Full manuscript title: Cutamesine (SA4503) Protects Retinal Ganglion Cells in an Ocular Hypertension Model of Glaucoma Determined Using DARC Technology and RBPMS Cell Marker

Short running title: Cutamesine-Induced RGC Protection

Deleted: Cutamesine-Induced Neuroprotection of RGCs

Authors: Najam A. Sharif,[1-9] Takashi Ota,[10]* Takazumi Taniguchi,[10] Masaaki Sasaoka[10], Li Guo,[2] Soyoung Choi,[2,11] Vy Luong[2] and M. Francesca Cordeiro[2,3,11]

[1] Ophthalmology Innovation Center, Santen Inc., Emeryville, CA, USA.
[2] Institute of Ophthalmology, University College London (UCL), London, UK.
[3] Imperial College of Science and Technology, St. Mary's Campus, London, UK.
[4] Eye-ACP Duke-National University of Singapore Medical School, Singapore.
[5] Singapore Eye Research Institute (SERI), Singapore.
[6] Department of Pharmacy Sciences, Creighton University, Omaha, NE, USA.
[7] Department of Pharmacology and Neuroscience, University of North Texas Health Sciences Center, Fort Worth, TX, USA.
[8] Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Texas Southern University, Houston, TX, USA.
[9] Global Research and Development, Nanoscope Therapeutics Inc., Dallas, TX, USA.
[10] Ophthalmology Innovation Center, Santen Pharmaceutical Co., Ltd., Nara, Japan.
[11] Novai Ltd., Work Life, The White Building, 33 King's Road, Reading, RG1 3AR, UK.

*** Correspondence to:** Takashi Ota, PhD
Ophthalmology Innovation Center, Santen Pharmaceutical Co., Ltd.
8916-16 Takayama-cho, Ikoma-shi, Nara 630-0101, Japan
takashi.ota@santen.com; nsharif@nanostherapeutics.com

Word count: 3982 (not including abstract, figure legends and references)

Formatted: Font: Bold

Key words: DARC; Cutamesine; Ocular hypertension; Sigma-1 receptor; Retinal ganglion cells; Rotenone.

Abstract

Purpose: This study aimed to evaluate the neuroprotective effects of cutamesine (SA4503), a potent sigma-1-receptor agonist (S1R-agonist), in rat models of retinal degeneration induced by elevated intraocular pressure (IOP) using the Detection of Apoptosing Retinal Cells (DARC) technology. A secondary aim was to test its effect in a rat retinal oxidative stress model.

Methods: Ocular hypertension (OHT) model was induced in Dark Agouti rats via episcleral vein injection of hypertonic saline, while a retinal oxidative stress was induced in Sprague-Dawley rats by intravitreal rotenone injection. In the OHT model, cutamesine (10 nmol) and recombinant human nerve growth factor (rh-NGF [positive control]; 0.09 nmol), were intravitreally administered. Their effects were evaluated using DARC technology and RNA-binding protein with multiple splicing (RBPMS) immunohistochemistry. In the oxidative stress model, cutamesine (10 and 300 nmol) was co-administered with rotenone, and neurofilament light chain (Nfl) gene expression was measured by RT-PCR.

Results: OHT induced a significant elevation of IOP over 3 weeks, peaked at day 1 ($p<0.001$), and gradually decreased by day 21. Cutamesine significantly reduced the number of DARC spots ($p<0.05$) and preserved retinal ganglion cells (RGCs) labeled with RBPMS ($p<0.01$), similar to rh-NGF ($p<0.01$). In the oxidative stress model, cutamesine preserved retinal Nfl expression levels in a dose-dependent manner.

Conclusions: Cutamesine demonstrated significant neuroprotective activity in rat models of OHT and oxidative stress using DARC and RBPMS labeling techniques. These findings provide further evidence that S1R-agonists possess substantial neuroprotective potential and may be beneficial for patients with OHT/glaucoma.

Commented [TO1]: Shortened to <250 words
Deleted: e
Deleted: primary
Deleted: of this study was
Deleted: novel
Deleted: the drug
Deleted: An o
Deleted: established
Deleted: by injecting hypertonic saline into the
Deleted: s
Deleted: of Dark Agouti rats,
Deleted: model
Deleted: (IVT)
Deleted: of rotenone in Sprague-Dawley rats
Deleted: IVT
Deleted: , and their
Deleted:).
Deleted: Rh
Deleted: also showed significant neuroprotective effects
Deleted: retinal degeneration induced by

1. Introduction

Major visual impairment is caused worldwide by a group of degenerative eye diseases, grouped under the term "glaucoma".[1] Several types of glaucoma prevail but their hallmark characteristic is the optic nerve damage that precedes retinal ganglion cell (RGC) demise and loss of their axonal connections to the visual centers in the brain.[1–4] The classic reduction of the optic disc and enlargement of the optic cup observed via ophthalmic fundus examination and optical coherence tomography (OCT) signifies and correlates with the peripheral, followed by peripheral-to-central, visual field loss experienced by glaucoma patients. Since over 75 million people suffer from the most prevalent types of glaucoma (open-angle glaucoma [OAG] and angle-closure glaucoma [ACG]),[5] it is imperative that research into and a better understanding of the etiological factors responsible for these disorders continues to be vigorously pursued. Furthermore, patients afflicted with neurodegenerative retinal diseases await novel treatment options to stave off further vision impairment and possible blindness.

One of the most prominent and common risk factors connected with OAG, ACG, and secondary forms of glaucoma is elevated intraocular pressure (IOP).[1–6] Indeed, preclinical rodent-based and clinical data have provided strong evidence that optic nerve damage is highly correlated with increases in IOP or ocular hypertension (OHT).[7–9] However, there are many patients who have normal IOPs who continue to experience RGC loss, optic nerve damage and vision deterioration.[1,6] Accordingly, many IOP-independent risk factors (e.g., neuroinflammation, immunologic attacks, protein misfolding, low retinal blood flow leading to oxidative stress, neurotrophin [e.g., nerve growth factor, NGF] deprivation, O_2 and energy depletion, excitotoxicity and structural stress factors) are increasingly thought to play a pivotal role in glaucoma pathology and suitable alternative therapeutic modalities need to be developed and approved for patient care.[1–6,10–12] Therefore, pharmaceutical, nutraceutical, electrical stimulation and gene therapy that directly provide protection and preservation of RGCs, their axons and retina-brain connections are being sought in order to combat glaucomatous optic neuropathy.[3,6,12–16] It is with this aim that we sought to use a novel diagnostic/prognostic technology (Detecting-Apoptosing-Retinal-Cell [DARC]) to evaluate the neuroprotective activity of two classes of compounds when delivered in close proximity to the retina via intravitreal (IVT) injections. DARC exploits annexin-5 labeled with fluorescent dyes (e.g. Alexa-Fluor 488 or DY-776 [ANX776]), which has been successfully utilized in several pre-clinical[17–20] and clinical[2,21] studies to quantify the number of cells in the retina that exhibit initial signs of cell stress and apoptotic cell death.[17–21] When coupled with cellular markers of RGCs (e.g., Brn3a or RNA binding protein with multiple splicing [RBPMS]), DARC technology has been previously shown to be a useful biomarker for measuring RGC death in models of retinal degeneration and RGC preservation by adenosine A3 agonists and other classes of compounds.[18,20,22] Additionally, many compound classes protect against oxidative stress where neurofilament light chain (Nfl) gene expression was used as a useful cell death biomarker.[23]

Test compounds have previously been administered topical ocularly[18,24,25] or intraperitoneally to determine their potential neuroprotective properties.[22] In the current study, the test compounds (a sigma-1 receptor agonist, cutamesine and recombinant human NGF [rh-NGF]) were administered IVT in a masked manner, and their effects were assessed with the DARC reagent which was delivered intranasally. This new delivery approach was of interest in order to refine the procedures and permit future screening efforts whereby the DARC reagent would be made available less invasively than in the past where it was injected intravenously.[18,20,22]

After obtaining positive efficacy data from the afore-mentioned DARC-based studies performed at University College London (UK), additional investigations with cutamesine were conducted in order to assess its ability to impart neuroprotection in a rat retinal oxidative stress model of glaucoma. Thus, we studied the ability of cutamesine to protect RGCs from retinal oxidative stress using IVT-delivered rotenone, an oxidizing agent[23], as a challenging insult since glaucoma is a multi-factorial disease where hypoxia/ischemia induced by low retinal blood perfusion are recognized as major culprits.[1,3,6,10,11] However, this study was considered confirmatory for evaluating the efficacy of cutamesine in another animal model of glaucoma. Unfortunately, due to cost, labor, time and geographic location-constraints of DARC technology availability, the rotenone-based study was conducted at Santen Pharmaceutical's facility in Japan, and Nfl gene expression measured by RT-PCR was used for RGC detection and quantification. The Nfl expression system was successfully utilized as a quantitative biomarker to assess protective efficacy of numerous classes of compounds in the rotenone-induced oxidative stress model of RGC death.[23] Since cutamesine is a highly selective sigma-1 receptor agonist with proven efficacy in a Phase II clinical trial of stroke[26] where neuronal death occurs, we wished to study its efficacy in two different animal models of glaucomatous retinal damage.

2. Materials and Methods

All animals were treated in compliance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. For the DARC technology experiments and OHT model, the use of animals was approved by the local ethics committee at the University College London Institute of Ophthalmology and adhered to the United Kingdom Home Office regulations for the care and use of laboratory animals, the United Kingdom Animals (Scientific Procedures) Act (1986). For the rotenone-induced oxidative stress model studies, the use of animals was approved and monitored by the Institutional Animal Care and Use Committee of Santen Pharmaceutical Co., Ltd. and adhered to "Basic Policies for the Conduct of Animal Experiments in Research Institutions" issued by the Ministry of Health, Labor and Welfare, Japan (2006), and "The Guidelines for Proper Conduct of Animal Experiments" published by the Science Council of Japan (2006). Every effort was made to avoid unnecessary use of laboratory animals. All studies were conducted in a masked manner.

2.1. DARC technology experiments and OHT model

Randomized experimental design: In total, 20 male Dark Agouti (DA) rats aged 8-10 weeks were used in this study. Fifteen rats were randomly divided into three blocks (n = 5 / group). Each block contained all three treatments, i.e., OHT-only (as negative control), OHT + rh-NGF (as positive control), and OHT + cutamesine. Three rats (six eyes) served as normal controls. Two additional rats were used to substitute for unexpected retinal abnormality and insufficient DARC dosing.

In Vivo Work: Each rat was anesthetized by intraperitoneal injections of ketamine (37.5%)/Domitor (25%; Pfizer Animal Health, Exton, PA) solution (0.75 mL ketamine, 0.5 mL Domitor, and 0.75 mL sterile water) at 0.1 mL/100 g.[17] All animals had surgically elevated IOP in the left eye by injection of 50 µL of hypertonic saline solution (1.85 M) into the episcleral veins.[27,28] Animals undergoing treatment (all groups except OHT-only) each received IVT drug administration (4 µL) in the left eye on the day of OHT surgery (day 1). All test compounds were dissolved in phosphate buffered saline (PBS; vehicle composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4; pH 7.4) and provided to the technician in a masked manner. Rats received a second intravitreal injection of the appropriate compound or vehicle eleven days later. IOPs were measured in both eyes before surgery (baseline) and day 1, weeks 1, 2, and 3 after surgery using a Tonolab tonometer, and 10 readings were collected from each measurement. In our experiments, IOP exhibited a rapid elevation within the first 24 hours followed by a gradual decline toward baseline over 21 days. This IOP profile differs from the classical hypertonic saline-induced ocular hypertension model,[29,30] which typically produces a sustained elevation of IOP for several weeks to months. This difference may be attributable to technical variation or strain-specific factors. Notably, the classical model employed Brown Norway rats, whereas we used Dark Agouti rats. However, similar IOP profiles have been reported in our previous studies using the same model,[31,32] supporting the reproducibility of this response in our experimental conditions. Despite the transient nature of the IOP elevation, histological and molecular analyses confirmed RGC stress and apoptosis, thereby validating the model for assessing the neuroprotective effects of various compounds. The rat eyes were imaged *in vivo*, using DARC for RGC apoptosis in both eyes at baseline and week 3, where fluorescently labeled annexin-5 was intranasally administrated 2 hours before imaging.[2,15,20,21,25] The retinal imaging was performed using the Heidelberg Retinal Angiograph Spectralis (HRA+OCT Spectralis, Heidelberg Engineering, Germany) as previously described utilized.[2,18,21,22,25] Animals were then sacrificed, their eyes enucleated and perfused in 4% paraformaldehyde (PFA) overnight then stored in PBS until retinal dissection for retinal histologic assessment.

The IVT doses of cutamesine (10 nmol; a high-affinity and potency S1R-A with half-maximal affinity constant [IC_{50}] of 17 nM) and rh-NGF (0.09 nmol; $EC_{50} < 1$ nM) were selected based on prior *in vitro* and *in vivo* experiments conducted by the investigators and based on the compound affinities, potencies and efficacies of the compounds from the literature (see ahead). Since the inner limiting membrane in the back of the eye is permeable to molecules up to 150 kDa molecular mass, it was anticipated that sufficient amounts of both cutamesine and rh-NGF would be able to reach the retina,[25] especially the inner most retinal neurons, RGCs, upon IVT injections.

The second IVT injection was contemplated to ensure that both compounds were present at sufficiently high concentrations to exert their biological functions. These aspects are described and discussed in more detail in the Discussion section.

Immunohistochemistry and imaging of retinae: RNA-binding protein with multiple splicing (RBPMS) is a highly selective marker of RGCs.[31,33] For detecting and correlating RGCs in the current studies, whole retinae were dissected from both eyes of each animal and immuno-stained with an anti-RBPMS antibody to assess RGC survival. Briefly, samples were washed in PBS and 0.5% Triton X-100 (Sigma-Aldrich, UK) then permeabilized through freezing at −80 °C and thawing at room temperature. Samples underwent a blocking process through incubation in 5% normal goat serum (Sigma-Aldrich, UK) in phosphate buffer (PB, 0.1 M). The guinea pig anti-RBPMS antibody was diluted at 1:250 in bovine serum albumin (BSA) solution. The samples were incubated for two days. Samples were then washed in PBS and 0.5% Triton X-100 and incubated in the secondary antibody solution (goat anti-guinea pig 647 nm, Alexa Fluor Invitrogen™ at 1:250 dilution in BSA solution. Samples underwent a final washing process and then were flat-mounted using Mowiol (Sigma-Aldrich, UK) and a coverslip (Merck, UK) onto microscope slides. These were then imaged using an automatic stage imaging set-up of a fluorescent microscope (Olympus BX40, Windsor, UK with a 10 × Olympus lens; 1 pixel = 0.636 μm) with the 647 nm filter. Several series of small, tiled areas from different regions of the retinae were image-captured and the labeled RGCs automatically quantified. The process was repeated for all the retinal samples from all treatment groups, and the mean ± SEMs calculated, and the data plotted.

Data Analysis: DARC spots on *in vivo* images were automatically counted by an algorithm, recently developed and validated in the Cordeiro lab.[2,18,21,22,25] The DARC count was defined as the number of annexin-positive spots seen in the retinal image at 120 minutes at Week 3 after baseline spot subtraction. The greater the DARC spot number means the greater the cell apoptosis. RBPMS^{+ve} RGCs were counted and analyzed as described above.

Statistical Analysis: All data were analyzed with a Student's t-test or one-way ANOVA with Dunnett's multiple comparisons tests, comparing treatments to control groups using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data were presented as means ± SEM or SDM as indicated in figure legends, and $p < 0.05$ was considered statistically significant.

2.2. Rotenone-induced oxidative stress model studies

Rotenone is a naturally occurring and broad-spectrum pesticide that inhibits the activity of NADH dehydrogenase in the mitochondrial respiratory chain complex I. Because of this unique biological activity, rotenone has been used as a versatile tool to study involvement of mitochondrial functions and oxidative stress in neuronal cell death including in the retina.[23]

Animals and intravitreal injections: Adult male Sprague-Dawley rats (6 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The environment was kept at 23 ± 3 °C with a 12-hour light and a 12-hour dark cycle. All rats were allowed food and water *ad libitum*, and they were acclimatized to the environment for at least 1 week prior to the experiment. Each rat was anesthetized with inhalation of isoflurane (3-4% for induction and 1-2% for maintenance). IVT injections were made via a 33-G needle connected to a 25 µL microsyringe (Hamilton company, Reno, NV, USA). The needle penetrated the eye from the nasal sclera at 1-2 mm posterior to the limbus, and was inserted toward the optic disc. Both eyes of each animal received a single injection of 5-µL solution containing vehicle or rotenone (2 nmol/eye). For concomitant injection of either rotenone or with cutamesine, both chemicals were premixed and a 5-µL aliquot of resultant solution was administered in the same way as described above. All injections were performed under a binocular microscope and care was taken not to injure the lens or retina during the procedure. As seen in our earlier studies, a bilateral approach was taken to minimize the number of animals used and sacrificed for this study. This study utilized 2-3 rats/group with bilateral IVT injections and thus data were obtained from n = 4-6 eyes/group. Twenty-four hours following IVT injection, the animals were sacrificed using intraperitoneally administered pentobarbital at a very high dose, and the eyes immediately isolated. They were subjected to further assays as described in the sections below.

Real-time PCR: The retinae were isolated and immediately immersed in RNA later® (Qiagen, Hilden, Germany). On the day of RNA extraction, each sample was transferred to a 2-mL tube containing a 0.5-mL QIAzol lysis reagent and a zirconia bead (Qiagen, Hilden, Germany), and rigorously homogenized for 5 min at 25 Hz using a TissueLyzer (Qiagen, Hilden, Germany). Total RNA was extracted individually from each retina, according to the rest of the protocol for a RNeasy 96 Kit provided by the manufacturer (Qiagen, Hilden, Germany). First strand cDNA was prepared using 0.2 µg of total RNA in a reagent mixture containing PrimeScript RT enzyme, oligo-dT primers and random 6-mers (PrimeScript RT reagent Kit, Takara, Shiga, Japan). An aliquot of resultant cDNA was added to a master mix of either QuantiTect or QuantiFast Multiplex PCR kit (Qiagen, Hilden, Germany), and real-time PCR was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City) according to the manufacturer's instructions. A pre-designed primer-probe mixture for Gapdh (Applied Biosystems, Foster City, CA, USA) or neurofilament light-chain (Nfl) (Sigma-Aldrich, St. Louis, MO, USA) was used for this reaction.[23] The sequences of forward and reverse primers used for Nfl were 5'-ACAAGCAGAATGCAGACATCA-3' and 5'-GGAGGTCCTGGTACTCCTTC-3', respectively, and the sequence of TaqMan® probe was [FAM] 5'- CCATCTCGCTCTTCGTGCTTCGC -3' [BHQ-1]. The sequences of primers and probe for Gapdh are not available, because they are kept

Deleted: Qiazol

undisclosed by the manufacturer. The real-time PCR conditions were the cycling conditions of 50 °C for 2 min and 95 °C for 15 min followed by 40 cycles of 94 °C for 1 min and 60 °C for 1 min. Fluorescence intensity at every annealing step was captured and threshold cycle time (C_T) values was determined using the 7500 software. The comparative C_T method was deployed to determine Nfl expression relative to that of Gapdh. The relative Nfl expression levels were further normalized to the vehicle group. All the latter procedures were performed according to previously reported methods.[23]

Statistical analysis: Each value represents the mean ± S.E.M. Statistical analyses were performed using EXSUS software version 10.0.7 and SAS version 9.4 (EPS Corporation, Tokyo, Japan) in accordance with the manufacturer's instructions. Student's t-test was performed to compare the values between two groups. For multiple comparison, Dunnett's multiple comparison test was used. Differences were assumed to be statistically significant when $p < 0.05$.

3. Results

3.1. DARC technology-based studies:

1. IOP profiles: The hypertonic saline-induced OHT resulted in significant elevation of IOP profiles of operated OS eyes in all animals over 3 weeks, compared to the contralateral OD controls (Fig. 1A). The levels of IOP peaked at day 1 ($p < 0.001$), and gradually decreased at day 7 ($p < 0.01$), and day 14 ($p < 0.01$) compared to the OD control eyes, and finally returned to baseline levels by day 21 (Fig. 1A). IOP profiles in Fig. 1B are presented as OS minus OD in different treatment groups and exhibited the same pattern as in Fig. 1A, with no significant difference between the groups. These data suggested that all compounds (test and positive control) did not

affect IOP in any group of animals and all subsequent treatments' effects were independent of IOP changes.

2. Neuroprotection: The computerized image analyses of the DARC-based neuronal loss in retinae of the rats subjected to OHT was successfully executed by the algorithm (see the un-annotated image in Fig. 2A, compared with the algorithm-based detection of the retinal cells undergoing apoptosis in Fig. 2B). Compared with the controls, there was a significantly increased number of DARC-labeled retinal spots in the OHT only group (Figs. 3A and 3C). IVT delivery of the positive control agent (rh-NGF), and cutamesine (SA4503), significantly reduced the DARC counts compared to the OHT only group, being 100% and 83% of controls, respectively (Fig. 3A), suggestive of protection from OHT-induced elevated retinal cell apoptosis. As expected, RBPMS^{+ve} RGCs were significantly reduced due to OHT as compared to naïve controls ($p < 0.01$) (Fig. 3B). Here, rh-NGF ($p < 0.01$) and cutamesine ($p < 0.01$) resulted in significantly higher RBPMS^{+ve} RGC density compared to the OHT-only group, suggestive of successful neuroprotection (90-98% of baseline control) from OHT-induced RGC loss (Figs. 3B and 3C). Representative images of RBPMS^{+ve} RGCs for these groups are shown in Fig. 3D, while the corresponding quantitative data for RGC counts are presented in Fig. 3B.

3.2. Rotenone-induced retinal degeneration studies:

Compared to vehicle-treated animals, IVT delivery of rotenone (2 nmol/eye) to rats decreased the expression level of Nfl to 45.1 ± 2.8%. Cutamesine at IVT doses of 10 and 300 nmol/eye preserved Nfl expression to 64.4 ± 4.9% and 95.4 ± 3.4% of vehicle-treated animals, respectively ($p < 0.01$ and 0.001, respectively) (Fig. 4). Here, the positive control agents, N-methyl-D-aspartate glutamatergic channel blockers (MK-801 and memantine; 10 and 100 nmol final, respectively; IVT delivered) protected/preserved Nfl expression by >90% akin to cutamesine (Fig. 4).[23]

4. Discussion

The current studies assessed the ability of a small molecule (cutamesine; SA4503; 1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl) piperazine dihydrochloride) and a medium sized polypeptide neurotrophic agent (rh-NGF) to penetrate the inner limiting retinal membrane and to protect RGCs from elevated IOP-induced RGC death. The hypertonic saline injection into the episcleral veins is a highly reproducible animal model of OHT and OAG.[27,28,31] We demonstrated its robust effectiveness to raise IOP in the rats and its temporal profile in our investigations (Figs. 1A and 1B). Our data obtained from nasally delivered DARC technology and immuno-staining procedures provided clear evidence of protection of retinal cells including RGCs by cutamesine and rh-NGF that were highly statistically significant relative to the untreated controls (Figs. 2 and 3). These results were further supported by the fact that cutamesine preserved RGCs in one of the non-IOP-induced rat retinal degeneration models where rotenone was used to induce oxidative stress (Fig. 4).

There are literature reports pertaining to sigma-1 receptor agonists (e.g., (+)-pentazocine; SKF-10,047; pridopidine) exerting neuroprotective actions on RGCs under different insult conditions,[34–39] and others such as fluvoxamine reducing the profibrotic effects of transforming growth factor-β2 in mouse trabecular meshwork cells.[40] However, cutamesine has not been tested previously in any of these assays or *in vivo* ocular investigations until our current studies, and it has a different chemical structure compared to other older generation sigma-1 receptor agonists (Fig. 6). Cutamesine is a selective high-affinity (IC_{50} = 17 nM) sigma-1 receptor agonist having a

100-fold lower affinity for the sigma-2 receptor.[41,42] It interacts with the sigma-1 receptors which serve as chaperones and which are located in the nuclear envelope and mitochondria of every retinal cell type and are abundant in the ganglion cell layer of the rat retina.[43] The neuroprotective mechanisms recruited by the activated sigma-1-receptor and the endoplasmic reticulum-based inositol trisphosphate receptor (ryanodine receptor) engagement encompass the reduction of intracellular Ca^{2+},[37,44–46] reduction of nitric oxide production,[47] decreasing glutamatergic ligand-induced neurotoxicity,[48,49] dampening of the caspase-activated production of inflammatory cytokines by optic nerve-head astrocytes,[49] and hence inhibition of local inflammation and cellular swelling.[50,51] Additionally, positive properties of cutamesine include its ability to enhance neurite outgrowth,[52–54] up-regulation of the early response kinase-1/2,[55] the protective transcription factor Bcl-2 and that of activation of the anti-oxidant transcription factor Nrf2 in the retina.[56] The ability of cutamesine to protect photoreceptors,[42,57] inner cochlear hair cells,[58] and cortical neurons from oxidative stress[59] and its neuroprotective functions against glutamate-induced cell death in retinal neurons[60] support its neuroprotective activity towards RGCs that we observed *in vivo* in two models of retinal degeneration (Figs. 3 and 4). Pridopidine and (+)-pentazocine also have recently been shown to exert similar characteristics to cutamesine in the rat microbead-induced model of OHT[61] and in other animal models of glaucoma, including optic nerve crush.[34,36,62] These comparative studies and the additional evidence that sigma-1 receptor agonists increase production of brain-derived growth factor,[63,64] which may contribute to the preservation of RGCs and their axons, highlight the potential of sigma-1 receptor agonists in preserving RGCs and suggest that cutamesine may offer similar or superior neuroprotective effects. Also, the beneficial effects of cutamesine in stroke patients have already been mentioned.[26]

Collectively, the described studies for cutamesine using elevated IOP-induced and oxidative stress-induced rat models of retinal pathology and using the DARC biomarker and RGC-specific labeling and Nfl gene expression readouts have provided new information about the role of sigma-1 receptor in directly protecting and preserving RGCs. The literature cited above suggests multiple mechanisms of action of this class of compounds at cellular and molecular levels (Figs. 5 and 6). Such data provide an impetus for creating novel compounds with multi-functional pharmacological properties via conjugation/hybrid generation.[65,66] These novel agents may offer higher potency and therapeutic indices than the existing singular compounds including ifenprodil and its analogs[67–69] and of course the other bona fide sigma-1 receptor agonists.[70–74] Likewise, drug formulation technologies that provide platforms to co-deliver multiple compounds[4,6,14,75] and to extend the duration of activity of the latter to mitigate retinal neurodegeneration should prove useful in the quest to halt RGC and thalamic/visual cortical neuronal demise.[2,75] Since sigma-1 receptor agonists appear to also lower IOP,[76,77] it is tempting to suggest that such compounds either on their own or when complexed with or co-delivered with other neuroprotective ocular hypotensive drugs such as betaxolol or brimonidine,[11,78] proteinaceous therapeutics,[79] and/or genetic cargos[80] may offer solutions to tackle both the elevated IOPs and the RGC loss experienced by OHT/glaucoma patients.[1,3,6,10–13] We await and hope for rapid progress in this realm since patients need novel drugs to help treat their diseases of the retina, especially glaucomatous optic neuropathy.[1–12]

Authorship contribution statement

N.A.S.: Conceptualization; Resources; Writing−original draft; Writing–review and editing; Supervision. T.O.: Validation; Data curation; Writing–review and editing; Visualization; Project Administration. T.T.: Validation; Data curation; Writing–review and editing; Visualization; Project Administration. M.S.: Methodology; Validation; Formal Analysis; Investigation; Data curation; Writing–review and editing; Project Administration. L.G., S.C. and V.L.: Validation; Formal Analysis; Investigation; Data curation; Writing–review and editing; Visualization. MFC: Resources; Methodology; Writing–review and editing; Supervision.

Funding Source

The DARC study was funded by Santen Pharmaceutical Co., Ltd. and conducted by Novai Ltd. at University College London (London, UK).

Declaration of Competing Interest

The authors declare that there is no known competing financial interest. At the time of the study, N.A.S. and M.S. were employees of Santen Inc. and Santen Pharmaceutical Co., Ltd., respectively. Currently, N.A.S. is an employee of Nanoscope Therapeutics Inc. (Dallas, TX), and M.S. is an employee of Shionogi & Co., Ltd. (Osaka, Japan).

Acknowledgement

Authors thank Tomomi Kohara for technical assistance in rotenone-induced oxidative stress model, Dr. Yasuko Yamamoto for preparing the cutamesine solution, and Dr. Takahiro Akaishi for supporting the statistical analysis.

References

1. Jonas JB, Aung T, Bourne RR, et al. Glaucoma. Lancet 2017;390(10108):2183–2193; doi: 10.1016/S0140-6736(17)31469-1.
2. Cordeiro MF, Normando EM, Cardoso MJ, et al. Real-time imaging of single neuronal cell apoptosis in patients with glaucoma. Brain 2017;140(6):1757–1767; doi: 10.1093/brain/awx088.
3. Weinreb RN, Aung T, Medeiros FA. The Pathophysiology and Treatment of Glaucoma. JAMA 2014;311(18):1901; doi: 10.1001/jama.2014.3192.
4. Anonymous. European Glaucoma Society Terminology and Guidelines for Glaucoma, 5th Edition. Br J Ophthalmol 2021;105(Suppl 1):1–169; doi: 10.1136/bjophthalmol-2021-egsguidelines.
5. Tham Y-CC, Li X, Wong TY, et al. Global Prevalence of Glaucoma and Projections of Glaucoma Burden through 2040. Ophthalmology 2014;121(11):2081–2090; doi: 10.1016/j.ophtha.2014.05.013.
6. Sharif NA. Glaucomatous optic neuropathy treatment options: the promise of novel therapeutics, techniques and tools to help preserve vision. Neural Regen Res 2018;13(7):1145; doi: 10.4103/1673-5374.235017.
7. Guo L, Moss SE, Alexander RA, et al. Retinal Ganglion Cell Apoptosis in Glaucoma Is Related to Intraocular Pressure and IOP-Induced Effects on Extracellular Matrix. Investig Opthalmology Vis Sci 2005;46(1):175; doi: 10.1167/iovs.04-0832.
8. Leske MC, Heijl A, Hyman L, et al. Factors for progression and glaucoma treatment: the Early Manifest Glaucoma Trial. Curr Opin Ophthalmol 2004;15(2):102–6; doi: 10.1097/00055735-200404000-00008.
9. Leske MC, Heijl A, Hyman L, et al. Predictors of Long-term Progression in the Early Manifest Glaucoma Trial. Ophthalmology 2007;114(11):1965–1972; doi: 10.1016/j.ophtha.2007.03.016.
10. Calkins DJ, Horner PJ. The Cell and Molecular Biology of Glaucoma: Axonopathy and the Brain. Investig Opthalmology Vis Sci 2012;53(5):2482; doi: 10.1167/iovs.12-9483i.
11. Howell GR, MacNicoll KH, Braine CE, et al. Combinatorial targeting of early pathways profoundly inhibits neurodegeneration in a mouse model of glaucoma. Neurobiol Dis 2014;71:44–52; doi: 10.1016/j.nbd.2014.07.016.
12. Levin LA, Patrick C, Choudry NB, et al. Neuroprotection in neurodegenerations of the brain and eye: Lessons from the past and directions for the future. Front Neurol 2022;13:964197; doi: 10.3389/fneur.2022.964197.
13. Bucolo C, Platania CBM, Drago F, et al. Novel Therapeutics in Glaucoma Management. Curr Neuropharmacol 2018;16(7):978–992; doi: 10.2174/1570159X15666170915142727.
14. He S, Stankowska DL, Ellis DZ, et al. Targets of neuroprotection in glaucoma. J Ocul Pharmacol Ther 2018;34(1–2):85–106; doi: 10.1089/jop.2017.0041.
15. Hill D, Compagnoni C, Cordeiro MF. Investigational neuroprotective compounds in clinical trials for retinal disease. Expert Opin Investig Drugs 2021;30(5):571–577; doi: 10.1080/13543784.2021.1896701.
16. Sharif NA. Therapeutic Drugs and Devices for Tackling Ocular Hypertension and Glaucoma, and Need for Neuroprotection and Cytoprotective Therapies. Front Pharmacol 2021;12:729249; doi: 10.3389/fphar.2021.729249.
17. Cordeiro MF, Guo L, Luong V, et al. Real-time imaging of single nerve cell apoptosis in

retinal neurodegeneration. Proc Natl Acad Sci 2004;101(36):13352–13356; doi: 10.1073/pnas.0405479101.
18. Davis BM, Tian K, Pahlitzsch M, et al. Topical Coenzyme Q10 demonstrates mitochondrial-mediated neuroprotection in a rodent model of ocular hypertension. Mitochondrion 2017;36:114–123; doi: 10.1016/j.mito.2017.05.010.
19. Guo L, Cordeiro MF. Assessment of neuroprotection in the retina with DARC. Prog Brain Res 2008;173:437–50; doi: 10.1016/S0079-6123(08)01130-8.
20. Nizari S, Guo L, Davis BM, et al. Non-amyloidogenic effects of α2 adrenergic agonists: implications for brimonidine-mediated neuroprotection. Cell Death Dis 2016;7(12):e2514–e2514; doi: 10.1038/cddis.2016.397.
21. Normando EM, Yap TE, Maddison J, et al. A CNN-aided method to predict glaucoma progression using DARC (Detection of Apoptosing Retinal Cells). Expert Rev Mol Diagn 2020;20(7):737–748; doi: 10.1080/14737159.2020.1758067.
22. Galvao J, Elvas F, Martins T, et al. Adenosine A3 receptor activation is neuroprotective against retinal neurodegeneration. Exp Eye Res 2015;140:65–74; doi: 10.1016/j.exer.2015.08.009.
23. Sasaoka M, Ota T, Kageyama M. Rotenone-induced inner retinal degeneration via presynaptic activation of voltage-dependent sodium and L-type calcium channels in rats. Sci Rep 2020;10(1):969; doi: 10.1038/s41598-020-57638-y.
24. Davis BM, Pahlitzsch M, Guo L, et al. Topical Curcumin Nanocarriers are Neuroprotective in Eye Disease. Sci Rep 2018;8(1):11066; doi: 10.1038/s41598-018-29393-8.
25. Guo L, Davis BM, Ravindran N, et al. Topical recombinant human Nerve growth factor (rh-NGF) is neuroprotective to retinal ganglion cells by targeting secondary degeneration. Sci Rep 2020;10(1):3375; doi: 10.1038/s41598-020-60427-2.
26. Urfer R, Moebius HJ, Skoloudik D, et al. Phase II trial of the sigma-1 receptor agonist cutamesine (SA4503) for recovery enhancement after acute ischemic stroke. Stroke 2014;45(11):3304–3310; doi: 10.1161/STROKEAHA.114.005835.
27. Morrison JC, Moore CG, Deppmeier LMH, et al. A rat model of chronic pressure-induced optic nerve damage. Exp Eye Res 1997;64(1):85–96; doi: 10.1006/exer.1996.0184.
28. Husain S, Ahmad A, Singh S, et al. PI3K/Akt Pathway: A Role in δ-Opioid Receptor–Mediated RGC Neuroprotection. Investig Opthalmology Vis Sci 2017;58(14):6489; doi: 10.1167/iovs.16-20673.
29. Tezel G, Yang X, Cai J. Proteomic identification of oxidatively modified retinal proteins in a chronic pressure-induced rat model of glaucoma. Invest Ophthalmol Vis Sci 2005;46(9):3177–87; doi: 10.1167/iovs.05-0208.
30. Morrison JC, Cepurna WO, Johnson EC. Modeling glaucoma in rats by sclerosing aqueous outflow pathways to elevate intraocular pressure. Exp Eye Res 2015;141:23–32; doi: 10.1016/j.exer.2015.05.012.
31. Taniguchi T, Akaishi T, Hata T, et al. Felodipine re-positioned as a neuroprotectant via improved optic nerve head blood circulation in retinal ischemic rabbits and ocular hypertensive rats. Sci Rep 2025;15(1):23811; doi: 10.1038/s41598-025-09733-1.
32. Guo L, Normando EM, Nizari S, et al. Tracking longitudinal retinal changes in experimental ocular hypertension using the cSLO and spectral domain-OCT. Invest Ophthalmol Vis Sci 2010;51(12):6504–13; doi: 10.1167/iovs.10-5551.
33. Rodriguez AR, de Sevilla Müller LP, Brecha NC. The RNA binding protein RBPMS is a

selective marker of ganglion cells in the mammalian retina. J Comp Neurol 2014;522(6):1411–1443; doi: 10.1002/cne.23521.

34. Geva M, Gershoni-Emek N, Naia L, et al. Neuroprotection of retinal ganglion cells by the sigma-1 receptor agonist pridopidine in models of experimental glaucoma. Sci Rep 2021;11(1):21975; doi: 10.1038/s41598-021-01077-w.
35. Ha Y, Dun Y, Thangaraju M, et al. Sigma Receptor 1 Modulates Endoplasmic Reticulum Stress in Retinal Neurons. Investig Opthalmology Vis Sci 2011;52(1):527; doi: 10.1167/iovs.10-5731.
36. Li L, He S, Liu Y, et al. Sigma-1R Protects Retinal Ganglion Cells in Optic Nerve Crush Model for Glaucoma. Investig Opthalmology Vis Sci 2021;62(10):17; doi: 10.1167/iovs.62.10.17.
37. Mueller BH, Park Y, Daudt DR, et al. Sigma-1 receptor stimulation attenuates calcium influx through activated L-type Voltage Gated Calcium Channels in purified retinal ganglion cells. Exp Eye Res 2013;107:21–31; doi: 10.1016/j.exer.2012.11.002.
38. Smith SB, Duplantier J, Dun Y, et al. In Vivo Protection against Retinal Neurodegeneration by Sigma Receptor 1 Ligand (+)-Pentazocine. Investig Opthalmology Vis Sci 2008;49(9):4154; doi: 10.1167/iovs.08-1824.
39. Smith SB, Wang J, Cui X, et al. Sigma 1 receptor: A novel therapeutic target in retinal disease. Prog Retin Eye Res 2018;67:130–149; doi: 10.1016/j.preteyeres.2018.07.003.
40. Tran MN, Medveczki T, Besztercei B, et al. Sigma-1 Receptor Activation Is Protective against TGFβ2-Induced Extracellular Matrix Changes in Human Trabecular Meshwork Cells. Life 2023;13(7):1581; doi: 10.3390/life13071581.
41. Matsuno K, Nakazawa M, Okamoto K, et al. Binding properties of SA4503, a novel and selective σ1 receptor agonist. Eur J Pharmacol 1996;306(1–3):271–279; doi: 10.1016/0014-2999(96)00201-4.
42. Shimazawa M, Sugitani S, Inoue Y, et al. Effect of a sigma-1 receptor agonist, cutamesine dihydrochloride (SA4503), on photoreceptor cell death against light-induced damage. Exp Eye Res 2015;132:64–72; doi: 10.1016/j.exer.2015.01.017.
43. Wang W-F, Ishiwata K, Kiyosawa M, et al. Visualization of Sigma1 Receptors in Eyes by ex vivo Autoradiography and in vivo Positron Emission Tomography. Exp Eye Res 2002;75(6):723–730; doi: 10.1006/exer.2002.2048.
44. Hayashi T, Su T-P. Sigma-1 Receptor Chaperones at the ER- Mitochondrion Interface Regulate Ca2+ Signaling and Cell Survival. Cell 2007;131(3):596–610; doi: 10.1016/j.cell.2007.08.036.
45. Srivats S, Balasuriya D, Pasche M, et al. Sigma1 receptors inhibit store-operated Ca2+ entry by attenuating coupling of STIM1 to Orai1. J Cell Biol 2016;213(1):65–79; doi: 10.1083/jcb.201506022.
46. Tchedre KT, Huang R-Q, Dibas A, et al. Sigma-1 Receptor Regulation of Voltage-Gated Calcium Channels Involves a Direct Interaction. Investig Opthalmology Vis Sci 2008;49(11):4993; doi: 10.1167/iovs.08-1867.
47. Vagnerova K, Hurn PD, Bhardwaj A, et al. Sigma 1 Receptor Agonists Act as Neuroprotective Drugs Through Inhibition of Inducible Nitric Oxide Synthase. Anesth Analg 2006;103(2):430–434; doi: 10.1213/01.ane.0000226133.85114.91.
48. DeCoster MA, Klette KL, Knight ES, et al. σ receptor-mediated neuroprotection against glutamate toxicity in primary rat neuronal cultures. Brain Res 1995;671(1):45–53; doi: 10.1016/0006-8993(94)01294-R.

49. Martin PM, Ola MS, Agarwal N, et al. The sigma receptor ligand (+)−pentazocine prevents apoptotic retinal ganglion cell death induced in vitro by homocysteine and glutamate. Mol Brain Res 2004;123(1–2):66–75; doi: 10.1016/j.molbrainres.2003.12.019.
50. Bogár F, Fülöp L, Penke B. Novel Therapeutic Target for Prevention of Neurodegenerative Diseases: Modulation of Neuroinflammation with Sig-1R Ligands. Biomolecules 2022;12(3):363; doi: 10.3390/biom12030363.
51. Vogler S, Winters H, Pannicke T, et al. Sigma-1 receptor activation inhibits osmotic swelling of rat retinal glial (Müller) cells by transactivation of glutamatergic and purinergic receptors. Neurosci Lett 2016;610:13–18; doi: 10.1016/j.neulet.2015.10.042.
52. Brimson JM, Safrany ST, Qassam H, et al. Dipentylammonium Binds to the Sigma-1 Receptor and Protects Against Glutamate Toxicity, Attenuates Dopamine Toxicity and Potentiates Neurite Outgrowth in Various Cultured Cell Lines. Neurotox Res 2018;34(2):263–272; doi: 10.1007/s12640-018-9883-5.
53. Kimura Y, Fujita Y, Shibata K, et al. Sigma-1 Receptor Enhances Neurite Elongation of Cerebellar Granule Neurons via TrkB Signaling. Hetman M. ed. PLoS One 2013;8(10):e75760; doi: 10.1371/journal.pone.0075760.
54. Rossi D, Marra A, Picconi P, et al. Identification of RC-33 as a potent and selective σ1 receptor agonist potentiating NGF-induced neurite outgrowth in PC12 cells. Part 2: g-Scale synthesis, physicochemical characterization and in vitro metabolic stability. Bioorg Med Chem 2013;21(9):2577–2586; doi: 10.1016/j.bmc.2013.02.029.
55. Mueller BH, Park Y, Ma H-YY, et al. Sigma-1 receptor stimulation protects retinal ganglion cells from ischemia-like insult through the activation of extracellular-signal-regulated kinases 1/2. Exp Eye Res 2014;128:156–169; doi: 10.1016/j.exer.2014.10.007.
56. Barwick SR, Siddiq MS, Wang J, et al. Sigma 1 Receptor Co-Localizes with NRF2 in Retinal Photoreceptor Cells. Antioxidants 2021;10(6):981; doi: 10.3390/antiox10060981.
57. Wang J, Xiao H, Barwick SR, et al. Comparison of Sigma 1 Receptor Ligands SA4503 and PRE084 to (+)-Pentazocine in the rd10 Mouse Model of RP. Investig Opthalmology Vis Sci 2020;61(13):3; doi: 10.1167/iovs.61.13.3.
58. Yamashita D, Sun G wei, Cui Y, et al. Neuroprotective effects of cutamesine, a ligand of the sigma-1 receptor chaperone, against noise-induced hearing loss. J Neurosci Res 2015;93(5):788–795; doi: 10.1002/jnr.23543.
59. Tuerxun T, Numakawa T, Adachi N, et al. SA4503, a sigma-1 receptor agonist, prevents cultured cortical neurons from oxidative stress-induced cell death via suppression of MAPK pathway activation and glutamate receptor expression. Neurosci Lett 2010;469(3):303–308; doi: 10.1016/j.neulet.2009.12.013.
60. Senda T, Mita S, Kaneda K, et al. Effect of SA4503, a novel σ1 receptor agonist, against glutamate neurotoxicity in cultured rat retinal neurons. Eur J Pharmacol 1998;342(1):105–111; doi: 10.1016/S0014-2999(97)01450-7.
61. Mysona BA, Zhao J, De Greef O, et al. Sigma-1 receptor agonist, (+)-pentazocine, is neuroprotective in a Brown Norway rat microbead model of glaucoma. Exp Eye Res 2023;226:109308; doi: 10.1016/j.exer.2022.109308.
62. Ellis DZ, Li L, Park Y, et al. Sigma-1 Receptor Regulates Mitochondrial Function in Glucose- and Oxygen-Deprived Retinal Ganglion Cells. Investig Opthalmology Vis Sci 2017;58(5):2755; doi: 10.1167/iovs.16-19199.
63. Fujimoto M, Hayashi T, Urfer R, et al. Sigma-1 receptor chaperones regulate the secretion of brain-derived neurotrophic factor. Synapse 2012;66(7):630–639; doi:

10.1002/syn.21549.
64. Mysona BA, Zhao J, Smith S, et al. Relationship between Sigma-1 receptor and BDNF in the visual system. Exp Eye Res 2018;167:25–30; doi: 10.1016/j.exer.2017.10.012.
65. Arena E, Cacciatore I, Cerasa LS, et al. New bifunctional antioxidant/σ1 agonist ligands: Preliminary chemico-physical and biological evaluation. Bioorg Med Chem 2016;24(14):3149–3156; doi: 10.1016/j.bmc.2016.05.045.
66. Estrada M, Pérez C, Soriano E, et al. New Neurogenic Lipoic-Based Hybrids as Innovative Alzheimer'S Drugs with σ-1 Agonism and β-Secretase Inhibition. Future Med Chem 2016;8(11):1191–1207; doi: 10.4155/fmc-2016-0036.
67. Hashimoto K, London ED. Interactions of erythro-ifenprodil, threo-ifenprodil, erythro-iodoifenprodil, and eliprodil with subtypes of σ receptors. Eur J Pharmacol 1995;273(3):307–310; doi: 10.1016/0014-2999(94)00763-W.
68. Sharif NA, Xu SX. Pharmacological Characterization of [3 H]-Ifenprodil Binding to Polyamine Binding Sites on Rabbit and Rat Retinal Homogenates: Role in Neuroprotection? J Ocul Pharmacol Ther 1999;15(3):271–281; doi: 10.1089/jop.1999.15.271.
69. Pernet V, Bourgeois P, Polo A Di. A role for polyamines in retinal ganglion cell excitotoxic death. J Neurochem 2007;103(4):1481–1490; doi: 10.1111/j.1471-4159.2007.04843.x.
70. Lachance V, Bélanger S-M, Hay C, et al. Overview of Sigma-1R Subcellular Specific Biological Functions and Role in Neuroprotection. Int J Mol Sci 2023;24(3):1971; doi: 10.3390/ijms24031971.
71. Linciano P, Sorbi C, Rossino G, et al. Novel S1R agonists counteracting NMDA excitotoxicity and oxidative stress: A step forward in the discovery of neuroprotective agents. Eur J Med Chem 2023;249:115163; doi: 10.1016/j.ejmech.2023.115163.
72. Malar DS, Thitilertdecha P, Ruckvongacheep KS, et al. Targeting Sigma Receptors for the Treatment of Neurodegenerative and Neurodevelopmental Disorders. CNS Drugs 2023;37(5):399–440; doi: 10.1007/s40263-023-01007-6.
73. Schmidt HR, Kruse AC. The Molecular Function of σ Receptors: Past, Present, and Future. Trends Pharmacol Sci 2019;40(9):636–654; doi: 10.1016/j.tips.2019.07.006.
74. Su T-PP, Hayashi T, Maurice T, et al. The sigma-1 receptor chaperone as an inter-organelle signaling modulator. Trends Pharmacol Sci 2010;31(12):557–566; doi: 10.1016/j.tips.2010.08.007.
75. Arranz-Romera A, Davis BMM, Bravo-Osuna I, et al. Simultaneous co-delivery of neuroprotective drugs from multi-loaded PLGA microspheres for the treatment of glaucoma. J Control Release 2019;297:26–38; doi: 10.1016/j.jconrel.2019.01.012.
76. Bucolo C, Campana G, Di Toro R, et al. Sigma1 recognition sites in rabbit iris-ciliary body: topical sigma1-site agonists lower intraocular pressure. J Pharmacol Exp Ther 1999;289(3):1362–9.
77. Campana G, Bucolo C, Murari G, et al. Ocular Hypotensive Action of Topical Flunarizine in the Rabbit: Role of ς1 Recognition Sites. J Pharmacol Exp Ther 2002;303(3):1086–1094; doi: 10.1124/jpet.102.040584.
78. Wood JPM, DeSantis L, Chao H-MM, et al. Topically Applied Betaxolol Attenuates Ischaemia-induced Effects to the Rat Retina and Stimulates BDNF mRNA. Exp Eye Res 2001;72(1):79–86; doi: 10.1006/exer.2000.0929.
79. Rebustini IT, Bernardo-Colón A, Nalvarte AI, et al. Delivery Systems of Retinoprotective

Proteins in the Retina. Int J Mol Sci 2021;22(10); doi: 10.3390/ijms22105344.
80. Mohanty S, Idigo C, Ayyagari A, et al. Optogenetic Approaches to Gene Therapy for Vision Restoration in Retinal Degenerative Diseases. In: Handbook of Basic and Clinical Ocular Pharmacology and Therapeutics Elsevier; 2022; pp. 581–606; doi: 10.1016/B978-0-12-819291-7.00004-6.
81. Tischler A, Riseberg J, Hardenbrook M, et al. Nerve growth factor is a potent inducer of proliferation and neuronal differentiation for adult rat chromaffin cells in vitro. J Neurosci 1993;13(4):1533–1542; doi: 10.1523/JNEUROSCI.13-04-01533.1993.

Figure Legends

Figure 1. IOP profiles among treatment groups. A. IOP profiles were compared between the OHT eyes (OS) and the contralateral eyes (OD) overtime (Day 0, 1, 7, 14, and 21 days). **B.** The IOP profiles were presented as the difference between the OS and OD eyes. The saline-induced OHT in the left eye induced a significant increase of IOP over the 3 weeks, compared to the contralateral OD eyes. Levels of IOP peaked at day 1 ($p < 0.001$) and gradually decreased over time (day 7 $p < 0.01$ and day 14 $p < 0.01$), reaching near baseline levels at day 21 in all OHT treatment groups. None of the test compounds (rh-NGF and cutamesine) significantly affected the IOP. Each value represents the mean ± S.E.M. of 5 eyes. ** $p < 0.01$, *** $p < 0.001$.

Figure 2. An automated, validated algorithm was used to compute the DARC count in each image. A. A raw image from an OHT eye with white spots visualized. **B.** Annotated spots identified by algorithm (green squares).

Figure 3. Effects of cutamesine on OHT-induced changes in DARC and RBPMS counts. A. DARC count (number of annexin positive spots) in OHT eyes with different treatments. DARC counts were presented as numbers of spots at week 3 minus that at baseline. OHT treatments significantly increased the DARC count compared to control group ($p < 0.05$). rh-NGF, as a positive control, and cutamesine resulting in a significant reduction of RGC apoptosis compared to OHT only group ($p < 0.05$ and 0.05, respectively). **B.** OHT treatment resulted in a significant reduction of RBPMS^{+ve} RGC density in OHT only compared to control ($p < 0.01$). rh-NGF, as a positive control, and cutamesine significantly preserved RBPMS^{+ve} RGC density compared to OHT only group ($p < 0.01$ and 0.01, respectively). Each value represents the mean ± SEM of 4 to 6 eyes. * $p < 0.05$, ** $p < 0.01$. Based on rat eye vitreous volume and the injection of cutamesine into each eye, even if only 5% of the injected compound reaches the retina, the ambient local concentration of cutamesine would be approx. 140 nM. This concentration, relative to its IC_{50} at the S1-R (17 nM) is more than sufficient to fully occupy and activate the S1-Rs on RGCs to permit the neuroprotective activity of cutamesine. Similarly, given that rh-NGF exhibits 0.7-1 nM potency at its neurotrophic receptor,[81] the expected local concentration of the IVT delivered rh-NGF (0.09 nmol) in the retina would be in the low nanomolar range, more than enough to activate NGF receptors on the RGCs. **C.** Representative DARC images at baseline and 2 hours

after intranasal administration of fluorescently labelled annexin-5 from control, OHT only, OHT+rh-NGF, and OHT+Cutamesine groups. **D.** Representative images of RBPMS labeling (shown in black and white) of retinal flat mounts from rats that underwent different treatments. The bar indicates 50 µm. These images are provided as examples, while the corresponding quantitative data for RBPMS^{+ve} RGC counts from 4-6 eyes are shown in Fig. 3B above.

Deleted: Example

Formatted: Font: Not Bold

Deleted:

Deleted: The retinal images obtained

Deleted: were analyzed by quantitative image analysis to yield the data

Figure 4. Retinal protective effects of cutamesine against retinal degeneration induced by rotenone as measured by neurofilament light chain (Nfl) gene expression. Vehicle or cutamesine was premixed with rotenone and simultaneously injected into the vitreous of rat eyes. Twenty-four hours following injection, the retina was isolated and Nfl expression was determined by real-time PCR. The Nfl expression level was normalized to that of Gapdh in an individual retinal sample and is shown as the percentage of the respective control. Closed column, vehicle (50% DMSO in distilled water, n = 6 from 3 animals); open column, rotenone alone (n = 5 from 3 animals); dark grey column, rotenone (2 nmol/eye) plus cutamesine (10 nmol/eye or 300 nmol/eye, n = 4 from 2 animals in each group). Each value represents the mean ± SEM of 4 to 6 eyes from 2 or 3 animals. $^{***}P < 0.001$, by Student's unpaired t-test, compared with vehicle. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, by Dunnett's multiple comparison test compared with rotenone alone. Memantine (100 nmol/eye) yielded >90% protection in this retinal oxidative stress model.[23]

Figure 5. A schematic that summarizes the current state of knowledge of the sigma-1 receptor related neuroprotective activities, for example, as it pertains to cutamesine. Adapted and modified from Smith et al. 2018.[39]

Figure 6. Chemical structures of cutamesine and older generation prototypic sigma-1 receptor agonists.