[b] $p < 0.05$ versus LUM or BIM at the same time point.

Table 2. Ophthalmic Artery Resistive Index (OA-RI) Changes After Repeated (30 µL/eye, BID) VEH, NCX 470, LUM, or BIM Treatments in ET-1-Treated Eyes

	Ophthalmic Artery Resistive Index (OA-RI)					
Time After ET-1 First Dose	Week 0 Baseline	Week 2 ET-1	Week 3 ET-1 + VEH or Test Items	Week 4 ET-1 + VEH or Test Items	Week 5 ET-1 + VEH or Test Items	Week 6 ET-1 + VEH or Test Items
VEH	0.36 ± 0.02	0.43 ± 0.03	0.49 ± 0.01	0.50 ± 0.02	0.52 ± 0.02	0.55 ± 0.01
NCX 470	0.34 ± 0.02	0.43 ± 0.02	0.38 ± 0.01[a]	0.34 ± 0.02[a,b]	0.33 ± 0.01[a,b]	0.33 ± 0.01[a,b]
LUM	0.38 ± 0.03	0.43 ± 0.03	0.41 ± 0.04	0.45 ± 0.02	0.44 ± 0.01[a]	0.43 ± 0.02[a]
BIM	0.31 ± 0.02	0.39 ± 0.03	0.43 ± 0.02[a]	0.41 ± 0.03[a]	0.41 ± 0.02[a]	0.44 ± 0.01[a]

Week 0 refers to baseline OA-RI; week 2 refers to OA-RI values registered in eyes treated for 2 weeks with ET-1 (twice/week, 200 µL of 250 nM); weeks 3, 4, 5, and 6 refer to OA-RI values registered in eyes treated with ET-1 + VEH, ($n = 7$) or test items (NCX 470, $n = 7$; LUM, $n = 4$; BIM, $n = 6$). Data are reported as mean ± SEM, multiple t-test with Welch correction.

[a] $p < 0.05$ versus VEH.

[b] $p < 0.05$ versus LUM or BIM at the same time point.

and BIM. Following 6 weeks of ET-1 (twice/week, 200 µL of 250 nM) injections, OA-RI increased significantly from 0.36 ± 0.02 to 0.55 ± 0.01 (Table 2). Conversely, in eyes treated with NCX 470, OA-RI did not increase (0.34 ± 0.02 and 0.33 ± 0.01, at baseline and week 6, respectively; see Table 2). LUM and BIM only partially reversed OA-RI changes elicited by ET-1 (see Table 2).

Electroretinogram Following Repeated NCX 470, LUM or BIM Dosing in Endothelin-1-Treated Rabbits

ET-1 treatment resulted in a marked decline in rod and cone function (as determined by ERG assessment) after 2 weeks of repeated ET-1 injection. The responses continued to decrease during the following weeks: 139.5 ± 17.8 and 89.1 ± 10.7 µV at time 0 (baseline) and week 6, respectively (Fig. 1). Eyes treated with NCX 470 (0.1%, 30 µL/eye BID) exhibited significantly less impairment: 129.8 ± 8.7 and 124.1 ± 11.3 µV, at time 0 (baseline) and week 6, respectively, in ERG wave amplitude at week 6 than those treated with vehicle (see Fig. 1A). Interestingly, these effects were not as evident in eyes treated (30 µL/eye BID) with LUM (see Fig. 1B) or BIM (see Fig. 1C). As in previous work,[18] NCX 470 as well as LUM and BIM did not change ERG peak latency in our experimental conditions.

Glutathione and 8-Hydroxy-2-Deoxyguanosine Levels in Retina After Repeated NCX 470, LUM or BIM Dosing in Endothelin-1-Treated Rabbits

When compared to naïve conditions, ET-1 treatment over 6 weeks in eyes also receiving 4 weeks of VEH treatment led to a significant decrease in retina GSH content (1.30 ± 0.04 and 0.37 ± 0.04 µmoL/mg protein in naïve conditions and VEH at week 6, respectively). Interestingly, in the eyes treated with NCX 470 (0.1%, 30 µL/eye BID), the levels of GSH were similar to those of naïve animals and significantly increased compared to VEH-treated eyes (1.31 ± 0.25 µmoL/mg protein; Table 3).

The levels of 8-Hydroxy-2-deoxyguanosine (8-OH2dG) were increased significantly in retina of VEH-treated eyes versus naïve, from 18.9 ± 0.7 pg/µg of DNA to 57.5 ± 6.3 pg/µg of DNA. By contrast, 8-OH2dG content was significantly reduced in eyes treated with NCX 470 (34.1 ± 5.0 pg/µg of DNA; see Table 3). LUM and BIM were also effective on these parameters (see Table 3).

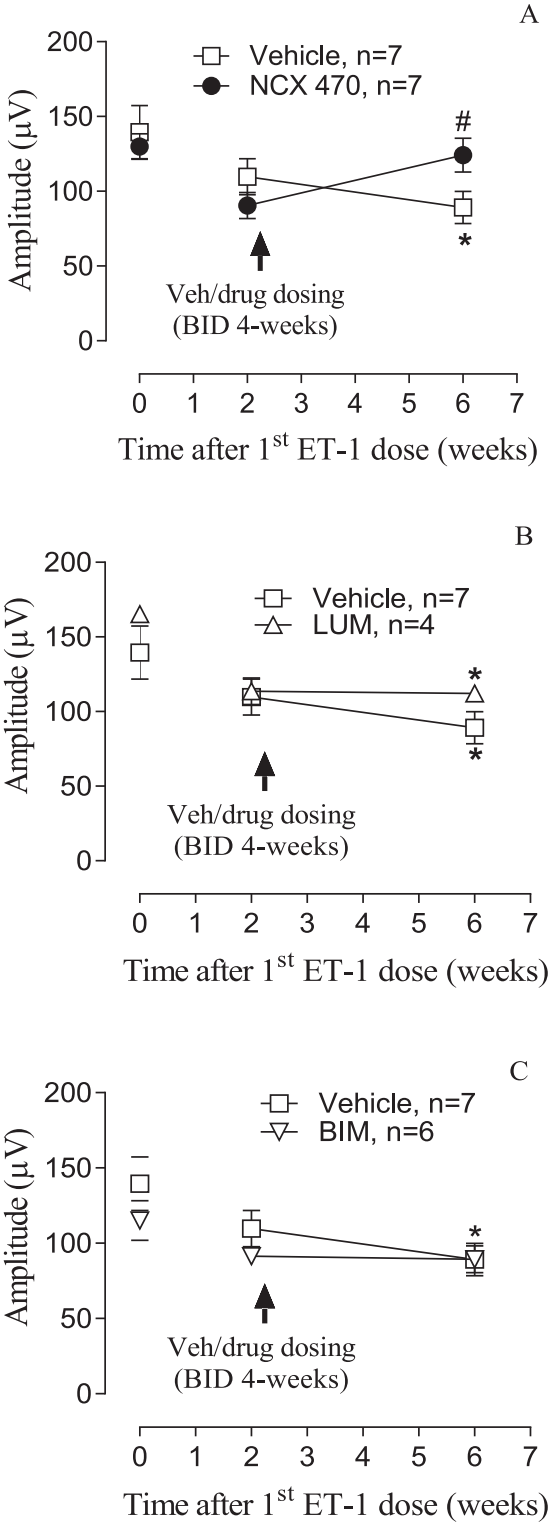

Figure 1. Rods and cones response prior to (baseline) and 2 and 6 weeks after ET-1-induced ischemia/reperfusion injury in eyes receiving (**A**) NCX 470 and VEH; (**B**) LUM and VEH; (**C**) BIM and VEH. Reported amplitude reflects the difference between b-wave and a-wave maximal intensities; data are reported as mean ± SEM, multiple *t*-test with Welch correction. **p* < 0.05 versus time 0 (baseline); #*p* < 0.05 versus vehicle at the same time point.

Table 3. Glutathione (GSH) and 8-Hydroxy-2-Deoxyguanosine (8-OH2dG) Levels in Retina After Repeated VEH, NCX 470, LUM, or BIM in ET-1-Treated Eyes

Group ID	Dose (% w/w)	GSH (µmol/mg Protein)	8-OH2dG (pg/µg DNA)
Naïve	–	1.30 ± 0.04 ($n = 4$)	18.9 ± 0.7 ($n = 3$)
VEH	–	0.37 ± 0.04[a] ($n = 6$)	57.5 ± 6.3[a] ($n = 6$)
NCX 470	0.1	1.31 ± 0.25[b] ($n = 6$)	34.1 ± 5.0[b,c] ($n = 6$)
LUM	0.01	1.09 ± 0.07[b] ($n = 4$)	51.8 ± 3.5 ($n = 5$)
BIM	0.072	1.39 ± 0.23[b] ($n = 4$)	41.5 ± 1.9 ($n = 6$)

GSH and 8-OH2dG levels in naïve eyes or at week 6 in eyes treated with VEH, NCX 470, LUM, and BIM. Data are mean ± SEM, multiple *t*-test with Welch correction.

[a] $p < 0.05$ versus naïve.

[b] $p < 0.05$ versus vehicle.

[c] $p < 0.05$ versus LUM or BIM.

Interleukin-1ß, Tumor Necrosis Factor Alpha, and Interleukin-6 Levels in Retina After Repeated NCX 470, LUM or BIM Dosing in Endothelin-1-Treated Rabbits

The treatment with ET-1 significantly increased IL-1β levels in retina from 4.7 ± 0.4 pg/mL (naïve) to 14.5 ± 0.4 pg/mL (ET-1 + VEH; Fig. 2A). TNFα content also increased in retina from 62.8 ± 6.6 pg/mL (naïve) to 128.9 ± 20.8 pg/mL (ET-1 + VEH; Fig. 2B). The treatment with NCX 470 (0.1%, 30 µL/eye BID) counteracted these effects (10.4 ± 0.7 pg/mL and 38.6 ± 4.2 pg/mL for IL-1β and TNFα, respectively) (see Figs. 2A, 2B). Conversely, IL-6 was unchanged in VEH-treated eyes (16.2 ± 4.3 pg/mL) compared to naïve conditions (20.4 ± 1.4 pg/mL; Fig. 2C) and partially increased in NCX 470-treated eyes (30.8 ± 3.9 pg/mL; see Fig. 2C). LUM and BIM diminished the levels of IL-1β (11.2 ± 0.9 and 11.6 ± 1.0 pg/mL, for BIM and LUM, respectively) and TNFα (58.7 ± 2.5 and 67.6 ± 12.2 pg/mL, for BIM and LUM, respectively; see Figs. 2A, 2B). Interestingly, however, neither LUM nor BIM affected IL-6 (19.0 ± 1.1 and 21.2 ± 1.5 pg/mL, for BIM and LUM, respectively; see Fig. 2C).

Caspase-3 Activity and Protein Content in Retina After Repeated NCX 470, LUM or BIM Dosing in Endothelin-1-Treated Rabbits

Caspase-3 activity was increased in retina 6 weeks post ET-1 treatment (35.9 ± 4.4 mU/µg) compared with that observed in naïve retina (17.8 ± 5.6 mU/µg; Fig. 3A). NCX 470 treatment significantly reduced ET-1-induced changes in Caspase-3 (5.1 ± 0.7 mU/µg; see Fig. 3A). LUM and BIM also diminished the levels of this marker although not as much as NCX 470 (20.5 ± 1.7 and 23.7 ± 3.6 mU/µg, for BIM and LUM, respectively; see Fig. 3A). A similar trend was observed when Caspase-3 protein was expressed as the percentage ratio between cleaved Caspase-3 and total Caspase-3 (cleaved + pro-Caspase-3) (Apoptotic Index reported in Figs. 3B, 3C) as determined by Western blot analysis.

Apoptotic Cell Determination in Retinal Sections After Repeated NCX 470 Dosing in Endothelin-1-Treated Rabbits

The degeneration of retinal cells in eyes undergoing ET-1 repeated injections was confirmed by TUNEL assay (Fig. 4) in retinal sessions counterstained with 7-amino actinomycin D (7-AAD)/RNase A. As shown in Figure 4, panels D to F, ET-1 treatment induced extensive retinal cell death with a large proportion of apoptotic nuclei present within the retinal ganglion cells (RGCs), inner nuclear layer (INL), and outer nuclear layer (ONL) as compared with naïve retina (see Figs. 4A-C). Four weeks repeated NCX 470 dosing significantly preserved retina tissue and reduced the apoptotic RGCs as well as apoptotic nuclei in the INL and ONL layers (Figs. 4G-I).

Discussion

NCX 470 is a novel NO-donating derivative of the prostamide bimatoprost which is esterified at the hydroxyl group in position 15 with a 6-(nitrooxy) hexanoic acid residue which serves as an NO-donating moiety[21] able to release functionally effective concentrations of NO (see Supplemental Fig. S1). The compound has demonstrated robust and dose-dependent IOP reduction in patients with ocular hypertension and glaucoma,[22] with IOP lowering properties recently confirmed within the first pivotal phase III

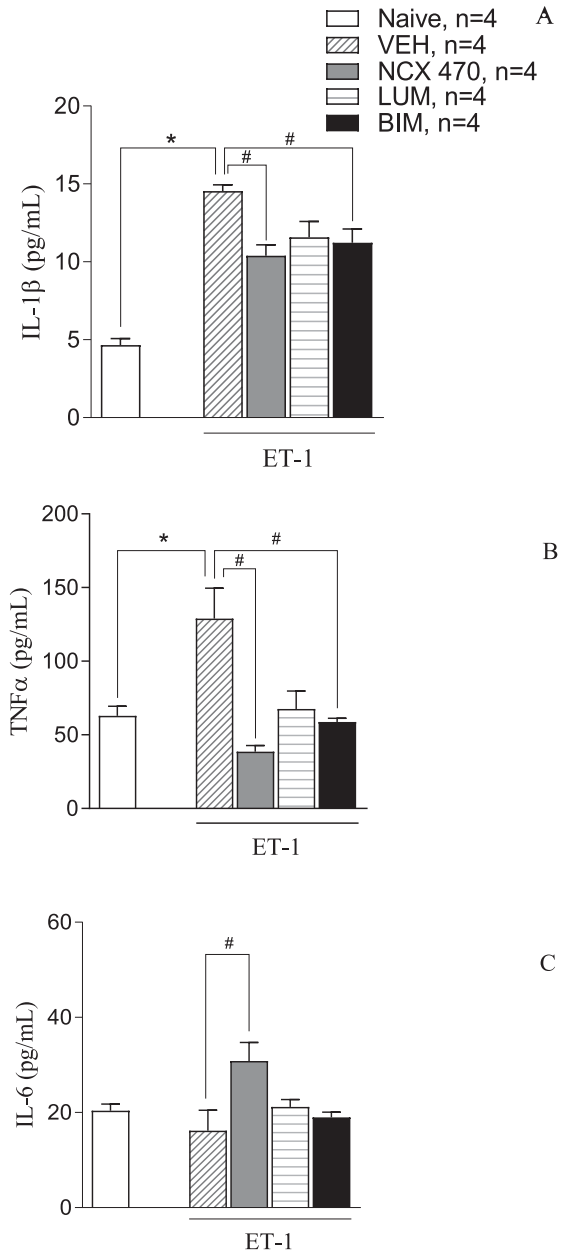

Figure 2. (**A**) Interleukin-1ß (IL-1ß), (**B**) tumor necrosis factor alpha (TNFα), and (**C**) interleukin-6 (IL-6) levels under naïve condition and 6 weeks after ET-1-induced ischemia/reperfusion injury in eyes treated with VEH, NCX 470 (0.1%), LUM (0.01%), or BIM (0.072%). Data are mean ± SEM, multiple *t*-test with Welch correction. *$p < 0.05$ versus naïve, #$p < 0.05$ versus vehicle.

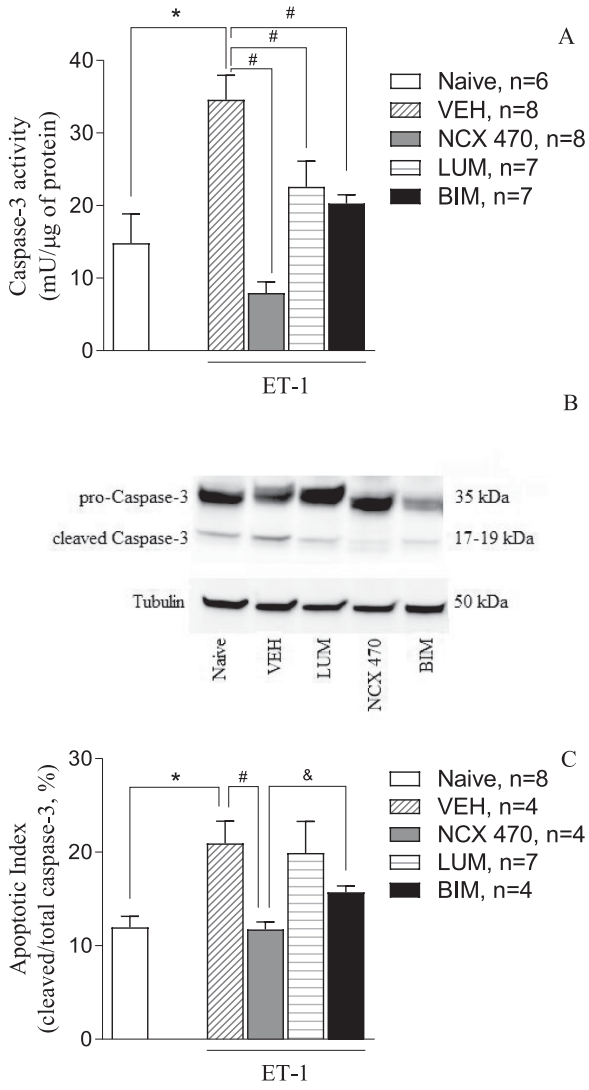

Figure 3. Caspase-3 activity (**A**), cleaved Caspase-3 protein expression (**B**), and Apoptotic Index (**C**) under naïve conditions and 6 weeks after ET-1-induced ischemia/reperfusion injury in eyes treated with VEH, NCX 470 (0.1%), LUM (0.01%), or BIM (0.072%). Apoptotic Index (%) was calculated as the percentage ratio between cleaved Caspase-3 protein expression (17-19 kDa) normalized to tubulin and total Caspase-3 (17-19kDa plus 35kDa [pro-Caspase-3]) normalized to tubulin. Data are mean ± SEM, multiple *t*-test with Welch correction. *$p < 0.05$ versus naïve; #$p < 0.05$ versus vehicle; $^{\&}p < 0.05$ versus BIM.

clinical trial where NCX 470 0.1% was tested versus latanoprost 0.005% ophthalmic solution in a similar patient population (https://clinicaltrials.gov/ct2/show/NCT04445519).

In addition, NCX 470 resulted as effective in reversing hemodynamic changes as well as retinal dysfunction in a rabbit model of ischemia/reperfusion injury of the optic nerve head and retina induced by repeated injection of ET-1.[18]

In the present study, we confirmed and extended previous results[18] by comparing the activity of NCX 470 (0.1% ophthalmic solution) to that of bimatoprost administered at an FDA-approved dose (Lumigan, bimatoprost 0.01% ophthalmic solution – LUM) and at a dose equimolar to 0.1% NCX 470 (0.072% - approximately 7 times the LUM dose – BIM). Both LUM and BIM were less effective than NCX 470 at reversing ET-1-mediated IOP changes as well as for hemodynamic end points suggesting that both active metabolites of NCX 470, bimatoprost acid and NO,

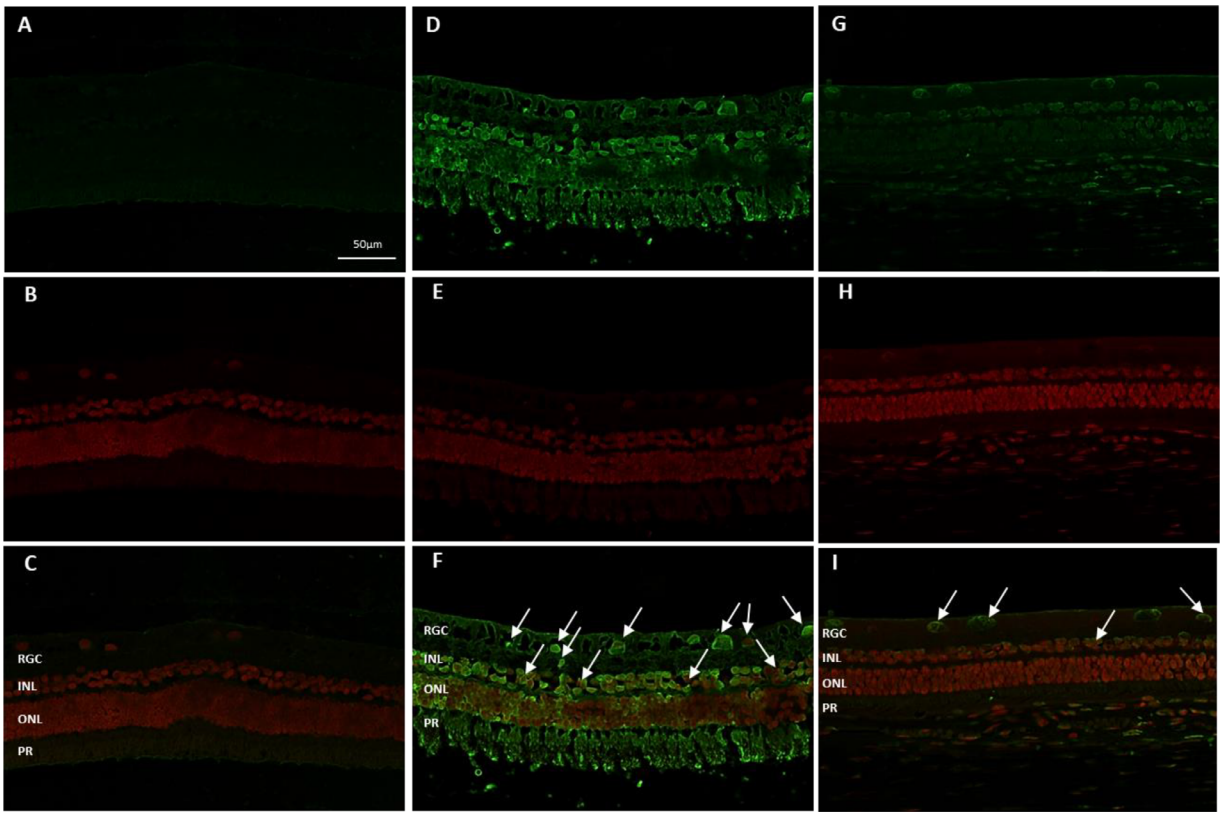

Figure 4. Representative retina sections immunostained for Br-dUTP (*green*, panels **A**, **D**, and **G** for naïve, ET-1 + VEH, and ET-1 + NCX 470, respectively) and 7-AAD/Rnase A (*red*, panels **B**, **E**, and **H** for naïve, ET-1 + VEH, and ET-1 + NCX 470, respectively) to counterstain the nuclei (magnification 20×). Panels **C**, **F**, and **I** show respective merging pictures. RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptors. *White arrows* indicate apoptotic bodies.

contribute to the overall effects of this drug on ocular hemodynamics. NO donors have been shown to diminish OA-RI in this model (unpublished results) and Vyzulta (Latanoprostene bunod ophthalmic solution, 0.024%) which shares similar NO-donating technology as NCX 470, has been shown to enhance ocular perfusion pressure in patients with glaucoma[15] and retinal vessel density in a similar patient population.[16] The effect of bimatoprost on ocular blood flow has not been established as clearly, with some authors reporting efficacy[23] and others suggesting no effect.[24] Our laboratory is not equipped to take fundus images; this is a clear limitation of our study as fundus images would have been important to understand whether vasculature anatomic changes account for NCX 470-mediated effects on ocular hemodynamics. These and other testing are needed to fully understand the contribution of bimatoprost and NO in the overall effect of NCX 470.

Confirming previous work,[18] NCX 470 enhanced rod and cone responses to scotopic stimulation as determined by an increased ERG wave amplitude in these eyes. Again, these effects were not as evident in eyes treated with LUM or BIM thus pointing to NO as the differentiating factor for NCX 470 activity.

NO has been associated with both neuroprotective and neurodegenerative effects, with the circulating amount of NO being the discriminating factor. Low nanomolar concentrations are essential for maintaining aqueous humor homeostasis,[11] ocular blood flow,[25] and promote rods' and cones' activity,[26] whereas excessive NO concentrations (µM – mM), as that produced by inducible nitric oxide synthase (NOS2), have the potential for cytotoxic effects.[27] In our study, NCX 470 diminished ET-1-induced changes in ophthalmic artery resistive index thereby suggesting that an increase in ocular blood flow likely contributed to the beneficial effect of NCX 470 on retina function; however, a bimatoprost-mediated contribution on this phenomenon also seems plausible, as indicated in previous work by Emre and coworkers,[28] where topical dosing of bimatoprost was found to protect rabbit retina from ischemia/reperfusion damage.

Little is known on the cellular and molecular mechanisms involved in the beneficial effects of NCX 470 in retina. We found that NCX 470 treatment prevented cellular apoptosis of RGCs as well as of cells in the INL and ONLs (INL and ONL, respectively) as highlighted by a decrease in Caspase-3 activity, a marker of the early phase of the apoptotic process.[29] Likewise, Caspase-3 protein expression in dissected retinas from NCX 470 eyes decreased compared to VEH-treated eyes suggesting that NCX 470 lowers the overall expression of this protein, and, as a result, its activity. Accordingly, in retinal slices from NCX 470-treated eyes, we found less TUNEL-positive cells showing DNA fragmentation compared to VEH-treated eyes, thus indicating that NCX 470 exerts protective effects on RGCs as well as on cells present within the INL and ONL by inhibiting apoptotic cascades. As to whether or not these latter effects are also shared by bimatoprost was not investigated in this study; however, a bimatoprost contribution could likely be a possibility. Indeed, BIM and LUM, albeit to lesser extent as compared to NCX 470, inhibited caspase-3 activity, and protein expression in treated eyes. These latter findings are in agreement with previous reports suggesting an anti-apoptotic activity of bimatoprost in cultured RGCs[30] and in vivo following topical dosing in a rabbit model where various prostaglandins, including bimatoprost, were found to reduce apoptotic cell nuclei in retinal layers following ischemia/reperfusion injury.[28]

Reduced glutathione (GSH), an anti-oxidant defensive marker[31] decreased after ET-1 plus VEH treatment. Likewise, pro-inflammatory cytokines, such as IL-1β and TNFα, increased after ET-1 plus VEH treatment, whereas IL-6 remained unchanged. Interestingly, IL-1β and TNFα were brought back to physiologic levels by NCX 470, an effect also observed in eyes treated with BIM or LUM, albeit to numerically lesser extent. On the contrary, IL-6 increased following NCX 470 treatment but not after BIM or LUM treatment. These findings once again suggest that the simultaneous contribution of NO- and prostaglandin-mediated mechanism/s on oxidative stress and inflammatory signaling pathways could have contributed to the overall effect of NCX 470. The role of IL-6 is somewhat controversial as a cytokine which is typically considered pro-inflammatory, however, with some reports attributing regenerative and/or anti-inflammatory activities to the molecule.[32] It is thus possible that the protective effects on retinal cells shown by NCX 470 compared with BIM or LUM are related to IL-6 production. Additional investigations are needed to provide more meaningful interpretation of this finding.

In conclusion, NCX 470 by virtue of its dual mechanism of action involving NO-mediated as well as bimatoprost-mediated effects improves ocular hemodynamics and retinal cell physiology in ET-1-induced ischemia/reperfusion injury model.

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