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NCX 470 Reduces Intraocular Pressure More Effectively Than Lumigan in Dogs and Enhances Conventional and Uveoscleral Outflow in Non-Human Primates and Human Trabecular Meshwork/Schlemm's Canal Constructs

Corinna Galli,[1] Elena Bastia,[1] Douglas A. Hubatsch,[2] Carol Toris,[3,4] Shan Fan,[3] Andrea Unser,[5] Feryan Ahmed,[5] Karen Y. Torrejon,[5] and Francesco Impagnatiello[1]

Abstract

Purpose: To determine NCX 470 (0.1%) and Lumigan® (bimatoprost ophthalmic solution, 0.01%—LUM) intraocular pressure (IOP)-lowering activity after single or repeated (5 days) dosing along with changes in aqueous humor (AH) dynamics.

Methods: Ocular hypotensive activity of NCX 470 and LUM was compared with vehicle (VEH) in Beagle dogs using TonoVet®. Non-human primates (NHP) and bioengineered three-dimensional (3D) human Trabecular Meshwork/Schlemm's Canal (HTM/HSC™) constructs exposed to transforming growth factor-β2 (TGFβ2) were used to monitor NCX 470 and LUM-induced changes in AH dynamics.

Results: NCX 470 (30 μL/eye) showed greater IOP reduction compared with LUM (30 μL/eye) following single AM dosing [maximum change from baseline (CFB_{max}) = -1.39 ± 0.52, -6.33 ± 0.73, and -3.89 ± 0.66 mmHg (mean ± standard error of the mean) for VEH, NCX 470, and LUM, respectively]. Likewise, repeated 5 days daily dosing of NCX 470 resulted in lower IOP than LUM across the duration of the study (average IOP decrease across tests was -0.45 ± 0.22, -6.06 ± 0.15, and -3.60 ± 0.22 mmHg for VEH, NCX 470, and LUM, respectively). NCX 470 increased outflow facility (Cfl) *in vivo* in NHP ($Cfl_{VEH} = 0.37 \pm 0.09$ μL/min/mmHg and $Cfl_{NCX470} = 0.64 \pm 0.17$ μL/min/mmHg) as well as *in vitro* ($C_{HTM/HSC}$) in HTM/HSC constructs ($C_{HTM/HSC\_VEH} = 0.47 \pm 0.02$ μL/min/mm^2/mmHg and $C_{HTM/HSC\_NCX470} = 0.76 \pm 0.03$ μL/min/mm^2/mmHg). In addition, NCX 470 increased uveoscleral outflow ($Fu_{VEH} = 0.62 \pm 0.26$ μL/min and $Fu_{NCX470} = 1.53 \pm 0.39$ μL/min with episcleral venous pressure of 15 mmHg) leaving unaltered aqueous flow ($AHF_{VEH} = 2.03 \pm 0.22$ μL/min and $AHF_{NCX470} = 1.93 \pm 0.31$ μL/min) in NHP.

Conclusions: NCX 470 elicits greater IOP reduction than LUM following single or repeated dosing. Data in NHP and 3D-HTM/HSC constructs suggest that changes in Cfl and Fu account for the robust IOP-lowering effect of NCX 470.

Keywords: aqueous humor dynamics, ocular hypertension, glaucoma, nitric oxide

[1]Nicox Research Institute, Bresso, Milan, Italy.
[2]Nicox Ophthalmics, Durham, North Carolina, USA.
[3]University of Nebraska Medical Center, Omaha, Nebraska, USA.
[4]The Ohio State University, Columbus, Ohio, USA.
[5]Humonix Biosciences Inc., Albany, New York, USA.

Introduction

Glaucoma comprises a variety of degenerative optic neuropathies characterized by retinal ganglion cell death and changes in the optic nerve head associated with irreversible vision loss.[1] Elevated intraocular pressure (IOP) is the predominant risk factor for the development and progression of glaucoma[2]; however, in certain patient populations, glaucoma and optic nerve head degeneration may occur despite normal IOP.[3]

NCX 470 is a novel, second-generation, dual-acting nitric oxide (NO)-donating prostaglandin (PGA) designed to concomitantly release the prostamide bimatoprost, and the NO-donating moiety 6-(nitrooxy) hexanoic acid that ultimately releases NO when exposed to a metabolically active environment. NCX 470 administered topically resulted in dose-dependent reductions of IOP in open-angle glaucoma patients[4] and, most recently, the dose of NCX 470 0.1% was shown to be ''non-inferior'' to generic latanoprost 0.005% ophthalmic solution in a large randomized phase III trial (Mont Blanc).[5,6] However, the completed Mont Blanc trial[6] as well as the ongoing Denali trial[7] compare NCX 470 with generic latanoprost 0.005% ophthalmic solution. No specific studies comparing clinically effective doses of NCX 470 with bimatoprost are available to date.

In an initial attempt to fill this knowledge gap we conducted a well-controlled head-to-head study in ocular normotensive Beagle dogs treated with NCX 470 at doses and regimen found to be effective in clinical settings or Lumigan® (bimatoprost ophthalmic solution, 0.01%—LUM). Beagles are ideal for explorative work of IOP-lowering efficacy of molecules holding PGA- and NO-mediated mechanism/s of action; indeed, NCX 470[8] as well as other NO-donating PGA[9] were previously shown to effectively decrease IOP in this species.

Under physiologic conditions, IOP homeostasis is achieved by the balance between the production of aqueous humor (AH) by the ciliary processes and the facility and rate of its drainage into the venous circulation through the uveoscleral outflow and trabecular meshwork (TM) pathways.[10] The episcleral venous pressure (EVP) also plays a major role in the determination of IOP as defined by the Goldmann equation.[11] Depending on the disease state, the conventional outflow pathway drains ~60%–96% of the AH, whereas the residual 4%–40% of the AH flows out through the unconventional pathway.[12,13]

The majority of Food and Drug Administration (FDA)–approved glaucoma therapies lower IOP by reducing AH production (e.g., β-receptor antagonists, alpha-2 receptor agonist, carbonic anhydrase inhibitors)[14] or by promoting the AH drainage *via* either the uveoscleral pathway (e.g., majority of PGA analogues including latanoprost, tafluprost, travoprost, and unoprostone), the conventional pathway (e.g., Rho kinase inhibitors),[15] or both (e.g. some PGAs like bimatoprost[16] and NO-donating derivatives such as latanoprostene bunod[17]). Other mechanisms such as the reduction of EVP shown for some Rho kinase inhibitors or the mechanical enlargement of the TM and the Schlemm's canal (SC) suggested for some miotic and IOP-lowering agents including the muscarinic receptor agonist pilocarpine, have also been described.[18,19]

The outflow pathway/s engaged by NCX 470 to lower IOP have not been directly investigated to date. Thus, the second objective of our work was to determine the relative contribution of the conventional and Fu pathways to the overall IOP-lowering effects of NCX 470. Therefore, we attempted to exploit this aspect *in vivo* using ocular normotensive non-human primates (NHP). To understand whether the findings could be translated to humans, we used an *in vitro* model with bioengineered three-dimensional (3D) human Trabecular Meshwork/Schlemm's Canal (HTM/HSC™) constructs stimulated with transforming growth factor-$\beta 2$ (TGFβ2) that not only mimics glaucomatous conditions[20,21] where TGFβ2 levels are increased[22] but is also known to be sensitive to both NO[23] and bimatoprost.[24]

NO has been shown to also affect AH production.[25] Therefore, we also determined in NHP whether NCX 470 affects AH flow as a consequence of repeated topical dosing.

Methods

In all experiments animals were cared for and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments were performed in accordance with protocols approved by the Animal Care and Use Committees at the Institution where the experiments were performed.

The studies in NHP were approved by the animal care and use committee at the University of Nebraska Medical Center and the experiments were conducted in this facility. The procedures on Beagle dogs were performed in compliance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123) and the Czech Collection of laws No. 246/1992.

IOP measurements in ocular normotensive Beagle dogs

Twelve healthy ocular normotensive male Beagle dogs between the ages of 40 and 44 months were used. Three groups of dogs were dosed daily in the morning (AM) in a cross-over manner with 3 weeks of washout between each study session. A total of three (six eyes/group, for the acute design) and four (six eyes/group, for the 5-day repeated design) consecutive sessions were performed. IOP measurements were recorded by an experienced investigator using a rebound tonometer (TonoVet®, Icare Finland Oy). Of interest were potential differences in the resulting diurnal IOP after single or repeated topical ocular administration. Change from baseline (CFB) was calculated as follows: (IOP_{Tx} – IOP_{T0}), where IOP_{Tx} and IOP_{T0} are IOPs at the time of interest and before dosing, respectively.

In experiments in which the IOP-lowering effect of NCX 470 was evaluated after an acute single challenge, NCX 470 (0.1%), Lumigan (bimatoprost ophthalmic solution, 0.01%—LUM), or vehicle (boric/phosphate buffer pH 6.0 added with macrogol 15-hydroxystearate, disodium ethylenediaminetetraacetic acid (EDTA), sorbitol, and benzalkonium chloride—VEH) were topically administered to animals in both eyes (30 μL/eye; $n = 18$ eyes/group). IOP measurements were taken before the treatment (0, baseline) and then 40, 80, 120, 180, 240, 300, 480, and 1,440 min postdosing.

In experiments where the IOP-lowering activity of NCX 470 was evaluated after repeated dosing, NCX 470, LUM, or VEH were topically administered in both eyes at the same dose as in the acute study (30 μL/eye; $n = 24$ eyes/group)

daily for 5 consecutive days. IOP measurements were taken before starting the study (day 0) and then before the daily dosing (0) and 3 h after starting on day 1 until day 5.

AH dynamics in ocular normotensive non-human primates

Experiments were carried out as described in a previous work[26] with minor modifications. The design of the study was randomized, crossover with VEH or NCX 470 administered (30 μL/eye) at 0.1% dissolved in the above vehicle. Female Cynomolgus monkeys ($n = 12$) between the ages of 13 and 22 years were used. Three to five topical drops of fluorescein (10%) were administered to animals starting at 5:00 AM on day 4 (measurement day). Central corneal thickness and anterior chamber depth were measured by ultrasound pachymetry (Sonomed), cornea diameter was measured with calipers (Fine Science Tools), and cornea and anterior chamber volumes were calculated from these biometric measurements. These measurements required no sedation.

Sedation was needed for the fluorophotometry scans. It was induced by intramuscular administration of ketamine HCl (15 mg/kg). The fluorescence of the cornea and the anterior chamber was measured with a scanning ocular fluorophotometer (Fluorotron™ Master; OcuMetrics, Palo Alto, CA). Scans were repeated 3 times at 45-min intervals for a total of 4 sets of scans.

The slopes of the cornea and anterior chamber fluorescein decay curves and the anterior chamber volume were used in the determination of aqueous flow (AHF). AHF is an estimate of AH production rate. The calculations were carried out with the Yablonski Routine in the Fluorotron Master Program. Immediately following the fourth set of scans, IOP was measured and acetazolamide (12.5–20 mg/kg depending on animal's responsiveness to acetazolamide) was given by intravenous or intramuscular injection to reduce IOP and AHF enabling the calculation of outflow facility (Cfl).

Animals were then dosed with a topical drop of NCX 470 (0.1%) or VEH. Forty-five, 90, and 135 min later, fluorophotometric scans and IOP measurements were repeated. Fluorophotometric Cfl was calculated as the ratio of the change in AHF to the change in IOP from the acetazolamide administration using the following equation:

$$\mathrm{Cfl} = \Delta\mathrm{AHF}/\Delta\mathrm{IOP}.$$

Fu was calculated using the modified Goldmann equation hereunder:

$$\mathrm{Fu} = \mathrm{AHF} - \mathrm{Cfl}(\mathrm{IOP} - \mathrm{EVP}),$$

where EVP is the episcleral venous pressure set to values between 14 and 15.5 mmHg.

AH outflow in 3D HTM/HSC constructs

The methods described originally by Torrejon et al.[27] were followed with minor modifications. In brief, HTM cells from five donors were isolated from discarded (postkeratoplasty) donor tissue rings or purchased (Cell Applications, San Diego, CA). HTM cells were plated in 75 cm^2 cell culture flasks with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO) in improved minimum essential medium (IMEM; Corning Cellgro, Manassas, VA) enriched with 0.1 mg/mL gentamicin. Fresh medium was supplied every 48 h and the cells were maintained at 37°C in a humidified atmosphere with 5% carbon dioxide until 90% confluence.[20] The cells were trypsinized using 0.25% trypsin/0.5 mM EDTA (Gibco, Grand Island, NY) and subcultured.

Similarly, HSC cells, isolated from two donors as previously described,[28] were initially plated in 75 cm^2 cell culture flasks with 10% premium select FBS (Atlanta Biologicals, Lawrenceville, GA) in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with penicillin (100 units/mL), streptomycin (0.1 mg/mL), and L-glutamine (0.292 mg/mL; Life Technologies). Cells were maintained at 37°C in a humidified atmosphere with 5% carbon dioxide until 80%–90% confluence which point cells were trypsinized using 0.25% trypsin/0.5 mM EDTA (Gibco) and subcultured.[29]

SU-8 2010 (Kayaku Advanced Materials, Westborough, MA) was used to develop free-standing biomimetic porous microstructures to serve as scaffolds on which primary HTM cells were cultured. Scaffolds were fabricated using standard photolithographic techniques.[27] To create HTM/HSC™ constructs, the individual microfabricated SU-8 scaffolds were seated on aluminum rings (15 mm diameter) and placed in a 24-well plate followed by seeding with 40,000–50,000 HTM cells. Once confluent, the HTM constructs were inverted and HSC cells (40,000 cells/sample) were cultured on the other side of the scaffold for 10 days. After the HTM/HSC™ constructs reached confluency, they were serum starved (1% FBS-IMEM) for 1 day before treatment with TGFβ2 (5 ng/mL for 6 days) to mimic glaucomatous conditions and reduce Cfl[22] followed by perfusion studies using a perfusion apparatus previously described.[27]

At day 7, the HTM/HSC™ constructs kept under normal and diseased conditions were placed in the perfusion chamber and perfused at various rates for 5 h per flow rate (2, 4, 8, and 16 μL/min) with medium containing dimethyl sulfoxide (DMSO; 1% final), NCX 470 (10 μM in 1% DMSO), or bimatoprost (10 μM in 1% DMSO). Samples were perfused in a basal-to-apical direction with respect to the HSC, with perfusion medium consisting of DMEM with 0.1% gentamicin containing the appropriate treatment. The temperature was maintained at 34°C, which simulates the temperature of the cornea and of the surrounding tissues and widely used for *ex vivo* perfusion studies of TM.[20] Pressure was continuously monitored and recorded. The ''outflow facility'' was calculated mathematically applying the following equation:

$$C_{HTM/HSC} = (\Delta f/\Delta p)/A$$

where $C_{HTM/HSC}$ is outflow facility, f is the flow of fluid through the construct, p is the pressure, and A is the area of the cultured cells subjected to f.

Results

IOP-lowering after single NCX 470 or Lumigan dosing in ocular normotensive Beagle dogs

This was designed as crossover, open-label study with 3 weeks washout between each experimental session; a total of three experimental sessions were performed each

including six eyes/group. Baseline IOPs did not differ significantly in eyes randomized for vehicle (30 μL/eye, $n=18$ eyes—VEH), NCX 470 (0.1%, 30 μL/eye, $n=18$), or Lumigan (bimatoprost ophthalmic solution, 0.01%, 30 μL/eye, $n=18$—LUM). Specifically, baseline IOPs were, respectively, 14.89 ± 0.46, 15.56 ± 0.86, and 14.28 ± 0.55 mmHg for VEH, NCX 470, and LUM (Fig. 1A).

NCX 470 and LUM dosing effectively reduced IOP in this model. Both compounds were largely effective starting from 120 min postdose to reach their maximal efficacy between 300 and 480 min [maximum change from baseline (CFB_{max}), -1.39 ± 0.52, -6.33 ± 0.73, and -3.89 ± 0.66 mmHg, for VEH, NCX 470, and LUM, respectively) with efficacy still present at 1,440 min (24 h) postdosing (CFB_{24h}, -0.89 ± 0.49, -5.33 ± 0.73, and -2.11 ± 0.54 mmHg, for VEH, NCX 470, and LUM, respectively; Fig. 1B). As given in Fig. 1B, NCX 470-mediated effects were largely greater than those of LUM at most time-points tested with a maximum difference of -3.28 ± 0.94 mmHg observed 300 min postdosing.

IOP-lowering after 5 days repeated NCX 470 or Lumigan qd dosing in ocular normotensive Beagle dogs

Naive male ($n=24$ eyes) Beagle dogs were treated in a crossover manner head-to-head with VEH, NCX 470 (0.1%), or LUM (bimatoprost ophthalmic solution, 0.01%) for 5 consecutive days in 4 consecutive sessions each including 6 eyes/group. The IOPs before treatments start were similar in all groups and did not differ substantially from baseline readings taken before the start of the experiments (Fig. 2A).

Average IOP change versus respective time 0 (baseline) across the 5 days of measurements was -0.45 ± 0.12 mmHg for VEH, -6.06 ± 0.15 mmHg for NCX 470, and -3.60 ± 0.22 mmHg for LUM (Fig. 2B). Although the overall effect was stable throughout the experimental period, the largest decrease in the NCX 470 group was

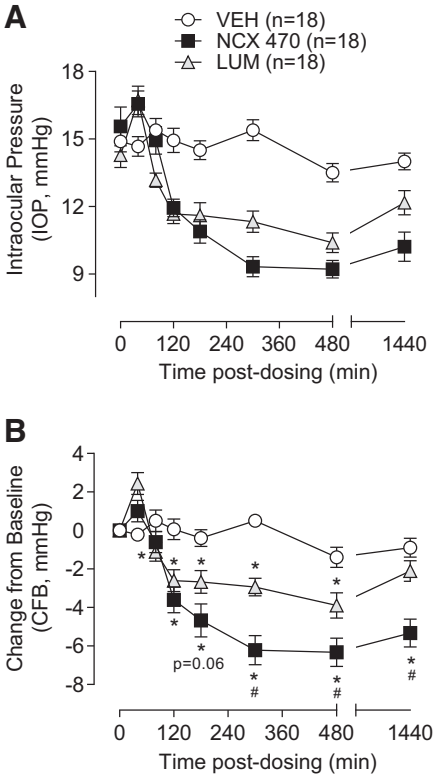

FIG. 1. NCX 470 and Lumigan® IOP-lowering efficacy in ocular normotensive Beagle dogs after single topical ocular administration. IOP **(A)** and CFB **(B)** after single ocular administration (30 μL/eye in both eyes) of NCX 470 ($n=18$), LUM ($n=18$) or VEH ($n=18$) in ocular normotensive Beagle dogs. CFB was calculated as: (IOP_{TX} – IOP_{T0}), where IOP_{TX} and IOP_{T0} are, respectively, the IOP at the indicated times and before dosing. Data are reported as mean ± SEM, $^{*}P<0.05$ versus VEH at the same time; $^{\#}P<0.05$ versus LUM at the same time, Multiple *t*-test with Welch correction. CFB, change from baseline; EDTA, ethylenediaminetetraacetic acid; IOP, intraocular pressure; LUM, Lumigan; SEM, standard error of the mean; VEH, vehicle.

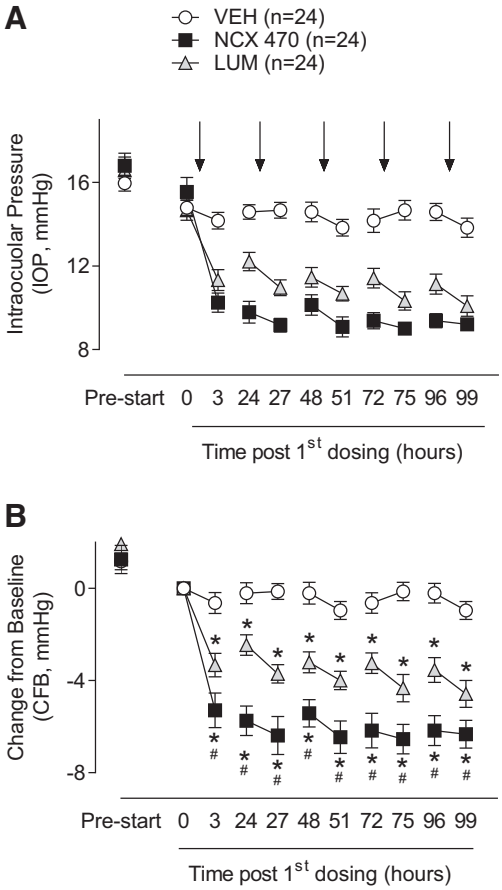

FIG. 2. NCX 470 and LUM IOP-lowering efficacy in ocular normotensive Beagle dogs after 5 days repeated daily topical ocular administration. IOP **(A)** and CFB **(B)** after 5 days repeated ocular administration (30 μL/eye in both eyes) of NCX 470 ($n=24$), LUM ($n=24$), or VEH ($n=24$) in ocular normotensive Beagle dogs. NCX 470, LUM, or VEH were administered once a day in the morning between 8:00 and 9:00 hours for 5 consecutive days. *Arrows* indicate VEH, NCX 470, or LUM dosing occasions. CFB was calculated as follows: (IOP_{TX}–IOP_{T0}), where IOP_{TX} and IOP_{T0} are, respectively, the IOP at the indicated times and before daily dosing. Data are reported as mean ± SEM, $^{*}P<0.05$ versus VEH at the same time; $^{\#}P<0.05$ versus LUM at the same time, Multiple *t*-test with Welch correction.

observed on day 4, 3 h postdose where the calculated CFB was −6.54 ± 0.64 mmHg. At the same time point, CFB for LUM was −4.33 ± 0.60 mmHg.

AHF, Cfl, and Fu in ocular normotensive NHP eyes after vehicle or NCX 470 topical ocular dosing

To calculate AHF, values for the cornea thickness and anterior chamber volume were obtained. Cornea thickness and anterior chamber volume were, respectively, 460 ± 20 μm ($n = 12$) and 105.8 ± 6.6 μL ($n = 12$) after VEH administration. Topical treatment with NCX 470 0.1% did not significantly change the cornea thickness (460 ± 10 μm) and anterior chamber volume (98.2 ± 4.2 μL).

AHF was 2.03 ± 0.22 μL/min, $n = 12$ after vehicle dosing confirming values already reported in literature.[26] Topical ocular dosing with NCX 470 did not modify AHF in our NHP (1.93 ± 0.31 μL/min, $n = 12$) (Fig. 3A).

Cfl increased substantially following NCX 470 0.1% dosing compared with that measured after VEH treatment. Specifically, Cfl in VEH-treated eyes was 0.37 ± 0.09 μL/min/mmHg, $n = 12$, whereas Cfl in NCX 470-treated eyes was 0.64 ± 0.17 μL/min/mmHg, $n = 7$ (Fig. 3B).

Finally, as given in Table 1, NCX 470 significantly increased Fu for EVP values ranging from 14 to 15.5 mmHg. Fu calculated at lower EVP values (<14 mmHg) resulted in negative Fu, whereas greater EVP values (>15.5 mmHg) gave an Fu higher than Fa thus were considered unsuitable.

Cfl ($C_{HTM/HSC}$) changes in TGFβ2-treated HTM/HSC constructs after vehicle, NCX 470, or bimatoprost exposure

Cfl ($C_{HTM/HSC}$) in naive 3D-HTM/HSC constructs was 0.73 ± 0.04 μL/min/mm^2/mmHg, $n = 5$ (Fig. 4). TGFβ2 treatment significantly diminished average $C_{HTM/HSC}$ to 0.47 ± 0.02 μL/min/mm^2/mmHg, $n = 5$ (Fig. 4). Of interest, NCX 470 (10 μM) effectively and significantly increased $C_{HTM/HSC}$ to 0.76 ± 0.03 μL/min/mm^2/mmHg, $n = 5$, thereby re-establishing physiological levels. Equimolar concentration of bimatoprost, although to a significantly lesser extent compared with NCX 470, also increased $C_{HTM/HSC}$ in this model (0.67 ± 0.04 μL/min/mm^2/mmHg, $n = 4$; Fig. 4).

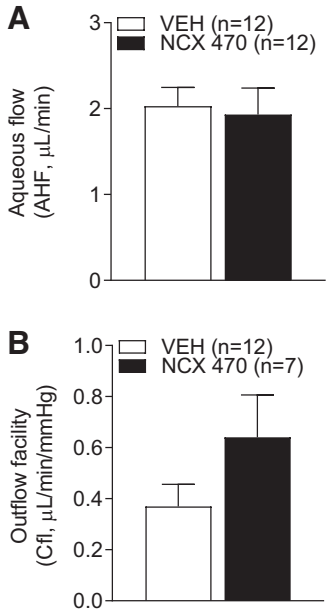

FIG. 3. Changes in AHF and Cfl after VEH or NCX 470 in ocular normotensive NHP. **(A)** and Cfl **(B)** were determined by fluorophotometry after twice daily dosing for 3 consecutive days with VEH or NCX 470 dissolved in the VEH above. Data are reported as mean ± SEM. AHF, aqueous humor flow; Cfl, outflow facility; NHP, nonhuman primates.

Discussion

Studies in patients with ocular hypertension or glaucoma have shown that NCX 470 reduces IOP in a dose-dependent manner.[4] Furthermore, repeated daily dose of this compound (0.1%) was recently found ''non-inferior'' to generic latanoprost in a large pivotal phase III study including $N = 328$ and $N = 333$ patients for the NCX 470 and latanoprost arm, respectively.[6]

In this study, clinically effective doses of NCX 470 dissolved in the same vehicle as in clinical phase III studies and administered topically as eye drops once a day in ocular normotensive Beagle dogs decreased IOP significantly more than Lumigan (bimatoprost ophthalmic solution, 0.01%—LUM). After a single AM dose, the largest IOP difference between NCX 470 and LUM was as high as −3.28 mmHg. NCX 470–mediated IOP reduction was −6.06 mmHg on average across testings with a peak effect recorded at 3 h on day 4 (CFB, −6.5 mmHg) making NCX 470 one of the most effective IOP-lowering agents in late clinical stage of development. This is particularly important in light of the relevance of each millimeter of mercury of IOP reduction at influencing the risk of developing a newly diagnosed glaucoma or the progression of a preexisting advanced glaucomatous pathological state.[30,31]

The robust IOP-lowering effect of NCX 470 observed in this and previous animal studies[8] as well as in human subjects[4] is hypothesized to involve (a) NO-mediated effects on TM/SC, pressure-dependent conventional Cfl, and (b) PGA-mediated effects on Fu. This study was structured to provide direct evidence of these effects as well as of potential activity of NCX 470 on AHF.

Table 1. Calculated Uveoscleral Outflow for Vehicle and NCX 470 as a Function of Increasing Episcleral Venous Pressure Values

	Uveoscleral outflow (Fu, μL/min)			
EVP (mmHg)	*Fu_{VEH} (n = 11)*	*$Fu_{NCX\ 470}$ (n = 6)*	*Fold increase*[a]	P
14	0.30 ± 0.29	1.01 ± 0.32	3.4	0.16
14.5	0.46 ± 0.27	1.27 ± 0.35	2.8	0.08
15	0.62 ± 0.26	1.53 ± 0.39	2.5	0.04
15.5	0.77 ± 0.26	1.79 ± 0.44	2.3	0.03

Data are expressed as mean ± SEM. *P*-value is calculated using Student's two-tailed paired *t*-test.

[a]Fold increase reflects the ratio between Fu_{NCX470} and Fu_{VEH}.

EVP, episcleral venous pressure; $Fu_{NCX\ 470}$, uveoscleral outflow for NCX 470; Fu_{VEH}, uveoscleral outflow for vehicle; SEM, standard error of the mean.

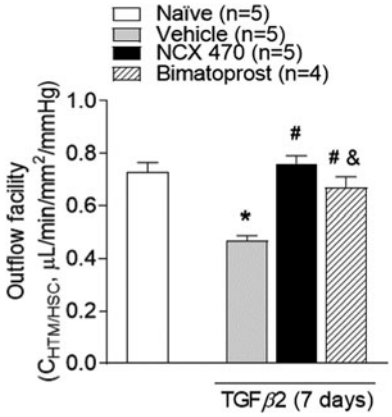

FIG. 4. Cfl ($C_{HTM/HSC}$) changes in TGFβ2-treated 3D-HTM/HSC™ constructs after treatment with vehicle, NCX 470, or bimatoprost. 3D-HTM/HSC constructs treated with TGFβ2 to mimic glaucomatous condition and decrease Cfl were exposed to vehicle (dimethyl sulfoxide, 1% final, $n=5$), NCX 470 (10 µM, $n=5$), or bimatoprost (10 µM, $n=4$) for 6 consecutive days (day 0, 3, and 6) and perfused on the day of testing (day 7). Data are reported as mean ± SEM, $^{*}P<0.05$ versus healthy; $^{\#}P<0.05$ versus vehicle; $^{\&}P<0.05$ versus NCX 470, Student's *t*-test.

As in previous studies,[32] Fu was derived from the Goldmann equation using various EVP values chosen to only have positive Fu values not exceeding the experimental AHF observed for these animals under our experimental conditions. This approach was undertaken because direct measurement of Fu is not possible in live NHP eyes as it would require surgery; likewise noninvasive measurement using venomanometry cannot be performed because the dense perilimbal pigmentation impedes visualization of the episcleral veins. Of interest, regardless of the EVP used, NCX 470 at an identical dose to that proposed for patients significantly increased Fu in our model. As mentioned previously, EVP was assumed constant over time regardless of NCX 470 or vehicle dosing in all our *in vivo* measurements; this is a clear limitation of our method as changes in EVP could have affected the entire AH dynamics and in particular, Fu, which is largely dependent on EVP.

NCX 470 is known to readily release bimatoprost and bimatoprost acid in target ocular tissues[8]; these compounds are able to stimulate PGA F2 alpha and/or prostamide receptors[33]; and modulate outflow resistance through the ciliary body by increasing matrix metalloproteinases in the tissue.[34] Various NO donors have been shown to relax the ciliary muscle[35] in various animal settings, thus suggesting that NO could have also affected NCX 470-mediated changes in Fu.

In this same series of experiments, although not significantly, Cfl was increased upon NCX 470 dosing likely via both NO- and bimatoprost-mediated mechanisms. NO has been shown to reduce IOP by stimulating AH drainage through the conventional outflow pathway specifically. NO relaxes TM cells via activation of soluble guanylyl cyclase (sGC)/cGMP signaling—indeed NO itself,[36,37] and sGC stimulators[38] have been shown consistently to lower IOP in various animal species and sGCα1 knockout mice that are unable to synthesize cGMP and have impaired conventional outflow were shown to have higher IOP compared with wild-type littermates.[39] NCX 470 was shown to progressively increase tissue cGMP content in eyes after topical ocular administration[8] making it possible that at least part of the overall effect of NCX 470 on Cfl is consequent to NO-mediated stimulation of this signaling pathway.

On the contrary, clinically effective doses of bimatoprost lower IOP partially via activation of TM/SC outflow pathway.[40,41] NCX 470 administered at the clinically effective dose (0.1%) used in this study releases about seven times the amount of bimatoprost available in commercial Lumigan (bimatoprost ophthalmic solution 0.01%); therefore, it is possible that part of NCX 470-mediated effects on Cfl can be attributed to bimatoprost. NCX 470-mediated effects on Cfl although pronounced, could not reach statistical significance; this might be attributable to insufficient sample size that we could not increase any further because of limited number of animals available for this particular study. Another possible explanation is that the anesthesia, which cannot be avoided in these experiments, could have blunted the response of TM cells.[42] In addition, the age of the animals used for this study could have also been a confounding factor as it is known that the overall outflow resistance varies as a function of age in living animals.[43]

In the bioengineered HTM/HSC constructs where $C_{HTM/HSC}$ can more easily be determined, we confirmed that NCX 470 increases $C_{HTM/HSC}$, an effect partially shared by bimatoprost, again suggesting that the effects of NCX 470 on this pathway counts on the concomitant action of bimatoprost and NO.

Previous studies were somewhat debatable on the effects of NO on the production of AH. Some studies reported a reduction of AHF in *ex vivo* preparation via stimulation of Na/K ATPase activity[44]; others reported a potential NO-mediated increase of AHF as a consequence of membrane potential depolarization.[25] In our study AHF measured *in vivo* remained unchanged following NCX 470 dosing suggesting that NO-mediated effects on AHF varies depending on the experimental conditions and thus it may not be the main pathway responsible for NCX 470-mediated IOP decrease.

In conclusion, our data point to NCX 470 as one of the most effective IOP-lowering agents currently in development with an efficacy exceeding that of other PGAs analogs. NCX 470-mediated IOP effects are attributable to the increase in Cfl and Fu with no effects on AH production.

Authors' Contributions

C.G.: Performed the experiment (equal); writing—original draft (equal); E.B.: Conceptualization (equal); writing—original draft (supporting); D.A.H.: writing—review and editing (supporting); C.T.: conceptualization (equal); S.F.: performed the experiment (supporting); A.U.: performed the experiment (supporting); F.A.: performed the experiment (supporting); writing—review and editing (supporting); K.Y.T.: writing—review and editing (supporting); F.I.: conceptualization (lead); writing—original draft (equal); data analysis (lead).

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Address correspondence to:
Dr. Corinna Galli
Nicox Research Institute
Via Ariosto 21
Bresso
Milan 20091
Italy

E-mail: galli@nicox.com