NCX 667, a Novel Nitric Oxide Donor, Lowers Intraocular Pressure in Rabbits, Dogs, and Non-Human Primates and Enhances TGFβ2-Induced Outflow in HTM/HSC Constructs

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Received: October 28, 2020
Accepted: February 1, 2021
Published: March 11, 2021

Citation: Bastia E, Toris CB, Brambilla S, et al. NCX 667, a novel nitric oxide donor, lowers intraocular pressure in rabbits, dogs, and non-human primates and enhances TGFβ2-induced outflow in HTM/HSC constructs. *Invest Ophthalmol Vis Sci.* 2021;62(3):17. https://doi.org/10.1167/iovs.62.3.17

Purpose. NCX 667, a novel nitric oxide (NO) donor with an isomannide core, was characterized for its IOP-lowering ability in animal models of ocular hypertension and glaucoma. Bioengineered human trabecular meshwork/Schlemm's canal (HTM/HSC) constructs were used to explore the mode of action.

Methods. Ocular normotensive New Zealand white (NZW) rabbits (ONT-rabbits), spontaneously ocular hypertensive pigmented Dutch-belted rabbits (sOHT-rabbits), hypertonic saline (5%)–induced transient ocular hypertensive NZW rabbits (tOHT-rabbits), ocular normotensive Beagle dogs (ONT-dogs), and laser-induced ocular hypertensive cynomolgus monkeys (OHT-monkeys) were used. NCX 667 or vehicle (30 µL) was instilled in a crossover, masked fashion and intraocular pressure (IOP) measured before dosing (baseline) and for several hours thereafter. The ONT-rabbits were used for cyclic guanosine monophosphate (cGMP) determination in ocular tissues after ocular dosing with NCX 667. Transforming growth factor-beta2 (TGFβ2) (2.5 ng/mL, six days)–treated HTM/HSC constructs were used to address changes in outflow facility.

Results. NCX 667 resulted in robust and dose-dependent IOP decrease in all models used. Maximal IOP-lowering efficacy at 1% was -4.1 ± 0.6, -12.2 ± 2.7, -10.5 ± 2.0, -5.3 ± 0.8, and -6.6 ± 1.9 mmHg, respectively, in ONT-dogs, sOHT-rabbits, tOHT-rabbits, ONT-rabbits, and OHT-monkeys. In ONT-rabbits NCX 667 (1%) increased cGMP in aqueous humor (AH) but not in retina and iris/ciliary body. NCX 667 concentration-dependently increased outflow facility in TGFβ2-treated HTM/HSC constructs (outflow facility, 0.10 ± 0.06 and 0.30 ± 0.10 µL/min/mmHg/mm^2, respectively, in vehicle- and NCX 667–treated constructs).

Conclusions. NCX 667 leads to robust IOP lowering in several animal models. Evidence in HTM/HSC constructs indicate that the IOP reduction likely results from NO-mediated increase of the conventional outflow pathway. Other mechanisms including changes in AH production and episcleral vein pressure may not be excluded at this time.

Keywords: nitric oxide, IOP, glaucoma, conventional outflow

Glaucoma comprises a set of optic neuropathies characterized by retinal ganglion cell loss and vision loss.[1] Increased intraocular pressure (IOP), typically resulting from an imbalance between aqueous humor (AH) secretion and its drainage through outflow pathways, remains the major risk factor for this disease.[2] However, the disorder can continue to progress even when IOP is lowered into the normal range,[3] making it important to test new potential therapeutic agents in both ocular normotensive and hypertensive conditions.

The ciliary body secretion of AH usually remains normal in glaucoma patients; whereas impaired AH drainage via the trabecular pathway caused by increased resistance of trabecular meshwork/Schlemm's canal pressure–dependent outflow (conventional pathway) is thought to be the primary cause of increased IOP in these patients.[4] Therefore novel

pharmacological agents that can improve the conventional outflow capacity are highly desirable.

Most glaucoma-approved medications either reduce AH secretion (i.e., β-blockers or carbonic anhydrase inhibitors, alpha$_2$ adrenergic agonists) or increase AH outflow via the uveoscleral outflow pathway (i.e., prostaglandin analogues), few (except netarsudil) directly target the conventional pathway.[5,6]

Converging evidence suggest that nitric oxide (NO), via activation of the soluble guanylyl cyclase (sGC) signaling pathway, reduces actomyosin contractility and cell adhesion. This in turn causes cell shape changes and relaxation of the trabecular meshwork (TM) and the inner wall of Schlemm's canal (SC), thereby leading to decreased resistance to AH outflow and reduction of IOP.[7] Furthermore, NO was shown to reduce AH formation and IOP via inhibition of the Na/K ATPase activity consequent to sGC stimulation.[8,9] Accordingly, a variety of drugs affecting NO/sGC signaling have been studied over the past few years, with some being registered for human use as IOP-lowering agents and others still in clinical development for the same ocular condition. These include Vyzulta (latanoprostene bunod ophthalmic solution, 0.024%), an NO-donating derivative of latanoprost that was first approved by the Food and Drug Administration in 2017[6] and is now available in many countries including Argentina, Canada, Hong Kong, Mexico, Columbia, Taiwan, Ukraine, and the United States, and NCX 470, a dual acting NO-donating derivative of bimatoprost currently in phase III clinical development for the reduction of ocular hypertension or glaucoma. Both compounds combine the IOP-lowering mechanism of action of a prostaglandin F2α analogue via enhancement of the uveoscleral pathway[10] with that of NO primarily increasing the TM/SC conventional outflow.[7] Latanoprostene bunod and NCX 470 are both largely safe and very effective IOP-lowering agents; these drugs were designed to release the actives on a 1:1 ratio where the amount of NO may not be adjustable independently from that of the prostaglandin F2α analogue. Likewise, sGC direct activators also were advanced into clinical development; MGV354 was shown to effectively reduce IOP in preclinical animal models[11] but was poorly efficacious when tested in humans.[12]

NCX 667 ((S)-((3R,3aR,6R,6aR)-6-hydroxyhexahydrofuro [3,2-b]furan-3-yl) 5,6-bis(nitrooxy)hexanoate), a New Chemical Entity[13] that comprises a NO-donating moiety esterified on the isomannide core, is currently under preclinical development as an IOP-lowering drug for the treatment of ocular hypertension and glaucoma. Here we report on the IOP-lowering ability of NCX 667 in various animal models comprising ocular normotensive models in rabbits (ONT-rabbits) and Beagle dogs (ONT-dogs), as well as ocular hypertensive [Dutch-belted rabbits; transient ocular hypertensive (tOHT) NZW-rabbits, and OHT-monkeys] models. In addition, albino and pigmented species were used to address potential differences caused by drug-melanin interaction.[14] All together these models are representative of different pathological conditions ranging from normotensive glaucoma to open-angle glaucoma and ocular hypertension or drug-mediated ocular hypertension. Furthermore, we investigated the molecular mechanism whereby NCX 667 lowers IOP by monitoring the accumulation of cGMP in ocular tissues of ONT-rabbits. Finally, we explored the effects of NCX 667 on conventional outflow using three-dimensional (3D) human trabecular meshwork/human Schlemm's canal (HTM/HSC) co-culture tissue constructs.[15] Despite being an in vitro model, the HTM/HSC was shown to be predictive of the modulatory activity of compounds on the conventional outflow pathway giving the opportunity to address the effects of new compounds directly in human tissues at early stages of development.[16,17]

Methods

NCX 667 Synthetic Pathway and Physicochemical Properties

NCX 667 ((S)-((3R,3aR,6R,6aR)-6-hydroxyhexahydrofuro [3,2-b]furan-3-yl)5,6-bis(nitrooxy)hexanoate) is a pale-yellow oil synthesized as follows: briefly, the carboxyl group of compound A in Figure 1 was protected as t-butyl ester; then compound B was submitted to enantioselective dihydroxylation with AD-mix-alpha (step 2, Fig. 1) to obtain the 5-(S) enantiomer (compound C). The nitration of the 5,6- diol with fuming HNO_3 in acetic anhydride as a solvent was used to make compound D (step 3, Fig. 1); compound E was then obtained by deprotection of the t-butyl ester (step 4, Fig. 1). At the same time, one of the two hydroxyl groups of commercial isomannide (compound F) was protected as tetrahydropyranyl ether (step 5, Fig. 1) affording compound G; finally, compound H was obtained by coupling compound E and compound G; NCX 667 was then obtained by deprotection with a yield of 10.7% (calculated on isomannide).

In Vitro Studies

Aqueous Humor Outflow in 3D HTM/HSC. The methods described originally by Torrejon et al.[15] were followed with minor modifications. Briefly, primary HTMs from various donors isolated from discarded (after keratoplasty) donor tissue rings were isolated and plated in 75 cm^2 cell culture flasks with 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO, USA) in improved MEM (Corning Cellgro, Manassas, VA, USA) with 0.1 mg/mL gentamicin. Similarly, the HSC cells were plated in 75 cm^2 cell culture flasks with 10% premium select FBS (Atlanta Biologicals, Lawrenceville, GA, USA) in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA) supplemented with penicillin (100 units/mL), streptomycin (0.1 mg/mL), and L-glutamine (0.292 mg/mL; Life Technologies) and maintained at 37°C in a humidified atmosphere with 5% carbon dioxide.

SU-8 2010 (MicroChem Corp., Westborough, MA, USA) was used to develop free-standing biomimetic porous microstructures to serve as scaffolds on which HTM cells were cultured. Scaffolds were fabricated as previously described using standard photolithographic techniques.[12] To create 3D-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 to 50,000 primary HTM cells. Once confluent, the HTM constructs were inverted and primary HSC cells (40,000 cells/sample) were cultured on the other side of the scaffold for 10 days. After the 3D-HTM/HSC constructs reached confluency, they were serum starved (1% FBS–Dulbecco's modified Eagle's medium) for one day before treatment with transforming growth factor-beta2 (TGFβ2, 2.5 ng/mL for six days to reduce outflow facility) followed by perfusion studies.[15] Constructs were perfused in an apical-to-basal direction with perfusion medium. The temperature

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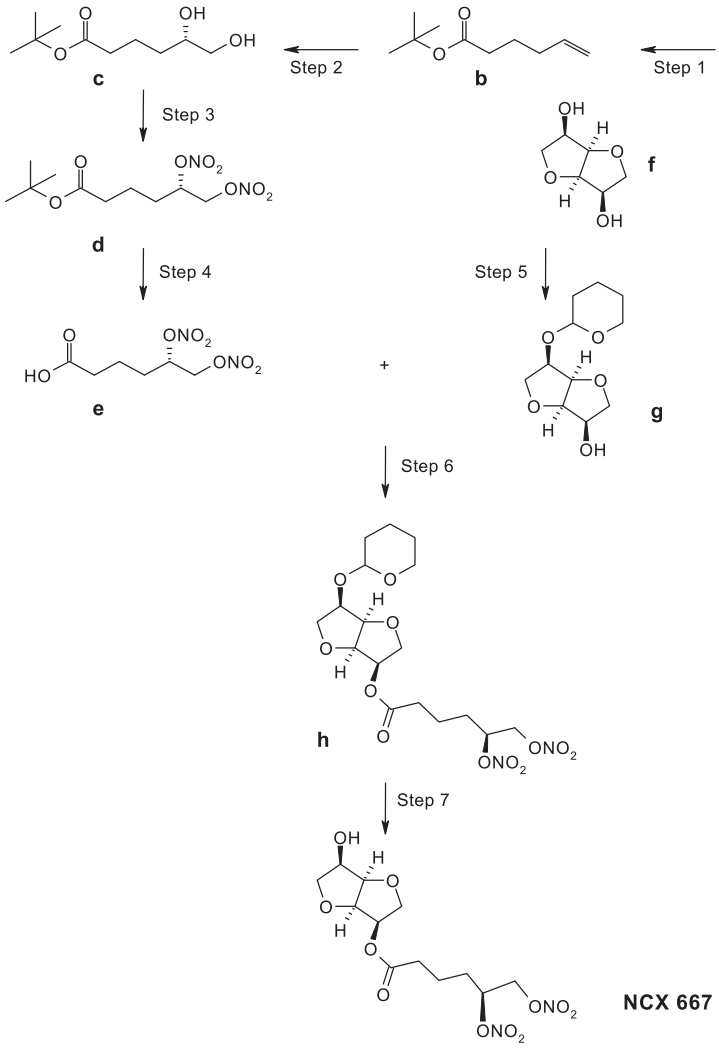

Figure 1. Synthetic pathway for NCX 667 ((S)-((3R,3aR,6R,6aR)-6-hydroxyhexahydrofuro[3,2-b]furan-3-yl) 5,6-bis(nitrooxy)hexanoate). Final yield of 10.7% (calculated on isomannide).

was maintained at 34°C throughout the experiment. Pressure was continuously monitored and recorded. After perfusion, the outflow facility of the constructs was calculated from the inverse of the slope of the pressure versus flow per unit surface area.

In Vivo Studies

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 various institutional Animal Care Committees, and all efforts were made to limit the number of animals and to minimize animal suffering. The procedures on New Zealand white (NZW) rabbits were approved by the animal Ethical and Care Committee of the University of Florence (Florence, Italy) and by the National Ethics Committee of the Italian Ministry of Health (Authorization number 318/2018-PR). These procedures were performed at the Center for Laboratory Animal Housing and Experimentation at the University of Florence. The non-human primate studies 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 dog studies were approved by the Institutional Animal Care and Use Committee and the committee for Animal Protection of the Ministry of Health of the Czech Republic. Experiments were conducted at the Meditox s.r.o. facility at Pod Zamken, Konarovice, Czech Republic.

NCX 667 was synthesized as summarized in Figure 1. Other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise specified.

Ocular Normotensive Rabbits (ONT-Rabbits) and Spontaneous Ocular Hypertensive Rabbits (sOHT-Rabbits). As in previous work,[18] male NZW rabbits weighting 1.5 to 2.0 kg or young (aged 30–40 weeks) male Dutch-belted rabbits weighting 1.0 to 1.5 kg were used. Animals were used for a single experiment and sacrificed by an overdose of sodium pentobarbital (Dolethal; Vetoquinol, MagnyVernois, France) at the end of each experimental session. IOP was determined using an applanation pneumatonometer (Model 30 Classic; Reichert, Depew, NY, USA). Measurements were taken before dosing (baseline) and at several time points thereafter by a masked investigator.

Vehicle [phosphate buffer pH 6.0, Kolliphor EL 5%, dimethyl sulfoxide (DMSO) 0.3%, benzalkonium chloride (BAC) 0.02%], and the actives were instilled into the conjunctival sac of the animals at the desired concentration in a 30 µL total volume. IOP changes versus vehicle and baseline (ΔΔIOP) were calculated as follows: (Drug IOP_{Tx} − Drug IOP_{T0}) − (Veh IOP_{Tx} − Veh IOP_{T0}) where IOP_{Tx} and IOP_{T0} are, respectively, the IOP at the time of interest and at baseline.

Ocular Normotensive Dogs (ONT-Dogs). Eight ocular normotensive (4 males, 4 females) beagles were used. Eyes of each animal were randomly treated with vehicle (30 µL) or actives at the indicated dose in a cross-over design. IOPs were determined in conscious animals using TonoVet (Icare Finland Oy, Finland) by a masked investigator. Measurements were made just before dosing and at 30, 60, 120, 240, 480, and 1440 minutes after dosing. IOP changes versus vehicle and baseline (ΔΔIOP) were calculated as described for experiments in sOHT-rabbits.

Transiently Ocular Hypertensive Rabbit Model (tOHT-Rabbits). A previously described method[19] was followed with minor modifications. Briefly, male NZW rabbits (N = 8/group) weighing 1.5 to 2.0 kg were anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg) intramuscularly and injected with 50 µL of hypertonic saline solution (5%, NaCl in water) into the vitreous body of both eyes. IOP was determined using a pneumatonometer (Model 30 Classic; Reichert) before injection of hypertonic saline (basal, time 0), as well as at 30, 60, 90, 180, and 240 minutes thereafter. Vehicle (phosphate buffer pH 6.0, Kolliphor EL 5%, DMSO 0.3%, BAC 0.02%), or NCX 667 at the indicated doses was instilled in one 30 μL drop into the conjunctival sac immediately after the injection of hypertonic saline into the eyes. Animals recovered to consciousness, and then IOP was monitored by an investigator masked to the treatment at the indicated time points. IOP changes versus vehicle and baseline (ΔΔIOP) were calculated as described for experiments in sOHT-rabbits.

Laser-Induced Ocular Hypertension in Non-Human Primates (OHT-Monkeys). Experiments were carried out as described in previous work.[20] Eight female cynomolgus monkeys between the ages of 10 and 22 years were used. All had unilateral laser treatment to the TM of the left eye made years earlier. IOP measurements were taken on separate sessions by investigators masked to the treatment groups. In a randomized masked fashion, the lasered eye of each animal was treated on day 1 with one 30 μL drop of vehicle or drug. One week later, the dosing order was reversed. Conscious animals were seated and treated topically with 30 μL of proparacaine HCl 0.13%. Tonometry was performed using a pneumatonometer (Model 30 classic; Reichert) connected to a PowerLab converter and a laptop computer. The treatment codes were broken only after all data were acquired and evaluations completed. IOP changes versus vehicle and baseline (ΔΔIOP) were calculated as described for experiments in sOHT-rabbits.

Cyclic Guanosine Monophosphate (cGMP) Quantitation in ONT-Rabbit Ocular Tissues. Levels of cGMP in AH, iris-ciliary bodies (ICB) and retina (RE) were determined as described previously with minor modification.[21] Briefly, REs, ICBs, and AHs were collected before treatment and at 30 and 60 minutes after vehicle or NCX 667 (1%) dosing; all animals were pre-treated (30 min) with subthreshold doses (0.03%) of YC-1, a soluble guanylyl cyclase stimulator, to enhance signal-to-noise ratio. The samples were processed according to the protocols specified in the cGMP ELISA kit (Catalog Number KA3389; Abnova Corporation, CA, USA). Specifically, ICBs and REs were homogenized in PBS buffer in presence of the PDE type-5 inhibitor avanafil (0.1 µM final concentration) and mixed with 95/5% water/trichloroacetate. The homogenates were centrifuged at 1500*g* and 48°C for 10 minutes to remove the precipitate. Similarly, AH samples were collected, avanafil (0.1 µM final concentration) added, and then diluted in five volumes of 95/5% water/TCA. The supernatants were later extracted with water-saturated ether, dried off at 70°C for five minutes, and assayed for cGMP content.

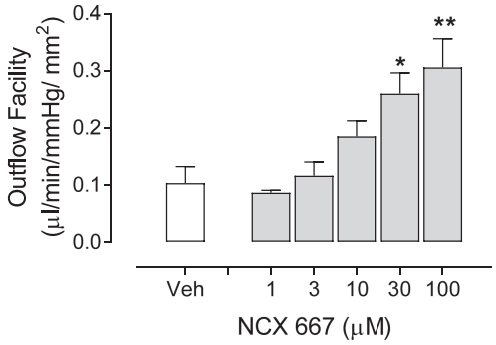

Figure 2. Concentration-dependent changes in outflow facility in 3D-HTM/HSC constructs. The "outflow facility" of the bioengineered 3D-HTM/HSC model was calculated mathematically from the flow rate and pressure data collected during the perfusion study. For each concentration n = 4–6 individual perfusion studies were conducted, and the data are shown as mean ± SEM. Differences between samples were analyzed using one-way analysis of variance followed by Tukey post-tests.

Results

In Vitro Pharmacology

Aqueous Humor Outflow Changes in 3D-HTM/HSC Constructs. The outflow facility of the bioengineered 3D-HTM/HSC was calculated mathematically from the flow rate, and pressure data were collected during the perfusion study. For each experimental group n ≥ 4 individual perfusion studies were conducted and the data reported as average ± SEM. As shown in Figure 2, in these series of experiments basal outflow in TGFβ2-stimulated conditions was 0.08 ± 0.04 µL/min/mmHg/mm^2. NCX 667 (1–100 µM) resulted in concentration-dependent increase of outflow facility reaching 0.31 ± 0.10 µL/min/mmHg/mm^2 at the highest concentration tested.

In Vivo Pharmacology

NCX 667-Mediated IOP-Lowering in Ocular Normotensive Conditions.

IOP-Lowering Effects in ONT-Rabbits. NCX 667 was first tested in ONT-rabbits for its ability to decrease IOP. Specifically, NCX 667 administered topically at 0.1% to 1% (N = 7–8) elicited a dose-dependent reduction of IOP (Fig. 3). Baseline IOP in these animals was indistinguishable among groups. The exposure to NCX 667 resulted in rapid IOP decrease reaching its maximum within 0.5 to 1 hour after dosing regardless of the dose used ($\Delta\Delta IOP_{60min}$, −5.3 ± 0.8,

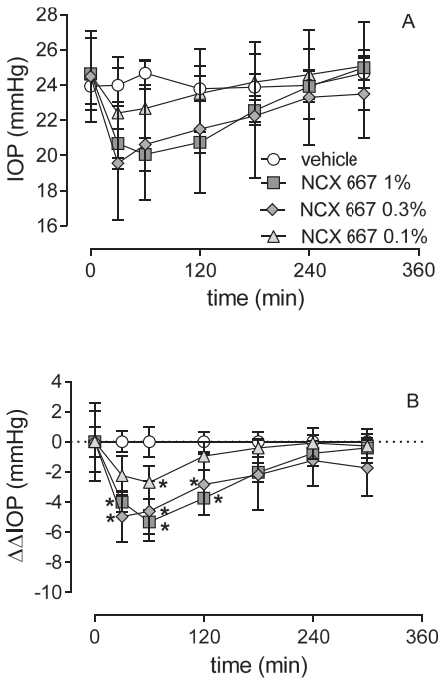

Figure 3. IOP-lowering effect of NCX 667 in ONT-rabbits. Intraocular pressures (**A**) and differences from vehicle (**B**) after instillation of NCX 667 (0.1%, N = 8; 0.3%, N = 8; 1%, N = 7) and the respective vehicle (phosphate buffer pH 6.0, Kolliphor EL 5%, DMSO 0.3%, BAC 0.02%, N = 5) were determined in ONT-rabbits. IOPs were determined using a pneumatonometer. Values reported at each time point result from two consecutive measurements taken one minute apart and averaged. The ΔΔIOP were calculated as follows: (Drug IOP_{Tx} − Drug IOP_{T0}) − (Veh IOP_{Tx} − Veh IOP_{T0}) where IOP_{Tx} and IOP_{T0} are, respectively, the IOP at the time of interest and at baseline. Data are reported as mean ± SEM. $^{*}P < 0.05$ versus the respective vehicle group, *t*-test multiple comparisons.

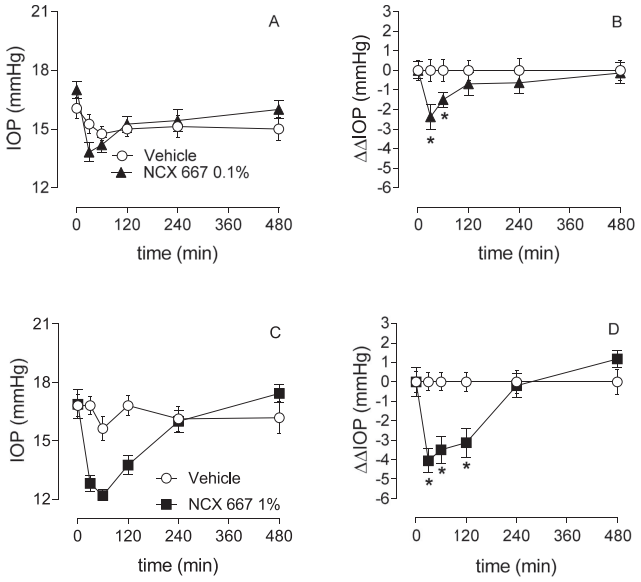

Figure 4. IOP-lowering effect in ONT-dogs. Intraocular pressure (**A, C**) and changes from vehicle and baseline (**B, D**) after instillation of NCX 667 (0.1%, and 1%) and the respective vehicle (phosphate buffer pH 6.0, Kolliphor EL 5%, DMSO 0.3%, BAC 0.02%) were determined in ONT-dogs (N = 8, four females and four males). ΔΔIOPs were calculated as follows: (Drug IOP_{Tx} − Drug IOP_{T0}) − (Veh IOP_{Tx} − Veh IOP_{T0}) where IOP_{Tx} and IOP_{T0} are, respectively, the IOP at the time of interest and at baseline. Data are reported as mean ± SEM. $^{*}P < 0.05$ versus the respective vehicle group, *t*-test multiple comparisons.

−4.6 ± 1.0, and −2.7 ± 0.4 mmHg after 1%, 0.3%, and 0.1% dose, respectively).

In parallel studies the dose of 1% was administered repeatedly to rabbits at one-hour intervals for several hours or twice daily for five days to address the desensitization and tolerance liability of this compound. Multiple dosing one hour apart resulted in a steady IOP decrease that lasted for the entire experimental session (ΔΔIOP, −3.1 ± 0.72, −3.4 ± 0.5, −2.6 ± 0.7, and −3.1 ± 0.7 mmHg at one, two, three, and four hours after dosing), suggesting that the response does not desensitize after the initial challenge. Likewise, repeated twice-daily dosing also resulted in similar responses over time ($\Delta\Delta IOP_{60min}$, −3.6 ± 1.0 and −3.4 ± 0.6 mmHg on day one and day five, respectively).

IOP-Lowering Effects in ONT-Dogs. The IOP-lowering activity of NCX 667 (1% and 0.1% 30 µL AM dosing) was compared to that of vehicle at 30, 60, 120, 240, and 480 minutes after dosing in ONT-dogs in a masked crossover design (N = 8; four males and four females). Regardless of the group, baseline IOP did not differ significantly (16.1 ± 0.5, 17.4 ± 0.4, and 17 ± 0.4 mmHg for vehicle, 1% and 0.1% dose, respectively). Similar to that observed in ONT-rabbits, in ONT-dogs NCX 667 elicited a dose-dependent decrease of IOP that was maximal between 30 and 60 minutes after dosing ($\Delta\Delta IOP_{30min}$, −3.2 ± 0.5 and −2.4 ± 0.6 mmHg for 1% and 0.1%, respectively) (Fig. 4).

NCX 667-Mediated IOP lowering in Ocular Hypertensive Conditions.

IOP-Lowering Effects in sOHT- and tOHT-Rabbits. Young (aged 30–40 weeks) sOHT-rabbits (N = 8/group) known to have high basal IOP,[22] and tOHT-rabbits (N = 8/group) were first used to address the ocular hypotensive effects of NCX 667 in ocular hypertensive conditions and the influence of melanin binding in the overall IOP-lowering effects of NCX 667. In this series of experiments, 1% NCX 667 was compared to vehicle at different time points after dosing.

In young sOHT-rabbits, baseline IOPs were similar between vehicle and treated eyes (33.0 ± 1.0 and 35.8 ± 1.5 mmHg in vehicle and NCX 667, respectively). The administration of 1% NCX 667 resulted in a robust IOP decrease (−26%) one hour after dosing that was not seen in vehicle treated eyes (Table).

The intravitreal administration of 50 µL hypertonic (5%) saline resulted in a transient IOP rise at 30 minutes (34.4 ± 0.7 and 34.8 ± 2.1 mmHg before administration of vehicle or NCX 667, respectively). The IOP slowly decreased over the following hours and reached baseline values at 300 minutes after injection of saline. The exposure to 1% NCX 667 significantly attenuated the saline−induced IOP rise throughout the experimental period. The effects were maximal at 60 minutes with an IOP difference versus vehicle of −10.5 ± 2.0 mmHg (Table).

IOP-Lowering Effects in Laser-Induced OHT-Monkeys. Efficacy studies in ocular hypertensive OHT-monkeys (N = 8, females) were conducted to address the IOP-lowering activity of AM dosing of NCX 667 (1%, 30 µL topical drop) in an advanced disease model of glaucoma and ocular hypertension. Measurements were recorded before dosing the animals (baseline) and 30, 60, 180, 300, 480, and 1440

Table. IOP-Lowering Effects of NCX 667 in Ocular Hypertensive Rabbits and Ocular Hypertensive Non-Human Primates

	sOHT-Rabbits (mmHg)	tOHT-Rabbits (mmHg)	OHT-Monkeys (mmHg)
Vehicle			
Baseline	33.0 ± 1.0	34.4 ± 0.7[a]	33.0 ± 2.4
ΔIOP_{60min}	2.8 ± 2.4	−4.6 ± 0.5[a]	−0.8 ± 1.3
$\Delta\Delta IOP_{60min}$	0.0 ± 2.4	0.0 ± 0.5	0.0 ± 1.3
NCX 667 (1%)			
Baseline	35.8 ± 1.5	34.8 ± 2.1[a]	33.4 ± 3.0
ΔIOP_{60min}	−9.4 ± 2.7*	−15.2 ± 2.0[a,*]	−7.4 ± 1.9*
$\Delta\Delta IOP_{60min}$	−12.2 ± 2.7*	−10.5 ± 2.0*	−6.6 ± 1.9*

ΔIOP were calculated as follows: $IOP_{Tx} - IOP_{T0}$ where IOP_{Tx} and IOP_{T0} are, respectively, the IOP at the time of interest and at baseline.
ΔΔIOP were calculated as follows: (Drug IOP_{Tx} − Drug IOP_{T0}) − (Veh IOP_{Tx} − Veh IOP_{T0}) where IOP_{Tx} and IOP_{T0} are, respectively, the IOP at the time of interest and at baseline.
[a] Values refer to IOP measured 10 minutes after the IVT injection (50 µL) of hypertonic (5%) saline.
* $P < 0.05$ versus the respective vehicle group, *t*-test multiple comparisons.

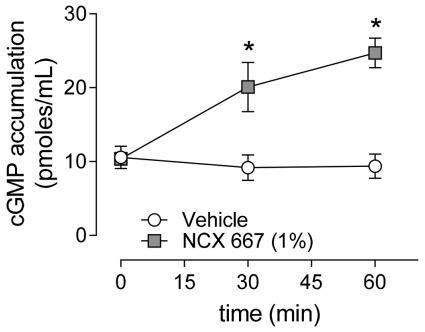

Figure 5. Changes in cGMP in ocular tissues after NCX 677 topical dosing in ONT-rabbits. The cGMP levels after topical dosing of NCX 667 (1%) were monitored in AH (**A**) or iris/ciliary body and retina (**B**) of ONT-rabbits at the indicated time points. Data are reported as mean ± SEM, n = 3 eyes/time point. *$P < 0.05$ versus respective vehicle; two-way analysis of variance followed by Dunnett's multiple comparisons test.

minutes thereafter. Baseline IOP readings did not differ significantly between eyes later assigned to vehicle or NCX 667 (33.0 ± 2.4 and 33.4 ± 3.0 mmHg for vehicle and NCX 667 groups, respectively).

In these animals NCX 667 was progressively effective starting 30 minutes after dosing to reach the maximum IOP-lowering activity between 30 and 60 minutes after dosing (Table). The effects were still evident 300 minutes after dosing and returned to baseline values at later time points.

NCX 667-Mediated cGMP Formation After Single Ocular Dosing in ONT-Rabbits. The levels of cGMP were determined as a surrogate marker of NO release in ocular target tissues. Little or no changes in cGMP levels were detected over time after vehicle treatment. Interestingly, cGMP doubled at 30 minutes after NCX 667 (1%) dosing (9.2 ± 1.8 and 20.1 ± 3.34 pmol/mL, vehicle and NCX 667, respectively) and continued to increase at 60 minutes (9.4 ± 1.3 and 24.7 ± 2.0 pmol/mL, vehicle and NCX 667, respectively) in AH. Conversely, cGMP levels were 0.78 ± 0.12 and 0.31 ± 0.01 pmol/mg protein, respectively, in retina and iris/ciliary body and remained virtually unchanged after NCX 667 treatment (Fig. 5).

Discussion

NCX 667, a new chemical entity[13] comprising a di-NO-releasing moiety anchored onto the isomannide core, is currently considered for the reduction of ocular hypertension and glaucoma. The compound is stable for several weeks when it is kept at room temperature in the dark and for up to two years in refrigerated conditions (data not shown); furthermore, NCX 667 is soluble at effective concentrations in various conditions consistent with a topical ophthalmic formulation. In ONT-rabbits and ONT-dogs, NCX 667 rapidly and effectively lowered IOP when dosed between 0.1% to 1%. Topical dosing of some NO-releasing molecules but not others was shown to result in tolerance liability and tachyphylaxis,[23] a characteristic that would greatly hamper the potential clinical use of this class of molecules in diseases requiring long-term dosing such as glaucoma. In ONT-rabbits the repeated administration one hour apart from a fully effective dose of NCX 667 (1%) retained its initial IOP-lowering effect, as did the repeated administration of the compound twice-daily for several days, suggesting that NCX 667 does not result in tachyphylaxis and tolerance under these experimental conditions. Indeed, latanoprostene bunod (Vyzulta), a dual-acting NO-donating prostaglandin using similar NO-based technology retained its IOP-lowering efficacy over a 12-month of daily administration in patients.[24]

In ONT-rabbits, the ocular administration of NCX 667 progressively increased the levels of cGMP in AH, whereas no changes were observed in other ocular tissues, including iris/ciliary body and retina, suggesting that TM or SC sGC activity could account for the observed changes in cGMP levels found in AH. Consistently, NO is known to elicit TM and SC relaxation.[25] Furthermore, NCX 667 diminished the transient ocular hypertensive response elicited by the intravitreal injection of hypertonic saline in eyes of tOHT-rabbits. A similar response was observed when NCX 667 was administered to sOHT-rabbits. Similar to findings in patients with glaucoma and elevated IOP,[26] ocular hypertension in these models is accompanied by diminished cGMP levels in ocular tissues compared to ocular normotensive conditions (data not shown), suggesting that NCX 667 may, indeed, lower IOP by direct stimulation of the sGC/cGMP signaling pathway in these animals.

We then studied NCX 667 in ONT-dogs and laser-induced OHT-monkeys, a model of ocular hypertension known to respond to the activation of the NO signaling cascade.[18,27] In ONT-dogs NCX 667 progressively and dose-dependently lowered IOP to reach its maximum effects between 30 and 60 minutes after dosing. In OHT-monkeys, NCX 667 effectively controlled IOP after morning dosing with a time-action reminiscent of that observed in other models and an overall efficacy comparable to that reported for other

NO-donating compounds including latanoprostene bunod[27] and NCX 470.[18] In all testing conditions and doses NCX 667 did not result in appreciable eye redness or ocular discomfort as assessed by visual inspection confirming that this, as other NO-donating compounds,[28] is well tolerated. High sustained concentrations of NO has been reported to cause neurotoxicity.[29] Although no specific investigations were made to address potential cytotoxicity concerns caused by NO release from NCX 667, retinal damage seems unlikely to occur given previous experience with similar compounds administered topically to patients over several months; however, additional studies are needed to better define the safety profile of this compound. Early investigations have shown that NO may reduce AH formation;[8,9] however, more recent studies suggest that NO lowers IOP predominantly via increased conventional outflow facility.[7,30,31] Human, bioengineered HTM/HSC constructs stimulated with TGFβ2 have proven to be an effective model retaining all biological and physiological features of HTM/HSC found in vivo[16]; moreover, this model seems to have many features relevant to glaucoma pathophysiology,[16] making it particularly relevant to study compounds presumably affecting, among other pathways, the TM/SC physiology at the cellular level. Here we provide evidence that NCX 667 increases TGFβ2-induced outflow facility in bioengineered HTM/HSC constructs in a concentration-dependent fashion, suggesting that the NO/soluble guanylyl cyclase signaling pathway stimulation resulting in TM and SC relaxation is likely a major mechanism involved in the acute IOP-lowering activity of NCX 667. The episcleral veins are in the distal part of the conventional outflow pathway that begins with the TM.[32] Consequently, episcleral venous pressure (EVP) has a significant role in the rate of AH drainage via this pathway and, hence, IOP control. In this study we did not address the effects of NCX 667 on EVP; thus changes in EVP cannot entirely be excluded at this point; however, given the well-established role of NO on the peripheral vascular bed, the possibility that NCX 667-mediated IOP changes depend on EVP modification seems unlikely because the application of NCX 667 should have caused a dilation of the episcleral vasculature, mostly of the arterioles, with a consequent increase in EVP and in IOP as shown for other NO donors.[33] Similarly, after exposing the animals to the nonspecific NO synthase inhibitor L-NAME,[34] vasoconstriction, decreased EVP, and decreased IOP were observed, further confirming the lack of involvement of this specific pathway on the overall effects of NCX 667. Moreover, the complete lack of hyperemia after single and repeated NCX 667 dosing confirms the concept that NCX 667–mediated effects on IOP are the result of its activity on TM/SC outflow rather than on the episcleral vasculature where vasodilation would have likely been accompanied by ocular hyperemia.

As mentioned above, an alternative pathway shown to be modulated by NO involves AH production.[8,9] The lack of cGMP changes in ciliary bodies would argue that NCX 667 has little, if any, effects on this pathway.

In summary, these data provide functional evidence that NCX 667 by virtue of its NO-donating ability lowers IOP regardless of baseline IOP values and of the animal species used, suggesting that this compound may be useful to treat ocular normotensive and hypertensive conditions in humans. Moreover, our data suggest that the IOP-lowering effect of this compound is mainly mediated via an increase in conventional outflow facility.

Acknowledgments

The authors thank Angela Kothe and José Boyer for comments and suggestions that greatly improved the overall manuscript.

Funding information specifying that no fundings were received for this work.

Disclosure: **E. Bastia,** Nicox (E); **C.B. Toris,** Nicox (F); **S. Brambilla,** Nicox (E); **C. Galli,** Nicox (E); **N. Almirante,** Nicox (E); **M.V.W. Bergamini,** Nicox (C); **E. Masini,** Nicox (F); **S. Sgambellone,** (N); **A.M. Unser,** Glauconix (E), **F. Ahmed,** Glauconix (E), Nicox (F); **K.Y. Torrejon,** Glauconix (E), Nicox (F); **T. Navratil,** Nicox, (E); **F. Impagnatiello,** Nicox (E)

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