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Histamine H_3 receptor (H_3R) antagonist-Nitric Oxide (NO) donor hybrid compounds as a new therapeutic strategy for the treatment of glaucoma and retinal neuroprotection

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Abstract

Glaucoma is a group of degenerative disorder of the optic nerve characterized by a malfunction of ciliary processes and trabecular meshwork (TM) in the anterior chamber of the eye. This damage causes an imbalance between production and outflow of aqueous humor (AH) generating an increase of intraocular pressure (IOP). The ocular hypertension (OHT), if untreated, provokes a reduction of local perfusion and chronic ischemia leading to a depletion of retinal ganglion cells (RGCs) by autophagy and/or apoptosis.

Currently, all treatment strategies are directed at lowering IOP; the two possible strategies are to reduce AH production or to increase the outflow. Unfortunately, a significant number of patients do not respond to these treatments and most of these compounds have side effects. In recent years, an approach consisting of multi-targeted compounds is emerging. This different strategy consists in design of hybrid compounds that incorporate moieties able to interact at different biological levels.

Nitric oxide (NO) is an endogenous messenger that plays a role in modulating the homeostasis of IOP. Further evidence shows that a dysregulation in NO production can contribute to increasing IOP levels by promoting the development or progression of glaucoma.

The histaminergic system also plays an important role in the regulation of IOP, in fact the presence of histamine H_1, H_3 and H_4 receptors in various ocular tissues has been demonstrated. In addition, the administration of H_3R antagonists has proven to be effective in reducing OHT, preventing RGCs loss by improving vascular performance of the ophthalmic artery, proposing this approach as a replacement therapy for the treatment of glaucoma.

Impaired ocular blood flow has been shown to result in insufficient oxygen and nutrient supply to the RGC axons crossing the optic nerve head (ONH), causing their degeneration. Indeed, unstable ocular blood flow produced, by chronically elevated and/or oscillating IOP, together with possible vascular dysfunction can lead to repeated hypoperfusion resulting in an ischemic retinal damage.

The present research was focused to evaluate the efficacy of three histamine H_3R antagonist-NO donor hybrid compounds in two animal model of OHT in rabbit. Moreover, I studied the capability of one of these molecules in ameliorating vascular

ocular performance and to prevent photoreceptor degeneration in a rabbit model of ischemia/reperfusion (I/R) induced by repeated injections of endothelin-1 (ET-1).

The acute or chronic OHT model were obtained respectively by injection of 50 µl of hypertonic saline (5% NaCl in sterile water) into the vitreous, or 100 µl of carbomer into the anterior chamber of New Zealand White rabbits' eye. The IOP was measured using a pneumatonometer at baseline, 60,120 and 240 minutes after OHT induction in the acute model or every day for 12 days before drug dosing in the chronic model. All animals received a topical single dose of 30 µl of studied compounds (ST-1989, ST-2126 and ST-2130) at a concentration of 1%; the molecules were compared with reference drugs ciproxifan (H_3R antagonist) and molsidomine (NO donor) administered alone and in combination.

The I/R model was obtained by repeated injections of ET-1 twice weekly for six weeks into the subtenon capsule of the eye. The animals were treated twice daily with vehicle or compound ST-1989 from the third week until the end of the protocol. IOP was measured every week after 36 hours of washout. Ophthalmic artery resistance index and the retinal function were assessed using an Echo Color Doppler ultrasound and Retimax Advance, respectively, at baseline, after 2 weekly injections of ET-1 and after 6 weeks. At the end of the experimental protocol, the animals were euthanized to collect AH, ciliary body, and retina tissues to perform biochemical evaluations such as the activation of NO-GC pathway (accumulation of nitrites and cGMP levels), the inflammatory response (IL-6 and TNF-α), the oxidative stress (GSH, MnSOD and 8OH2dG), and the apoptotic process (Caspase 3 activity). Furthermore, whole eyes were harvested to perform morphological and histopathological evaluations (Haematoxylin/eosin staining and TUNEL assay).

Among the hybrid compounds, ST-1989 has proven to be the most responsive molecule, able to reduce the IOP in both acute and chronic OHT model demonstrating a long-lasting effect. In addition, treatment with ST-1989 was able to reduce the inflammatory response, oxidative stress, and apoptotic process in chronic OHT and attenuate hypertension, improve ocular perfusion and prevent photoreceptor degeneration in the retinal I/R model. In conclusion, these hybrid compounds could be potential therapeutic drugs for glaucoma management and retinal neuroprotection.

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Abbreviations

AC: adenylate cyclase

ACG: angle-closure glaucoma

AH: aqueous humor

CAs: Carbonic Anhydrase

CAIs: Carbonic Anhydrase Inhibitors

CB: ciliary body

cGMP: cyclic guanosine monophosphate

CNS: central nervous system

CREB: cAMP- response element-binding protein

DMSO: dimethyl sulfoxide

EDV: end diastolic velocity

EDVFs: endothelium derived vasoactive factors

ERG: electroretinogram

ET: Endothelin

FAAH: fatty acid amide hydrolase

GFAP: glial fibrillary acidic protein

GPCRs: G protein-coupled receptors

GSH: reduced glutathione

HDC: histidine decarboxylase

HxR: histamine receptor

IOP: intraocular pressure

IP3: inositol-1,4-5-trisphosphate

I/R: ischemia/reperfusion

ISCEV: International Society for Clinical Electrophysiology of Vision

JOAG: juvenile open angle glaucoma

LBN: latanoprostene-bunod

MYOC: myocilin

MLT: melatonin

MMPs: matrix metalloproteinases

MnSOD: Manganese Superoxide Dismutase

NMDA: N-methyl-D-aspartate

NO: nitric oxide

NOS: nitric oxide synthase

NTG: normal-tension glaucoma

NZW: New Zealand White

OAG: open-angle glaucoma

OA-RI: Ophthalmic artery-resistance index

OHT: ocular hypertension

ONH: optic nerve head

PACG: primary angle-closure glaucoma

PDE5: phosphodiesterase 5

PG: Prostaglandin

PKA: protein kinase A

PKC: protein kinase C

PLC: Phospholipase C

POAG: primary open-angle glaucoma

PSV: peak systolic velocity

RGCs: retinal ganglion cells

RNFL: retinal nerve fiber layer

ROCK: Rho kinase

ROS: reactive oxygen species

sGC: soluble guanylate cyclase

SDOCT: spectral domain optical coherence tomography

TDOCT: time domain optical coherence tomography

TM: trabecular meshwork

TNF-α: Tumor necrosis factor- alpha

VEGF: vascular endothelial growth factor

1. Introduction

1.1 Definition

Glaucoma is the second most recurrent source of sightedness after cataract [1], afflicting more than 60 million people worldwide [2] with a discouraging estimate of 111 million in 2040 [3]. It is a degenerative disorder of the optic nerve characterized by a malfunction of ciliary processes and trabecular meshwork (TM) in the anterior chamber of the eyes. This damage causes an imbalance between production and outflow of aqueous humor (AH) and this situation generates an increase of intraocular pressure (IOP). The elevated pressure within the eye, if untreated, causes a reduction of local perfusion, chronic ischemia leading to a depletion of retinal ganglion cells (RGCs) by autophagy and apoptosis [4]. When the number of RGCs is no longer adequate to ensure correct neuronal transmission, the field of view becomes narrower. The damage to the optic nerve leads to a progressive and permanent loss of vision, from imperceptible blind spots at the edges of the field of vision, to tunnel vision, and then blindness [5].

1.2 Classification of glaucoma

Glaucoma-related optic neuropathy is characterized by a specific pattern of abnormalities of the optic nerve complex (optic nerve head, retinal nerve fiber layer, and peripapillary region) and corresponding damage to the visual field [6].

It can be classified into two main categories, open-angle glaucoma (OAG) and angle-closure glaucoma (ACG), based on the obstruction of AH outflow and the configuration of the anterior chamber drainage angle [7]. Both can occur with no identifiable cause, resulting in idiopathic or primary glaucoma. Secondary glaucoma refers to any form of glaucoma in which there is an identifiable cause of increased IOP, resulting in optic nerve damage [6] (Figure 1).

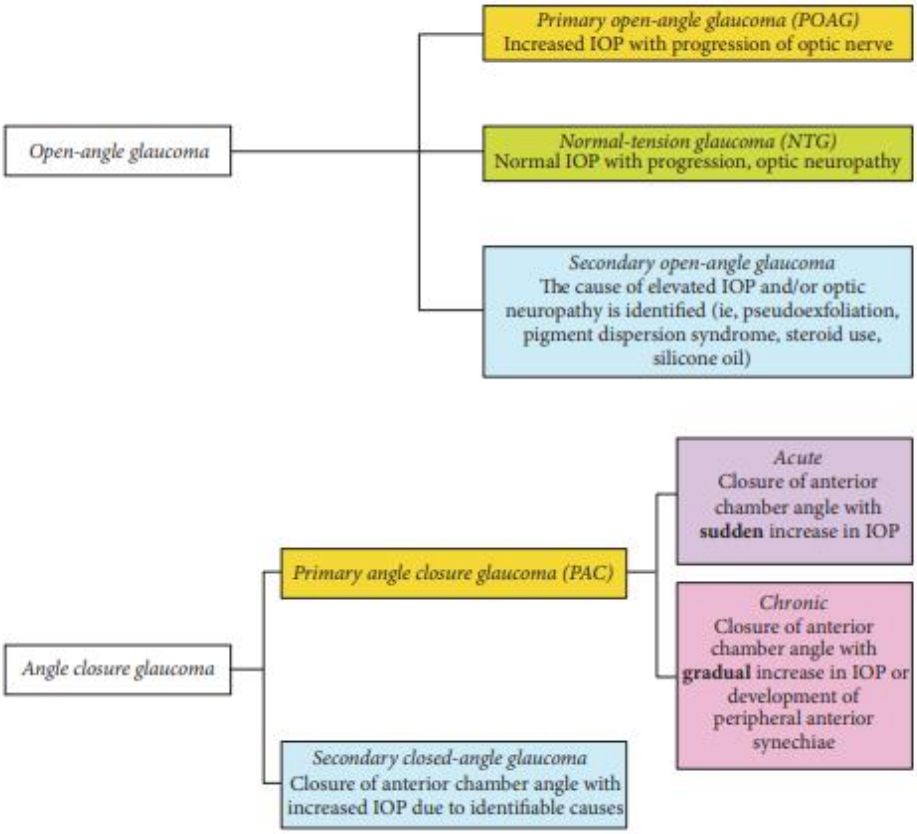


Figure 1: Classification of glaucoma subtypes (Harasymowycz *et al.*, 2016).

Among the different OAGs, primary open-angle glaucoma (POAG) is the most common form; this type of disease includes two subtypes: hypertensive and normal-tension glaucoma (NTG). In the first one, the drainage angle formed by the cornea and iris remains open, but the TM is partially blocked (Figure 2 panels A-B). This causes an imbalance between production and outflow of AH and pressure increases. As the disease progresses, patients have impairment in peripheral vision and sometimes in central vision. If OAG is left untreated, eventually central vision may be lost, resulting in irreversible blindness.

In NTG, there is a degeneration of the optic nerve due to a reduction in ocular perfusion although IOP is within the normal range. The pathogenesis of NTG remains unclear; however, several mechanisms involved in the onset and progression of this disease have been proposed [8]. It probably represents a heterogeneous or multifactorial group of etiologies, with a common final pathway of RGCs loss. Despite IOP in the statistically normal range, there is evidence that an IOP-dependent mechanism plays a role in the etiology of the disease [9]. One of the proposed IOP-independent mechanisms include

vascular insufficiency at the optic nerve head (ONH), metabolic and neurodegenerative disorders, oxidative stress, and abnormal biomechanics of the lamina cribrosa [10].

Angle-closure glaucoma can be classified into primary angle-closure glaucoma (PACG) and secondary closed-angle glaucoma. PACG is further classified into acute (closure of anterior chamber angle with a sudden increase in IOP) and chronic (closure of the anterior chamber angle with a gradual increase in IOP or development of peripheral anterior synechiae). Secondary closed-angle glaucoma is the closure of the anterior chamber angle with increased IOP due to identifiable causes [6].

Primary angle-closure glaucoma occurs when the iris is located forward of its normal position and blocks the angle between iris and cornea (Figure 2C). As a result, the access to the TM is physically obstructed, the AH cannot circulate through the eye and pressure increases. Three main mechanisms hypothesized to be responsible for PACG are pupillary block, anterior iris rotation, and plateau iris [2]. In the former case, contact between the iris and the lens results in increased resistance to AH flow in the anterior chamber. When the pressure in the posterior chamber exceeds that in the anterior chamber, the iris moves forward leaning against the TM blocking the AH drainage system at two levels: the pupillary margin and the TM [6]. Pupillary block occurs when the flow of AH from the posterior chamber to the anterior chamber is obstructed by a functional block between the pupillary portion of the iris and the lens [11]. In plateau iris configuration, the ciliary body (CB) is anteriorly positioned resulting in the anterior displacement of the peripheral iris into the angle [12].

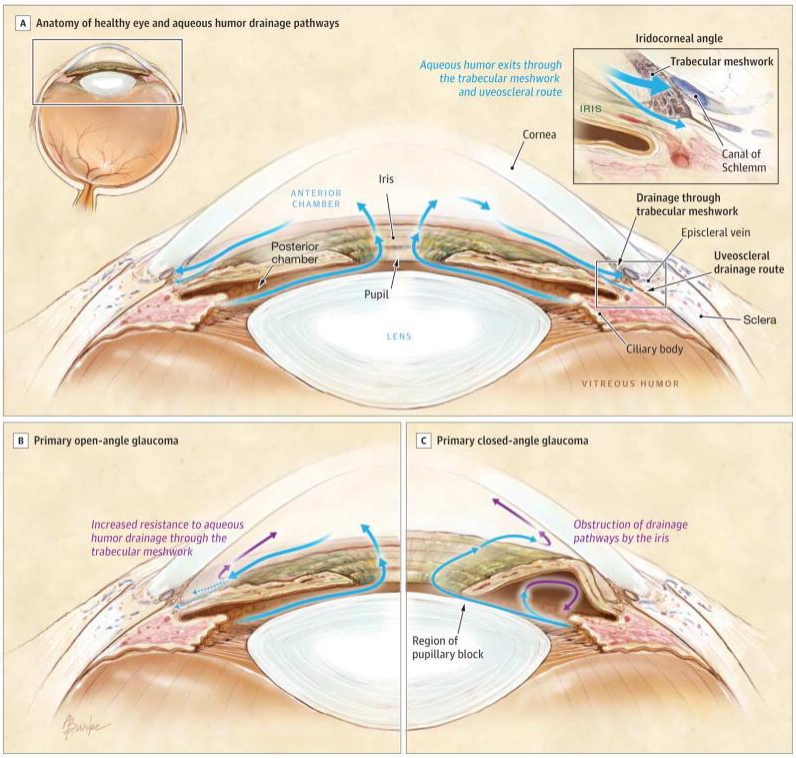


Figure 2: Anatomy of the eye. A) Physiological aqueous humor (AH) drainage pathways, B) Primary open-angle glaucoma, C) Primary angle-closure glaucoma (Stein *et al.*,2021).

Secondary glaucoma can be divided as open-angle or angle-closure and can occur in one or both eyes. In clinical settings, it is defined as the occurrence of IOP above 21 mmHg requiring the prescription of IOP-managing drugs [13]. It may be caused by an eye injury, inflammation, certain drugs such as steroids and advanced cases of cataract or diabetes. The type of treatment will depend on the underlying cause, but usually includes medications, laser surgery, or conventional surgery [14].

There are also early-onset forms of glaucoma associated with developmentally normal eye structure; one of the main is the juvenile open-angle glaucoma (JOAG). It is a rare form of POAG that generally affects individuals between the ages of 5 and 35 years. JOAG is a rare condition associated with altered expression of the protein myocilin due to genetic alterations in the MYOC gene [15]. Typically, patients affected by JOAG develop a severe form of glaucoma characterized by very high IOP that can be difficult to control by current therapies [16]. Existing therapies are most effective at early disease stages; however, patients may be asymptomatic and not seek medical attention early in the disease, limiting the opportunity for early intervention [17]. Glaucoma also affects infants; this variant is called congenital glaucoma, a rare but high-impact condition on child's development and quality of life [18]. Angle dysgenesis is caused by incomplete

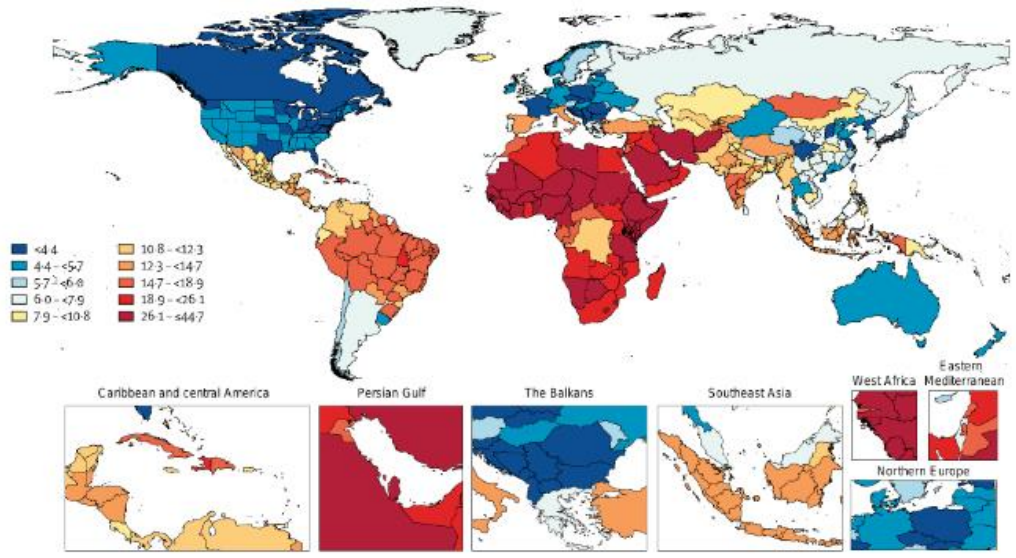
development of the TM before and/or after birth, this results in a decrease in AH outflow causing a significant increase in IOP [19].

1.3 Epidemiology

Glaucoma represents a significant public health problem. It is the second leading cause of blindness after cataracts, and this blindness is usually irreversible [20].

The number of POAG cases in adult population (40–80 years old) was estimated 52.68 million in 2020 and 79.76 million in 2040 [3]. The population-based prevalence of POAG varies widely across individual studies, due to variations in risk factors such as age, gender, and population geographic location [21,22]. Age is known to be the main risk factor for POAG, as the prevalence increases with advancing age [23,24]. In fact, the population over 80 years of age has the highest risk of POAG (9.2%) due to thinner central corneal thickness and higher IOP, which are major responsible for the higher prevalence of POAG in the elderly population [25]. Gender is also a significant risk factor: several studies suggested that men are more likely to develop POAG than women because they have greater axial length and anterior chamber depth [26]. Among the continents, Africa is found to have the highest prevalence of POAG (4.0%), while Oceania had the lowest (1.8%). North America ranked second highest POAG prevalence (3.4%) [27]. Asia accounts about 60% of global glaucoma population [28]. The prevalence of POAG also varies in different Asia regions. South-central Asia was considered to have highest burden of POAG and overall glaucoma than other regions, while the East Asia is reported to have higher prevalence of PACG [30] (Figure 3 panels A-B). In Europe, POAG prevalence was 2.60% with a prevalence rate in men and women of 3.05% and 2.22% respectively [29]. In addition, it was noted that people living in urban areas were 58% more likely to have POAG than people in rural areas. This may be explained by the higher prevalence of myopia in urban areas [3].

A



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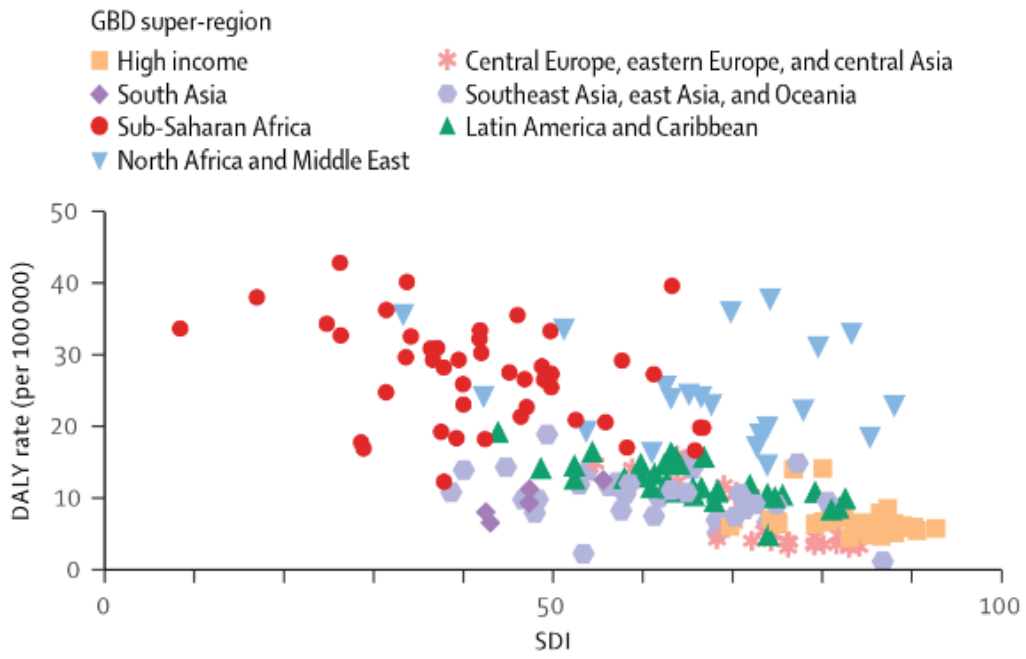


Figure 3: Worldwide prevalence of glaucoma: A) Age-standardized disability-adjusted life year (DALY) rates (per 100 000) by location, both sexes combined, 2019. B) Age-standardized DALY rates for each location by socio-demographic index (SDI), both sexes combined, 2019. Institute for Health Metric and Evaluation.

The vision loss associated with glaucoma is largely irreversible. Those afflicted with the disease and living in developing countries are at a particular disadvantage; they have a higher risk of progressing to blindness, present with the more advanced disease, and often have a higher incidence of disease than developed nations [30,31].

Everyone is at risk for glaucoma from children to senior citizens. Older people are at a higher risk for glaucoma, but children can be born with glaucoma (approximately 1 out of every 10,000 babies born in the United States). Young adults can get glaucoma, too. African Americans are susceptible at a younger age. Many risk factors are considered to be involved in the pathogenesis of glaucoma: ocular hypertension (OHT) is commonly considered one of the major risk factors [32,33].

1.4 Pathophysiology

Glaucoma is a collection of disease processes that result in a common pathology. The biological basis of this condition is poorly understood and the factors contributing to its progression have not been fully characterized [34,35]. Clinically, glaucoma is classified according to characteristic changes in the ONH, in which a typical hollowed-out, so-called "cupped" appearance determined by the reduction of RGC axons is observed (Figure 4). Because the progression of visual field damage is slow and typically painless, patients with glaucoma commonly do not experience any problems with their vision until they have a significant level of visual loss [34].

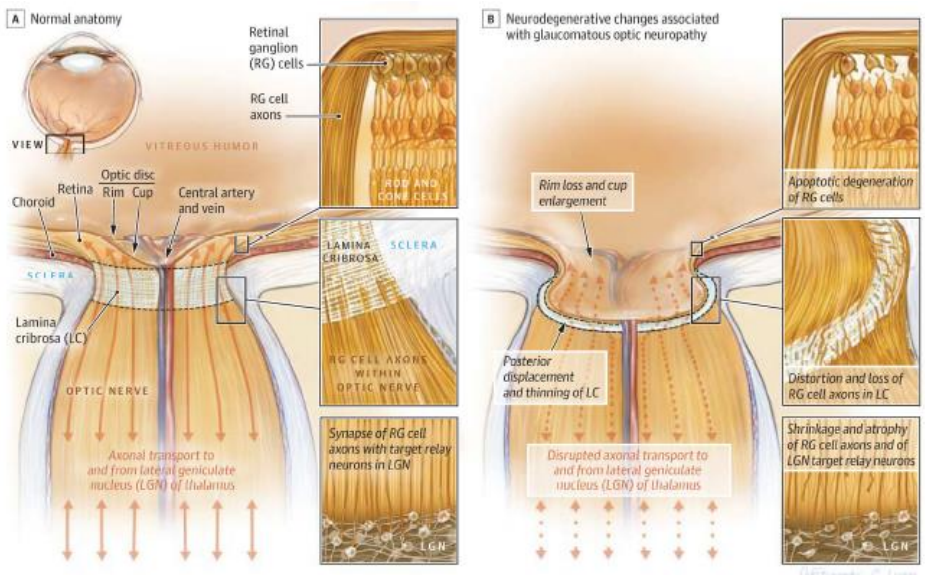


Figure 4: Normal anatomy and neurodegenerative changes associated with glaucomatous optic neuropathy (Weinreb *et al.*,2014).

Higher IOP at baseline is a strong risk factor for glaucoma [36]. The relative risk conferred to both the development and progression of glaucoma is so high that elevated IOP, in combination with other characteristics, is often used to classify individuals as

affected in large genetic and genomic studies. Physiologically, IOP is determined by the production, circulation, and drainage of AH. The major drainage pathways are the trabecular outflow pathway (conventional pathway) and uveoscleral outflow pathway (unconventional pathway). AH draining through the trabecular outflow system will traverse the TM, through the juxtacanalicular connective tissue, into Schlemm's canal and the collecting channels, and finally into the aqueous veins which then drain into the episcleral venous system. AH draining through the uveoscleral route passes through the ciliary muscle bundles into the suprachoroidal space and then through the sclera into the orbital vessels [37,38]. In patients with OAG, there is increased resistance to aqueous outflow through the TM.

1.4.1 Primary Open-angle glaucoma

In POAG, IOP can cause mechanical stress and strain on the posterior structures of the eye, notably the lamina cribrosa and adjacent tissues [39]. The sclera is perforated at the lamina where the optic nerve fibers (RGC axons) exit the eye. The lamina is the weakest point in the wall of the pressurized eye. IOP–induced stress and strain may result in compression, deformation, and remodeling of the lamina cribrosa with consequent mechanical axonal damage and disruption of axonal transport [40,41] that interrupts retrograde delivery of essential trophic factors to retinal ganglion cells from their brainstem target (relay neurons of the lateral geniculate nucleus). Studies involving cats and monkeys with experimentally induced OHT have demonstrated a blockade of both orthograde and retrograde axonal transport at the level of the lamina cribrosa [42]. Disrupted axonal transport occurs early in the pathogenesis of glaucoma in experimental systems resulting in collections of vesicles and disorganization of microtubules and neurofilaments in the pre-laminar and post-laminar regions [35].

Glaucomatous optic neuropathy can occur in individuals with IOP within the normal range. In such patients, there may be an abnormally low cerebrospinal fluid pressure in the optic nerve subarachnoid space resulting in a large pressure gradient across the lamina [43,44]. Impaired microcirculation, altered immunity, excitotoxicity, and oxidative stress may also cause glaucoma. Primary neural pathological processes may cause secondary neurodegeneration of other retinal neurons and cells in the central visual pathway by altering their environment and increasing susceptibility to damage [45].

Several genes, including myocilin (MYOC, GLC1A) [46], optineurin (OPTN, GLC1E) [47] and WD repeat domain 36 (GLC1G) [48] are associated with a monogenic,

autosomal dominant trait; however, these genes account for less than 10% of all glaucoma cases [49]. The first reported locus for POAG was located on chromosome 1 (GLC1A). The relevant gene at the GLC1A locus is MYOC, which encodes the protein myocilin [35]. Disease-associated mutations of myocilin generally occur in the juvenile or early adult form of POAG, usually characterized by very high levels of IOP. In populations of adults with POAG, the prevalence of myocilin mutations varies from 3% to 5%. The mechanism of myocilin-related glaucoma has not been fully elucidated [49]. It appears that mutations alter the myocilin protein in a way that disrupts normal regulation of IOP. Disease-associated forms of myocilin interfere with protein trafficking and result in intracellular accumulation of misfolded protein. Failure to adequately secrete the protein is thought to somehow cause an IOP increase. In contrast to individuals with the MYOC gene, those with the OPTN gene have normal levels of IOP [47]. Although the mechanism relating the OPTN gene variants to glaucoma have not been elucidated, there is evidence suggesting that optineurin may have a neuroprotective role by reducing the susceptibility of RGCs to apoptotic stimuli.

1.4.2 Primary angle-closure glaucoma

The main feature distinguishing PACG from POAG is that the angle between iris and cornea, is obstructed by apposition of the iris, resulting in an anatomically closed angle and this can happen acutely, intermittently, or chronically [50]. Like OAG, ACG is predominantly an asymptomatic disease with individuals often unaware they have the disorder until advanced visual loss has occurred [35]. Primary closed-angle glaucoma is caused by disorders of the iris, the lens, and retro-lenticular structures. Pupillary block is the most common mechanism of ACG, and it is caused by resistance to AH flow from the posterior to anterior chambers at the pupil. AH accumulates behind the iris increasing its convexity causing angle closure. Non-pupil block mechanisms such as a plateau-like iris configuration may be responsible for a significant proportion of angle closure in Asian patients [51]. Angle-closure glaucoma may also be caused by dynamic physiological factors, such as an increase in iris volume with pupil dilation and choroidal effusion [52]. Primary angle-closure glaucoma can be categorized into three different subtypes: acute, sub-acute or intermittent and chronic. Acute primary angle-closure is caused by a sudden block of the TM by the iris. Symptoms include blurred vision, pain, colored halos around lights, nausea, emesis, and frontal headaches. Patients with acute PACG may present clinically with increased IOP, a mid-dilated, non-reactive, or irregular pupil. Patients with intermittent PACG have periodic episodes of blurred vision, halos, and mild pain. These

patients often have normal IOP in between episodes, and episodes often resolve during sleep due to miosis of the pupils. Chronic PACG occur after an episode of acute angle closure, or due to frequent intermittent angle closures [50].

Open-angle and closed-angle glaucoma are distinct genetic entities with different genes associated with each disease in fact, a genome-wide association study involving more than 20000 individuals from several countries found 3 new genetic loci for angle closure: rs11024102 at PLEKHA7, rs3753841 at COL11A1 (HGNC:2186), and rs1015213 located between PCMTD1 (HGNC:30483) and ST18 (HGNC:18695) on chromosome 8q [53].

1.5 Diagnostic tests

Glaucoma progresses without causing symptoms until the disease is advanced with substantial amounts of neural damage. When symptoms do occur, the disease results in vision loss with concomitant reduction in quality of life and the ability to perform daily activities, such as for example, driving. Early intervention is essential to slow the progression of the disease.

Despite the strong association between OHT and glaucoma, several numbers of people with elevated IOP never develop the disease [54]. In fact, several population-based studies found that IOP was lower than 22 mmHg in 25%-50% of glaucomatous patients [40,54]. With RGCs death and optic nerve fiber loss in glaucoma, characteristic changes in the appearance of the ONH and retinal nerve fiber layer (RNFL) occur [54]. These changes are the most important aspect of a glaucoma diagnosis and can be identified during ophthalmoscopic examination of the ONH. The importance of conducting an appropriate ophthalmologic examination of the eye cannot be overstated with respect to early detection of glaucoma. The depletions of RGCs causes progressive deterioration of visual fields, which usually begins in the midperiphery and may progress in a centripetal manner until there remains only a central or peripheral island of vision.

Albeit there are no current gold-standard criteria to diagnose and most important, for detecting and monitoring structural damage in the continuum of glaucoma, diagnostic tests most commonly utilized include perimetry (visual field test), gonioscopy, IOP measurement, the corneal thickness measurement (which helps to estimate the true IOP versus measured IOP, which can be affected by the cornea), the evaluation of the ONH,

assessment of RNFL thickness with spectral domain optical coherence tomography (SDOCT) or time domain OCT (TDOCT) and fundus photography [7,55].

Challenges in the accurate diagnosis of glaucoma progression include the difficulty in discriminating true disease-related changes from measurement variability or natural age-related changes in structural measurements. Although the use of OCT in glaucoma has most commonly been based on the assessment of the circumpapillary RNFL thickness, recently there has been increased interest in the macular region. Advantages of assessment of the macula for glaucoma evaluation include the fact that a large proportion of total macular thickness is composed of the RNFL, RGC layer, and inner plexiform layer, structures that are lost and thin as glaucoma damage develops and progresses. Additionally, at least in eyes without other macular pathology, there appears to be less variability and a lower likelihood of the presence of anomalous structural characteristics than are present in the optic disc and peripapillary region [55]. Preservation of macular function until late in the disease process may suggest that macular assessment would not be sensitive for glaucoma detection. Preservation of central vision until later in the disease course is a result of the fact that the macular RGC layer is more than one cell thick and up to seven cells thick and contains more than 50% of the RGCs of the entire retina [56]; thus, scanning of the macula allows sampling of the majority of the RGCs. Although glaucomatous macular changes are difficult to detect clinically, OCT allows quantitative assessment of either the entire macular thickness or the thickness of specific layers of importance in glaucoma.

1.6 Treatments for glaucoma

Slowing disease progression and improving quality of life are the primary goals for glaucoma treatment. The decrease in quality of life associated with glaucoma may occur earlier than previously thought, underscoring the importance of early diagnosis and treatment [57]. IOP reduction is the only effective method to treat glaucoma. The initial goal aims for a pressure reduction of 20% to 50%; however, baseline pressure should be continuously monitored during the patient's follow-up, depending on the evolution of the disease [58]. Current American Academy of Ophthalmology Preferred Practice Pattern management guidelines recommend lowering IOP toward a target level, which is a value or range of values at which the clinician believes the rate of disease progression will be slowed sufficiently to avoid functional impairment of the eye. At present, there are three

different approaches for lowering hypertension associated with the disease: pharmacological therapy, laser therapy and surgical management [59,60].

1.6.1 Pharmacological therapies

Medication choice may be influenced by cost, adverse effects, and dosing schedules [35]. Nowadays there are six classes of agents clinically used for the treatment of glaucoma: adrenergic agonists and antagonists [61,62]; systemically or topically acting carbonic anhydrase inhibitors (CAIs) [59]; cholinergic agonists [62]; prostaglandin analogs [63] and the recently introduced in clinical practice Rho kinase (ROCK) inhibitors. It is important to note that other targets have been focused, such as the melatonin receptors [64], the fatty acid amide hydrolase (FAAH) [64], the adenosine receptors [65] and nitric oxide (NO) donors, alone or in combination with other pharmacological agents to validate new therapies for the management of glaucoma.

1.6.1.1 The Adrenergic Agonists and Antagonists

a) α_2 agonists

The-α-adrenoceptors are abundantly present in the eye, especially in iris smooth muscle cells, in the blood vessels of the conjunctiva and CBs and in the aqueous outflow tract [66,67]. These drugs, through stimulation of α receptors induce a constriction of blood vessels resulting in decreased AH production and thus diminished IOP. Adrenaline has been the first representative of this group of drugs. Unfortunately, it has been observed that tolerance develops in prolonged treatments; in addition, side effects and low bioavailability limit the use of adrenaline in the clinic. Selective α_2-adrenergic agonists are generally used. Clonidine, a selective α_2 agonist, with some α_1 effect, decreases IOP by reducing AH production; clonidine induces systemic hypotension and lowered the pressure in the ophthalmic artery, inducing visual field defects, for these reasons its clinical use has been abandoned [68]. Therefore, a clonidine derivative, apraclonidine, which has lower lipophilicity than clonidine, was introduced into therapy; this drug is able to reduce IOP by decreasing AH production and increasing trabecular blood flow and is mainly used after post-laser and post-surgical IOP elevation [69]. Also, this drug presents several side effects such as allergic reaction, blepharo- and follicular-conjunctivitis, hyperaemia, itching and tearing [70]. Brimonidine, the last of this class of drug, is highly α_2 selective, it reduces IOP decreasing AH production and increasing uveoscleral outflow. It is manly used to prevent hypertension after laser trabeculoplasty

and for chronic treatment of OAG patients. The patient compliance for this drug is not very good for its short pharmacological action: eye drops must be applied 3-4 times daily. The side effects are similar to apraclonidine. In Figure 5 are reported the structures of α_2 agonists.

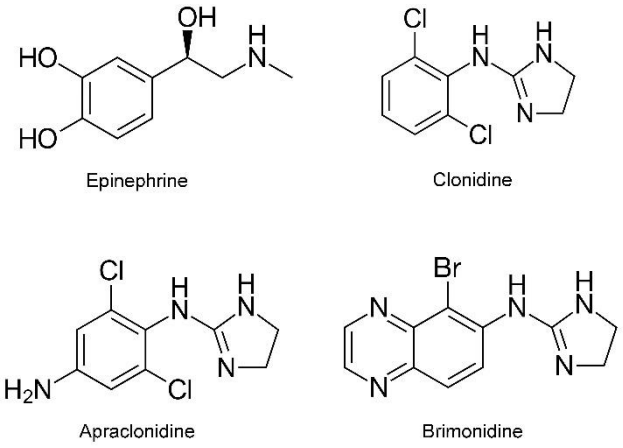


Figure 5:Structure of α_2 agonists (Sgambellone *et al.*, 2020)

b) β-blockers

Topical β-blockers reduce IOP by blockade of sympathetic nerve endings in the ciliary epithelium causing a fall in AH production. Two types of topical β-blockers are available: non-selective, which blocks both β_1- and β_2-adrenoceptors; and cardioselective, which blocks only β_1 receptors. Among the β-blockers commercially available, timolol, levobunolol, metipranolol and carteolol belong to the first type, and betaxolol to the second one [67]. Timolol, the most active compound, frequently used as reference drug for the development of novel treatments, requires at least twice a day administration to maintain the pharmacological effect, although levobunolol is equally effective and can be used once daily with little difference in effect. Timolol has few local side effects, such as hyperaemia, but it may induce systemic adverse effect by blocking β_1-adrenoceptors of the heart, thus inducing bradycardia, arrhythmia, congestive heart failure and Adam-Stokes syndrome. Moreover, blocking the β_2 receptor at lung level, timolol can induces bronchospasm in asthmatic patients [71]. Betaxolol has an effect comparable to timolol in lowering IOP but is less effective in some patients. Local stinging can be a problem in some patients with betaxolol, although it seems relatively free of adverse respiratory effects, but this may be dose-related and extreme caution should still be exercised in patients with any history of respiratory illness [72]. Because of the lower risk of side

effects, betaxolol is probably the β-blocker of first choice for use in glaucoma; timolol or levobunolol are reserved for patients who do not respond satisfactorily to betaxolol and are quite free of respiratory disease [67]. Moreover, recent evidence demonstrates that the use of β-blockers, which modulate the vasoproliferative retinal process, may reduce the progression of retinopathy of prematurity (ROP) [73]. Oral propranolol is effective in reducing the progression of the disease, although not safely; while propranolol 0.2% eye micro-drops is well-tolerated and reduce ROP progression [74].

1.6.1.2 Carbonic Anhydrase Inhibitors

Carbonic anhydrases (CAs) are ubiquitous metalloenzymes present in prokaryotes and eukaryotes with different catalytic activity, subcellular localization, and tissue distribution. CAs catalyse a physiological reaction that converts CO_2 into bicarbonate ion and protons. Many of these isoenzymes are important therapeutic targets for the treatment of a range of diseases including glaucoma. The main constituent of AH is bicarbonate and CA in ciliary processes is responsible of bicarbonate secretion and after the discovery of this information [75]. The first effective drug in reducing IOP both in animals and humans and used for the treatment of glaucoma was acetazolamide, a sulfonamide CAI. The use of systemic inhibitors, such as acetazolamide is useful for decreasing elevated IOP characteristic of many glaucoma forms, however their use leads to several side effects, including metallic taste, depression, fatigue, weight loss, decreased libido, gastrointestinal irritation, and metabolic acidosis [76]. Dorzolamide and brinzolamide are nanomolar CA II/CA XII inhibitors, possess good water solubility, and are enough liposoluble to penetrate through the cornea. The incidence of ocular discomfort, burning and stinging, on instillation of brinzolamide, twice daily, was significantly less compared with the treatment with dorzolamide. The most common side effects are stinging, burning of the eye, pruritus, and bitter taste. These two formulations are still in use; dorzolamide is often selected as reference drug for the development of new CAIs. Novel type of sulfonamide CAIs with good water solubility and IOP-lowering effects have been developed [67]. These new drugs are 2 or 3 times more effective than dorzolamide, possess good water solubility, good penetrability through the cornea, inhibition in the low nanomolar range against hCA II and hCA IV, and very good IOP-lowering properties in both normotensive and glaucomatous rabbits [77].

1.6.1.3 Cholinergic Agonists

Cholinergic drugs, such as pilocarpine or physostigmine, were the first class of agents used for the treatment of glaucoma and, still now they are useful for the short-term management of ACG associated with pupillary block. Cholinergic agonists cause contraction of the longitudinal ciliary muscle, tighten the iris, decrease the volume of iris tissue in the angle and pull the peripheral iris away from the TM, increasing outflow of AH. This results in a 15-25% reduction in IOP. If the IOP is quite elevated, i.e., about 40-45 mmHg, the pupillary sphincter may be ischemic and may not respond to cholinergic stimulation [78]. Systemic side effects of pilocarpine are rare; however, ocular side effects are common and are brow ache, induced myopia, miosis, leading to decreased vision, shallowing of the anterior chamber, retinal detachment, corneal endothelial toxicity, breakdown of the blood-brain barrier, hypersensitivity, or toxic reaction, cicatricial pemphigoid of the conjunctiva and atypical band keratopathy. Indirect-acting para-sympathomimetics inhibit the enzymatic destruction of acetylcholine by inactivating cholinesterase, leaving acetylcholine free to act on the effector cells of the iris sphincter and ciliary muscle, causing pupillary constriction and spasm of accommodation [67]. Anti-cholinesterase agents are generally more potent than pilocarpine, but they have more intense side effects. Finally, prolonged respiratory paralysis may occur during general anaesthesia in some patients who are in treatment with cholinesterase inhibitors because of their inability to metabolize paralytic agents such as succinylcholine [79].

1.6.1.4 Prostaglandin Analogs

Prostaglandin (PG) receptors are expressed in different tissues including the eye [80]. There are nine different types of PG receptors, they are G-protein-coupled receptors modulating a large number of biological responses. All four PGE_2 receptors are expressed in human cornea, conjunctiva, TM, iris, CB, and retina [81], but the first molecules acting on these receptors had several side-effects; however, the IOP-lowering action of PGs attracted the attention of the scientific world and led to investigate different PG receptors and to develop the first clinically used $FGF_{2\alpha}$ derivatives, such as latanoprost, bimatoprost, travoprost and tafluprost for the treatment of glaucoma (Figure 6).

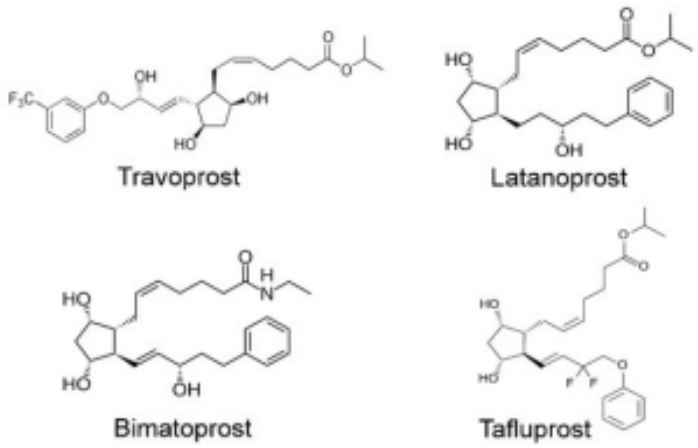


Figure 6: Structure of prostaglandin analogs (Sgambellone *et al.,* 2020)

These molecules activate both TM and ciliary muscle cells, increasing the outflow of AH [82]. The mechanism by which PGs increase uveoscleral outflow is not yet completely understood. Several studies suggest that the antiglaucoma effect is probably due to two different mechanisms: the first is the unconventional pathway, increasing AH outflow by binding to prostaglandin E and F receptors in the ciliary muscle, resulting in ciliary muscle relaxation and increasing AH outflow [83]. The second is the conventional pathway, where PG analogs modulate outflow pathway through the increase of TM cell contractility and the decrease of cell contractility of the inner wall of Schlemm's canal [84]; of note PG analogs up-regulate metalloproteinases, enzymes involved in extracellular matrix remodelling, making the area more permeable to AH [67]. Latanoprost has become the most popular drug for the treatment of glaucoma in the world, showing good ocular tolerability.

In clinical studies, travoprost once daily produced an IOP reduction similar to bimatoprost or latanoprost, with little diurnal fluctuation and results in a large percentage of patients the most active compound [85]. Bimatoprost is a synthetic prostamide analog that reduces IOP by increasing the outflow of AH through the TM and uveoscleral routes. It provides a constant level of IOP control throughout a 24-hour period. Bimatoprost was more effective than timolol at maintaining overall IOP control throughout the day, and significantly more bimatoprost than timolol recipients achieved target IOP levels [86]. Tafluprost is the last prostaglandin analog introduced in the clinical therapy. It is used topically to control the progression of OAG and in the management of OHT, alone or in

combination with other medication. As all the other PGs, it reduces IOP by increasing the outflow of AH from the eyes [67].

1.6.1.5 Rho-Kinase Inhibitors

Rho-kinase (ROCK) is one of the best characterized effectors of RhoA, a small GTPase belonging to the subfamily of Ras protein superfamily. The activation of the binding of Rho to its GTP protein is controlled by GTPase activating proteins and by guanine nucleotide exchange factors which are controlled by Transforming Growth Factor- β (TGF-β) and endothelin-1 (ET-1). The crucial role of Rho/ROCK pathway in cell proliferation, cell migration and cellular contraction makes ROCK inhibitors promising therapeutic agents for the treatment of several diseases including glaucoma [87]. Relaxing TM tissue, ROCK inhibitors directly decrease resistance in the conventional AH outflow, thus resulting in a significant IOP-lowering effect [88]. Ripasudil was shown to lower IOP within two hours after instillation of the drop solution and was proven to do so consistently over a period of a full year; however, this drug caused conjunctival hyperaemia in the majority of subjects in each clinical trial reviewed. This is a dose-dependent side effect, and it is seen in the majority of patients treated with ripasudil [89]. Netarsudil, decreases IOP within two hours of instillation maintaining this decrease for a 24-hour period after dosing [90]. It uses two mechanisms to lower IOP: by acting as both a ROCK inhibitor and a norepinephrine transport inhibitor. The latter helps to prolong reduction in IOP by constriction of vascular structures in the eye. This reduces blood flow to the ciliary processes, inhibiting production of AH. Two phase-3 clinical trials compared the safety and efficacy of netarsudil to timolol in patients with elevated IOP and as ripasudil the most commonly seen side effect is conjunctival hyperaemia [91].

1.6.1.6 Combined therapies and multi-target strategies

Combining ocular hypotensive drugs is indicated when the target pressure for a patient cannot be reached with a monotherapy. The first fixed combination product was a PG with a β-blocker (latanoprost 0.005% plus timolol 0.5%) followed by other PGs like travoprost, bimatoprost, or tafluprost with timolol. These combines therapies were effective in controlling IOP for 24 hours and had a similar effect on diurnal and nocturnal IOP variation. Combination therapy of β-blockers is also addictive with miotics, topical CAIs and α-agonists; in fact, it has been demonstrated that timolol 0.5% plus dorzolamide 2% induced an increased reduction of IOP of 13 to 29% in comparison to timolol alone [92].

In recent years, an approach consisting of multi-targeted compounds is emerging: the design of hybrid molecules incorporating moieties able to interact at different biological levels for lowering IOP. Derivatives of PGs, agonists of $PGF_{2\alpha}$ receptors, with several linkers bearing nitric oxide (NO)-releasing moieties have been patented [64]. The first NO-releasing PG analog was latanoprostene-bunod (LBN) formed by latanoprost acid linked to a NO-donating moiety. This compound reduced myosin light chain phosphorylation, induced cytoskeletal rearrangement, and decreased resistance to current flow to a greater extent than latanoprost in TM cells, indicating that NO released from LBN elicited TM cells relaxation [93]. These data indicate that LBN has a dual mechanism of action, increasing AH outflow through both the uveoscleral (using LA) and TM/Schlemm's canal (using NO) pathways.

The neurohormone melatonin (MLT) has been shown to decrease IOP. In mammals, the majority of MLT actions are driven by the activation of the two G-protein coupled receptors named MT1 and MT2 expressed in the central nervous system (CNS) and in the periphery. Moreover, MLT exerts direct antioxidant effects at high concentrations, it is produced locally in the eye and its receptors have been identified in several areas, such as retina, CB, cornea, lens, and sclera [94]. Topical and systemic administration of MLT has been shown to transiently reduce IOP in normotensive and hypertensive/glaucomatous animals [95,96]. Hybrid compounds, activating melatonin receptors and inhibiting FAAH have been recently synthesized [64] and the topical administration of these molecules reduced elevated IOP in rabbits, with a longer action and improved efficacy compared to the reference compounds. Adenosine-based analogs have been recently patented acting as selective A_1 agonists [97] and A_3 antagonists [98]. The compounds can be used alone or in combination therapy and have shown IOP reduction in New Zealand White (NZW) rabbit.

1.6.2 Non-pharmacological interventions

Unfortunately, drug therapy is not always the most effective strategy, for this reason when pharmacological treatment does not achieve adequate IOP reduction with acceptable adverse effects, laser or incisional surgeries are the first-line therapy. Laser trabeculoplasty lowers the IOP by inducing biological TM modifications resulting in an increase of AH outflow. Although substantial IOP reductions can be achieved in the majority of patients, the effect decreases gradually over time with a failure rate of about 10% per year [99–101].

Trabeculectomy is the most common surgical procedure to lower IOP. It consists of excision of a small portion of TM and or adjacent corneoscleral tissue to provide a drainage route for AH. Devices that drain AH to an external reservoir are an alternative to trabeculectomy that are similarly effective in lowering IOP [102].

The first-line treatment of ACG is laser peripheral iridotomy, a procedure in which a full thickness hole is created in the iris to eliminate pupillary block. Rare complications of iridotomy include transient increases of the IOP, cornea decompensation, adhesions of iris to lens (posterior synechiae formation), and optically induced visual disturbances. Eyes treated with iridotomy may still develop increased pressure over time; thus, it is essential to have periodic follow-up after the procedure. If pressure remains high after iridotomy, long-term pharmacological treatment can be instituted, similar to the management of OAG [35].

1.6.3 Neuroprotection as a new therapeutic approach

Unfortunately, IOP management may not be sufficient to prevent the development of glaucoma or the associated progressive vision loss. There are novel therapeutic interventions that purpose to interfere with the molecular mechanisms causing neuronal damage, acting on targets that are not concerned with IOP control, although enhancing the survival of retinal cells [103]. By observing the complexity of these mechanisms, numerous molecules have been identified that could have the potential to block neurodegenerative events induced by glaucoma. Since the advent of the concept of neuroprotection, a lot of molecules have been tested with very low success rates in the translation from the laboratory to patients. Accordingly, more than 100 neuroprotective drug candidates have failed to demonstrate efficacy, acceptable safety, or patient benefit. Most of them, in fact, despite successful preclinical data, failed to pass most of the Phase 2 and virtually all the Phase 3 clinical trials. For instance, memantine, a non-competitive N-methyl-D-aspartate (NMDA) subtype of glutamate receptor antagonist, already in use in the treatment of Alzheimer's disease, showed convincing neuroprotective effect in animal models of glaucoma. Similar outcome was yielded by brimonidine, preclinical studies have shown a neuroprotective effect on RGCs in animal models of optic nerve injury relevant to glaucoma [104]. Moreover, the Low-Pressure Glaucoma Treatment Study has shown that, over a 30-month period, patients treated with brimonidine had a significantly lower rate of visual field defect progression compared to subjects treated with timolol (9 vs. 30%), thus supporting the dual action of the molecule [104]. However,

the study and, therefore, neuroprotective efficacy in humans, were questioned in two reviews suggesting the need for further studies [103].

It is widely known that oxidative stress is involved in the aetiology of glaucoma [105–111]. Understanding the mechanisms of reactive oxygen species (ROS)-induced oxidative stress is very crucial for designing strategies in prevention of damage caused by ROS. Based on the involvement of ROS in the aetiology of glaucoma, there are several strategies to control the balance between ROS and antioxidants. They include the following: control endogenous antioxidants and antioxidant enzymes; administration of antioxidants; control of ROS production; and regulating the expression of antioxidative factors or genes.

There are a variety of antioxidants being applied in animal studies and clinical studies, including vitamin C, vitamin E, lutein, CoQ10, and flavonoids, *Ginkgo biloba*, *Lycium barbarum*, α-lipoic acid, and ghrelin. Although a number of reports have shown beneficial effects of antioxidative strategies in animal glaucomatous models and cell cultures, additional studies are needed to demonstrate that these effects would be useful approaches in clinical therapeutics [112].

1.7 Ocular blood flow and ischemic damage in glaucoma

The regulation of blood flow in the eye, is different in different tissues. The regulation of retinal blood flow is very similar to the regulation of blood flow in the brain, with the exception that retinal vessels have no autonomic innervation and therefore its regulation depends even more on the activity of endothelium cells. These cells release several factors, the so-called endothelium derived vasoactive factors (EDVFs) [113] which on one hand regulate the size of the vessels by influencing vascular smooth muscle cells locally, and on the other hand, via intraluminal release of these factors influence the size of the vessels globally. The regulation of blood flow of the choroid is very different from that of retinal blood flow. The choroidal vessels are extensively autonomically innervated, and the capillaries are fenestrated. Besides providing oxygen and other molecules to the retina, the choroid regulates the temperature of the back of the eye and most contributes to the fine tuning of accommodation by regulation of volume [114].

It has been hypothesized that low ocular perfusion pressure led to alterations in blood flow at the ONH and contribute to progressive glaucomatous optic nerve damage. Population-based studies in African Americans, non-Americans of African descent, Hispanics, and non-Hispanic whites have provided evidence that low diastolic perfusion

pressure (<50 mmHg) is associated with a higher prevalence of POAG [23,115–118]. In addition, in the Early Manifest Glaucoma Treatment Study, low systolic perfusion pressure (≤ 125 mmHg) was associated with a higher risk of glaucoma progression over an 8-year period [119]. More recent data suggest that nocturnal mean arterial pressure 10 mmHg lower than daytime mean arterial pressure may predict progression of NTG and increased risk of visual field loss [120]. Recent evidence suggests that low diastolic perfusion pressure is associated with increased risk for glaucoma only in patients taking treatment for systemic hypertension [121]. However, statistical analysis is unable to determine whether perfusion pressure is associated with glaucoma because of its individual components (systolic blood pressure, diastolic blood pressure, or IOP), a combination of these components, or an interaction between these components [122].

Impaired ocular blood flow has been shown to result in insufficient oxygen and nutrient supply to the RGC axons crossing the ONH, causing their degeneration [123]. In fact, unstable ocular blood flow produced, by chronically elevated and/or oscillating IOP, together with possible vascular dysfunction (e.g., decreased perfusion pressure, autoregulation deficit, or vasospasm) can lead to repeated hypoperfusion resulting in ischemic damage. This causes a massive inflammatory response and chronic oxidative stress resulting in free radical formation [114,124,125]. The ROS formed attack RGCs cell structure leading to their depletion by autophagy and/or apoptosis, resulting in a degeneration of the whole retina and ONH [126].

In addition to those already mentioned, there are other factors that contribute to generating retinal damage subsequently an ischemic insult, such as mitochondrial dysfunction, endothelins, glutamate and tumor necrosis factor-alpha (TNF-α).

1.7.1 Mitochondrial dysfunction

The retina is one of the most energy and oxygen demanding tissues in the body [127]. In this context, mitochondria are essential for retinal homeostasis, and substantial evidence has shown that retinal damage is triggered and perpetuated by mitochondrial dysfunction. Mitochondria produce ROS as a by-product of electron leak along the electron transport chain during cellular respiration [128]. This activity is essential to maintain the energy requirements for neuronal function, and RGCs have an absolute requirement for optimal mitochondrial function to maintain survival [129]. Mitochondrial activity may be impaired by ischemia/hypoxia or by oxidative stress, both associated with glaucoma. A lack of nutrients or oxygen can not only result in bioenergetically

compromised RGC axons, but can also dramatically increase ROS production, which leads to further oxidative stress and retinal damage [125,130–132].

1.7.2 Endothelins

Endothelins are vascular factors closely related to ischemia and blood-retinal barrier disruption. The ET receptors have been shown to mediate RGC loss and ONH degeneration [133,134]. ET-1 is a potent vasoconstrictor produced by vascular endothelial cells, but also by microglia/macrophages that plays a role in local vascular tone [133,135]. Increased plasma levels of ET-1 have been found in POAG patients and this may be linked to vasoconstriction, decreased ocular blood flow and ischemia/hypoxia [136]. Acute intraocular injection of ET-1 in rats has been shown to increase glial fibrillary acidic protein (GFAP) expression in Müller cells, indicating glial cell hyperactivity and RGC death [137]. Both ETA and ETB receptors were upregulated during the early stage of retinal neurodegeneration. In an ocular hypertensive rat model, elevated IOP resulted in overexpression of ETA and ETB in the retina and was associated with cell death [138]. Finally, Wang et al. reported upregulation of ETB expression in ONH astrocytes in an ET-1-induced chronic optic neuropathy, causing RGC loss [125,139].

1.7.3 Glutamate

Accumulation of glutamate is the result of a number of insults, including inappropriate electrical activity, ischemia/reperfusion injury and neurotrophin deprivation. This may initiate the death of neurons containing ionotropic glutamate (NMDA) receptors [140]. Activation of NMDA receptors on RGC induces the influx of calcium and the generation of free radicals, leading to cell death. It has been suggested that Müller cells may increase RGC susceptibility to stress signals in response to elevated IOP, thereby contributing to disease progression [141]. In support of this, it has been shown that Müller cells have a reduced ability to regulate glutamate homeostasis upon release of TNF-α and other inflammatory molecules [142–144].

1.7.4 Tumour necrosis factor-alpha (TNF-α)

TNF-α is found in high levels in the retina and ONH of glaucoma patients and appears to play a key role in the regulation of neuroinflammation [145–147]. This cytokine is primarily produced by glial cells, including microglia, astrocytes, and Müller cells, and its production seems to be upregulated in glaucoma in response to vascular

(ischaemic) or oxidative stress as previously described [146,148,149]. It has been shown that in a rat model of glaucoma, the OHT determined a dramatic increase in TNF-α levels produced by microglial cells around the ONH within a few days, associated with axonal degeneration and a 38% loss of RGCs after several weeks [150]. TNF-α may affect RGC survival through different pathways, including the activation of nitric oxide synthase (NOS) expression and NO production [145], mitochondrial dysfunction [151], modulation of tissue remodelling via the synthesis and secretion of matrix metalloproteinases (MMPs) including MMP-9 [152], and stimulation of ET-1 synthesis in several ocular cell types, including ONH astrocytes [153]. There is evidence demonstrated that, on RGCs, soluble TNF-α, induces apoptosis, via activation of the caspase (caspase 8) signalling cascade, loss of mitochondrial membrane potential and generation of ROS [144]. These processes are consistent with the significant decrease in RGC death due to neutralisation of TNF effects with anti-TNF-α antibodies [154]. In addition, inhibition of TNF-α activity, by a soluble TNF-α receptor antagonist, inhibited microglial response and prevented axonal degeneration and loss of RGCs in a rat model of hypertensive glaucoma [150]. Some contradictory studies have also shown that TNF-α released by activated microglia causes astrocytes to produce neuroprotective factors in response to relatively mild hypertensive glaucomatous injury [155].

It was suggested that TNF-α acts as an important regulatory cytokine with differential signalling through the two distinct receptors that determine its contribution to degenerative or regenerative processes. Therefore, depending on the type and stage of glaucoma, environmental/external factors, TNF-α may function to aggravate or ameliorate the disease [156].

1.8 Histaminergic system

1.8.1 Biological effects of histamine

Histamine [2-(4-imidazolyl)-ethylamine; β-imidazolylethylamine] is an endogenous, short-acting biogenic amine that is ubiquitously distributed in several tissues at various levels [157]. It is synthesized by decarboxylation of the amino acid L-histidine via the catalytic activity of histidine decarboxylase (HDC). Histamine elicits its pleiotropic functions by activating four types of class A rhodopsin-like G protein-coupled receptors (GPCRs), named histamine H_1, H_2, H_3 and H_4 receptors (H_xR) [158], which are 7-transmembrane chain proteins that mediate the effect of several molecules (Figure 7).

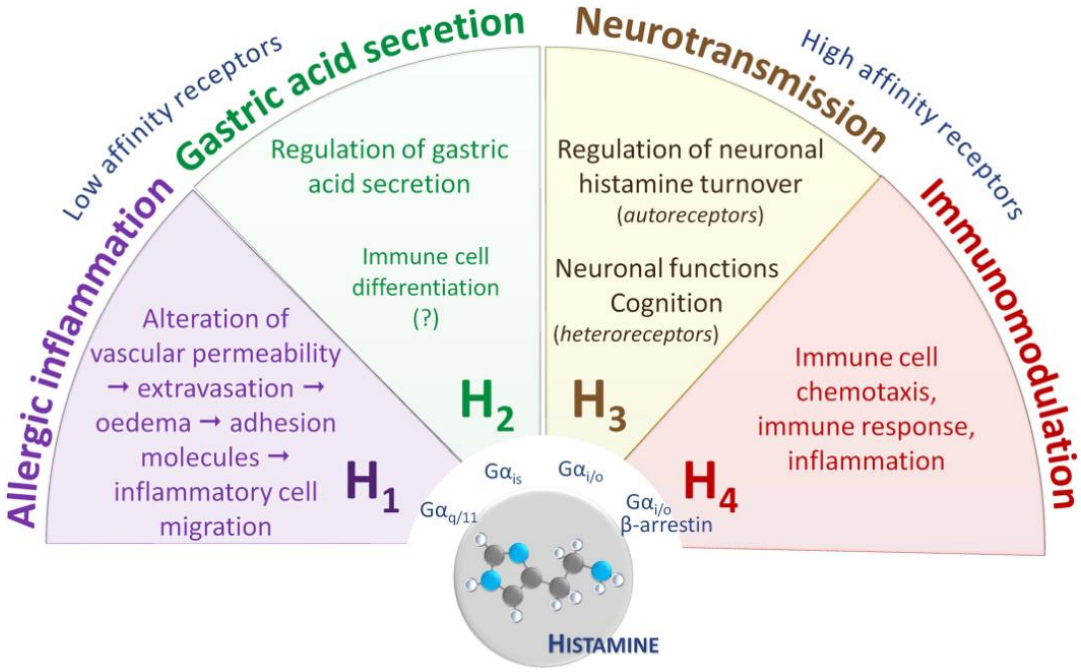


Figure 7: Histamine receptors and their functions (Tiligada & Ennis, 2020).

1.8.2 Histamine H_1 receptor

H_1 receptor (H_1R) is widespread throughout the body, including neurons, smooth muscle cells of the airways, and blood vessels. It preferentially couples to Gαq/11 proteins, causing phospholipase C (PLC) and protein kinase C (PKC) activation as well as inositol-1,4-5-trisphosphate (IP3) formation and intracellular Ca^{2+} release [159]. As a consequence, through the activation of H_1R, histamine elicits the contraction of smooth muscle of the respiratory tract, increases vascular permeability stimulating NO availability, and induces the production of prostacyclin and platelet activating factor. Histamine, H_1R regulates sleep-wake cycles, food intake, thermal regulation, emotions/aggressive behavior, locomotion, memory, and learning [160]. Antagonists and inverse agonists of this receptor, including mepiramine, fexofenadine, loratadine, diphenhydramine, and astemizole are the most important anti-allergic drugs used [161].

1.8.3 Histamine H_2 receptor

H_2R is highly expressed in various cells and tissues, such as dendritic cells, gastro-parietal cells, smooth muscle cells, brain and cardiac tissues, and B and T cells. The H_2R is coupled to Gαs protein triggering adenylate cyclase (AC), its stimulation enhances the amounts of cAMP and downstream effects mediated by protein kinase A (PKA) and the

transcription factor cAMP- response element binding protein (CREB). However, using a different GTP-dependent mechanism, H_2R also modulates phosphoinositide second messenger system [159]. Many of the H_1R-mediated effects can be balanced by the H_2R, including the relaxation of smooth muscle cells, causing vasodilation. Moreover, the activation of this receptor causes marked chronotropic and inotropic effects in the heart and induces gastric acid production from parietal cells in the gastric mucosa. Most H_2R antagonists/inverse agonists including cimetidine, famotidine, and nizatidine are clinically used to inhibit histamine-induced gastric acid secretion [162,163]. Experiments with *knockout* mice have shown that the histamine H_2R is implicated in modifying the immune responses, specifically in the modulation of Th1- or Th2-cell polarization [164,165].

1.8.4 Histamine H_3 receptor

The H_3R is a Gαi/o-coupled protein receptor mainly expressed in neurons acting as a pre-synaptic auto- and hetero- receptor. Its activation leads to inhibition of cAMP formation, accumulation of Ca^{2+}, and stimulation of the MAP-kinase pathway [166]. It inhibits the release of histamine [167,168] but also of other neurotransmitters such as acetylcholine, noradrenaline, dopamine, or glutamate [169]. It is important for homeostatic regulation of energy levels, sleep-wake cycle, cognition, and inflammation [170]. H_3R-deficient mice exhibit altered behavior and locomotion [171] and display a metabolic syndrome characterized by obesity, hyperphagia, and increased leptin and insulin levels [172,173]. Similarly, several studies suggest that H_3R *knockout* can also lead to an increase in severity of neuro-inflammatory diseases and can enhance the expression of interferon (IFN)-inducible protein 10, MIP 2, and CXCR3 in T cells [174]. This receptor has also been associated with rhinitis [160], likely because it is expressed on presynaptic nerves in the peripheral sympathetic adrenergic system and also on nasal sub-mucosal glands. Indeed, as mentioned above, the stimulation of H_3R suppresses norepinephrine release at presynaptic nerve endings and stimulates nasal sub-mucosal gland secretion [175]. There is increasing evidence showing that histamine H_3R is also expressed post-synaptically [176], in particular in the basal ganglia and within the dorsal and ventral striatum, for this reason it is considered a potential target for treating cerebral disorders [177]. Pitolisant is a first-in-class FDA-approved agent for the treatment of daytime sleepiness in adults with narcolepsy by acting as an antagonist/inverse agonist at the H_3 receptor. In addition, H_3R antagonists are effective for the treatment of other

psychiatric diseases such as attention-deficit/hyperactivity disorder, schizophrenia, and neurological conditions like, Parkinson's disease, pain, Alzheimer's disease, and epilepsy. There is also evidence showing efficacy of H_3R antagonists in the management of allergies and alcohol dependence [178,179]. Other conditions that may be treated with H_3R antagonists are Tourette syndrome [180], multiple sclerosis[181], depression [182], Huntington disease [183] and autism [184].

1.8.5 Histamine H_4 receptor

Histamine H_4R, discovered in 2000, is the most recently identified histamine receptor. The structure of H_4R is almost 40% identical with that of H_3R in terms of sequence identity and 60% similar when the transmembrane domains are compared [185]. It is a Gαi/o-coupled protein receptor that is predominantly expressed in cells of the immune system (basophils, mast cells, eosinophils, dendritic cells, monocytes, NK, iNK T and γδ cells, CD8+ T cells, Treg, and Th2 cells) and it is involved in immune-modulatory pathways. The expression of this receptor has been detected in various tissues including the spleen, thymus, lung, small and large intestines, and also cancer cells [186]. Histamine H_4R antagonism prevents histamine-induced $[Ca^{2+}]i$ increase, mast cell chemotaxis, and submucosal mast cell accumulation in the trachea of mice after histamine inhalation. It has been demonstrated that the administration of H_4R antagonist, such as JNJ7777120 prevents bleomycin-induced pulmonary fibrosis alone and in association with naproxen [187,188]. In recent years, more information about this receptor has been highlighted; in fact, it has been found that H_4R is able to interact with adenosine A_3 receptor (A_3AR) by modulating neuropathic pain via T cell-mediated IL-10 production, suggesting a role of the histaminergic system, and in particular of this receptor, in the mechanism of A_3AR-mediated neuropathic pain relief through IL-10 up-regulation [189]. Moreover, it has been shown that H_4R *knockout* diabetic mice developed more severe hyperglycaemia and a higher 24h urine volume hypothesizing that the effect on the urinary volume could be due to an imbalance in histamine receptors due to the histamine H_4R deletion [190].

1.8.6 The histaminergic system at ocular level

Histamine is an important neurotransmitter acting at different levels, but its influence on ocular physiology and pathology has not yet been fully elucidated. In the retina, no histamine-forming cells have been identified to date; however, retinopetal axons arising from the tuberomamillary nucleus extend across the inner plexiform layer,

eliciting responses in a range of inner retinal neurons [191]. The synthesis and release of this neurotransmitter are controlled by presynaptic histamine H_3 auto-receptors located in the CNS [167,168]. The presence of histamine H_1, H_2 and H_3R in the inner layer of ganglion cells in rodents and primate retinae has been established [191,192]; in particular, it has been shown that circuits involved in scotopic vision may be altered by histamine release. In the macaque retina, the stimulation of histamine H_3R increases the delayed rectifier component of the voltage-dependent potassium conductance in ON bipolar cells [193], and in dark-adapted baboon retinas, histamine decreases the rate of maintained firing and the amplitude of the light responses of ON ganglion cells [194]. The primate retina receives input from histaminergic neurons that are active during the day in the posterior hypothalamus and receive input from the brain via axons emerging from the optic nerve. It is known that histamine reduces the amplitude of light responses in monkey RGCs, a finding consistent with a role for retinopetal axons in light adaptation in these diurnal animals [195]. These findings suggest that histamine acts primarily via volume transmission in the primate retina, increasing the operating range of cones and conserving ATP in bright, ambient light [165]. The histaminergic system is deeply implicated in circadian rhythm and fulfils a significant role in maintaining wakefulness [196], hence, it could play a role in maintaining IOP balance. Indeed, it has been shown that histamine tone is decreased at night, and nocturnal IOP is higher than diurnal pressure [197]. This balance is entirely controlled in healthy subjects, with three crucial mechanisms: the rate of AH formation, the resistance to the outflow, and episcleral venous pressure. These factors change during the night, the AH production decreases significantly in diurnal mammalians, as does the drainage; thus, the IOP increases [198]. Histamine regulates ciliary muscle contraction in human eyes and, therefore, it controls IOP reduction [199].

Regarding histamine receptors localization, it is well known that H_1Rs are situated on horizontal cells and in a small number of amacrine cells, whereas H_2Rs appear closely associated with synaptic ribbons inside cone pedicles [192]. Both these receptors in the iris arterioles, and specially H_2R in the iridal venules, modulate vascular tone in rats [200]. Moreover, several ocular hypertensive effects have been reported in chronic glaucoma patients following the use of cimetidine and ranitidine, two H_2R antagonists, for peptic ulcer treatment [201]; conversely, recent studies have failed to demonstrate the significant action of topical administered H_2 blockers on IOP in humans [202]. Histamine H_1 and H_2R antagonists possess anticholinergic activity that may induce glaucoma.

Promethazine, an antipsychotic with antihistamine activity, has been shown to produce an idiopathic swelling of the lens that could increase the risk of ACG. Topical administration of ranitidine produces vasoconstriction in both the arterioles and the venules of the iris, suggesting a predominant role of H_2R in the vasculature of the iris [200]. As previously mentioned, H_4Rs are expressed by various inflammatory cells, including eosinophils and Th2 cells, in allergic disorders [203], it was also observed that infiltrating inflammatory cells in subconjunctival tissues of kerato-conjunctivitis patients strongly express H_4R [204].

The expression of histamine receptor in the eye is well documented. A previous work of our group [205] reported the expression of histamine receptors in rabbit eyes. In this work H_1R and H_4R expression were found in the retina and optic nerve at a higher concentration, high H_3R protein expression levels were found in the retina, optic nerve, and CB, whereas histamine H_2R was found only in the stomach [205]. In the same work, the effectiveness of several compounds, mainly histamine H_3R antagonists, was analysed, demonstrating that a chronic treatment with these compounds reduced IOP, improved ocular vascular tone and prevented the death of neurons in the RGC layer of hypertensive rabbit eye [205] suggesting a crucial role of histamine H_3 receptor at retinal level.

1.9 The role of Nitric Oxide in the eye

Nitric Oxide is an endogenous messenger produced, by oxidation of L-arginine under the action of the two NOS isoforms, neuronal and endothelial NOS. Under normal conditions, they generate relatively small amounts of NO (picomolar or low nanomolar range) when activated by the calcium/calmodulin complex after an increase of calcium.
With the knowledge that NO and its second messenger cyclic guanosine monophosphate (cGMP) are distributed in most tissues with a diverse array of biological effects it has become evident that the NO pathway is also important for multiple functions in the eye.
Moreover, in the vasculature, shear stress generated by blood flow plays an important role in eNOS regulation [206]. The vasodilating action of NO, mediated by stimulation of soluble guanylate cyclase (sGC), leads to elevation of intracellular cGMP levels. Cyclic guanosine monophosphate then interacts with various cyclic-nucleotide–gated channels, protein kinases, and proteins phosphodiesterase to produce physiological effects. Other biological actions occur through cGMP-independent pathways such as post-translational modification of proteins by S-nitrosylation [207]. Through S-

nitrosylation of proteins, NO has been shown to regulate apoptosis, vascular tone, and inflammatory responses [208].

In pathological conditions, stimuli such as those generated by infectious diseases, inflammation, or ischemia induce and activate a third NOS isoform, inducible NOS (iNOS) producing a large amount of NO. Reaction of these high NO levels with superoxide free radicals in the local milieu can then lead to the formation of peroxynitrite and other reactive nitrogen species [209]. Under physiological conditions, low levels of peroxynitrite are removed rapidly by endogenous antioxidant mechanisms; however, the high levels formed in conditions of oxidative stress can bind to many molecules including lipids and proteins, thus altering biological function and potentially mediating oxidative damage. In the ocular system, reactive nitrogen species may lead to pathophysiological actions such as inflammation and optic nerve degeneration [210].

Several studies have shown that NO plays a key role in modulating the dynamic balance between the inflow and outflow of AH. Indeed, the increased intracellular content of cGMP is metabolized by phosphodiesterase 5 (PDE5), causing a relaxation of the smooth muscle resulting in an elevation of AH outflow and therefore a decrease in IOP.

There are increasing evidence that a deficit of NO/sGC pathway is directly associated with glaucoma. In fact, the stimulation of this pathway directly or via administration of NO donors lowers IOP through relaxation of the TM [211]. In addition, NO levels were decreased in the AH of patients with POAG [212].

NO donating anti-glaucoma drugs have been attracted many attentions and achieved great advances. NO donating drugs including nitroglycerin, isosorbide dinitrate, sodium nitrite, and sodium nitroprusside, can lower IOP after topical administrations [213]. Recently, antiglaucoma drugs consisting of a conventional drug to which a NO-releasing group is covalently bound via a linker have been developed. After administration via eye drops, NO-donating anti-glaucoma drugs would decompose to regenerate parent anti-glaucoma drugs and NO, generating additional or synergistic anti-glaucoma effect. In this context, NO donor molecules linked to phosphodiesterase inhibitors, aimed not only at treating ocular hypertension but also at improving blood flow at ocular level, are recently in pre-clinical study [214].

2. Aim of the research

The aim of my research has been to evaluate the capability of histamine H_3R antagonist-NO donor hybrid compounds acting at different biological levels in ameliorating glaucoma condition and to study their neuroprotective action, in three different animal models. The present project was focused:

1. to study the effects of hybrid compounds formed by a histamine H_3R antagonist bound to a NO donor in two different OHT model in NZW rabbit. The first was the transient OHT model achieved by injection of a hypertonic saline solution into the posterior chamber of the eye, the second was the chronic OHT model, performed by injection of carbomer into the anterior chamber of the eye.
2. to evaluate the effectiveness of the histamine H_3R antagonist-NO donor hybrid compound ST-1989 in a chronic OHT model and to understand its effect in the control of inflammation, oxidative stress, and apoptosis in ocular tissues.
3. to investigate the capability of this hybrid compound to reduce the IOP, to ameliorate the hemodynamic of ophthalmic artery and to preserve the degeneration of photoreceptors induced by the ischemic damage, in a rabbit model of retinal ischemia/reperfusion (I/R).

The molecules used in this project are three histamine H_3R antagonists-NO donors hybrid compounds synthesized by a research group at the University of Düsseldorf and named ST-1989, ST-2126 and ST-2130. These compounds were compared with reference drugs such as Ciproxifan, an H_3R antagonist, and molsidomine, an NO donor, to see whether the compounds under study, had the same efficacy as reference drugs, administered alone and in combination, or whether the newly synthesized molecules had a better pharmacodynamic profile than the reference drugs and fewer side effects. First, in an acute model of OHT I performed an efficacy screening of compounds to select the most responsive molecule, then I tested this compound in a chronic model of OHT to investigate its action in a long-term period. In addition, in chronic OHT I decided to evaluate the efficacy of this molecule in controlling the inflammatory response, oxidative stress and apoptotic process, the main factors responsible for the progression of glaucomatous disease and retinal degeneration.

Abnormalities in blood flow in the ONH and retina are commonly observed in multiple ocular diseases, including glaucoma. Recent studies have also suggested that perfusion instability, rather than a progressive decline in ocular blood flow, may contribute to the

development of glaucoma. It is well established that circadian rhythms play an important role in maintaining homeostasis in the human body [215]. In this context, glaucoma progression is not necessarily related to the overall level of blood pressure, but an abnormal vascular reaction to a reduction in blood pressure or increase in IOP during non-awake hours [216]. It has long been known that IOP varies throughout the day, but not until recently has it been shown that other vascular risk factors such as systemic blood pressure [217], ocular perfusion pressure [218], and ocular blood flow [219] also follow circadian patterns. There are several evidence showings that the histaminergic system is also involved in circadian rhythm, so the final part of this project has been directed:

4. to investigate the interaction between the histaminergic and nitrergic systems in improving the hemodynamic profile at the ocular level and to investigate their potential neuroprotective action in preventing RGCs loss and photoreceptor degeneration in a model of retinal ischemia/reperfusion induced by repeated ET-1 injections in rabbit.

3.Materials and Methods

3.1 Animal models

The experimental protocols were carried out in NZW rabbits. All the experimental procedures were conducted in accordance with the Italian regulation on protection of animals used for experimental and other scientific purpose (Italian Legislative Decree 26, March 13, 2014) and with the EU Regulations (Council Directive of the European Community 2010/63/EU), upon authorizations of Italian Ministry of Health (number 318/2018-PR and 110/2021-PR).

Male albino rabbits (body weight 1.8-2.0 kg) were kept in individual cages; food and water were provided *ad libitum*. The animals were maintained on 12-12h light/dark cycle in a temperature-controlled room (22°-23°). Animals were identified with a tattoo in the ear, numbered consecutively. All selected animals underwent ophthalmic and general examinations before the beginning of the study.

In the present study were use three different animal models:

3.1.1 Acute ocular hypertension model was obtained by injection of 50 µl of sterile hypertonic saline (5% NaCl dissolved in sterile water) into the vitreous bilaterally in locally anesthetized rabbits with one drop of 0.2% oxybuprocaine hydrochloride [220]. In this model, the IOP rises after about 10-20 minutes and the hypertension is maintained for 4 hours (Figure 8).

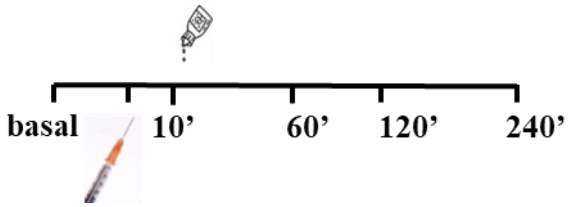


Figure 8: Acute ocular hypertension model with IOP measurements at different time points. IOP was measured under basal conditions, then 50 µl of sterile hypertonic saline was injected into the posterior chamber of the eye. When maximum IOP was reached, usually after 10 minutes, treatments were administered, and IOP values were measured at 60-, 120-, and 240-minutes post-dosing.

3.1.2 Chronic ocular hypertension model was obtained by injection of 100 µl of carbomer 0.25% (Siccafluid, Farmila THEA Pharmaceutical) bilaterally into the anterior chamber of NZW rabbits pre-anesthetized with xilazine (Xilor 2%, 5 mg/kg) plus

ketamine (Lobotor 20 mg/kg) injected intramuscularly [221]. In this model the IOP rises after about two days and the hypertension is maintained for at least 12 days (Figure 9).

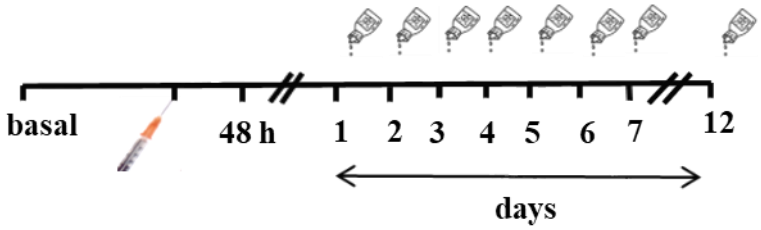


Figure 9: Chronic ocular hypertension model with IOP measurements. The IOP was measured at basal conditions, then 100 µl of carbomer 0.25% was injected into the anterior chamber of the eye. After the hypertension stabilization, the IOP was detected every day for 12 days before drug dosing.

All animals received a single topical dose of 30 µl of the studied compounds. During the experimental protocol, IOP was measured with a Pneumatonometer (Model T30, Reichert Technologies, Depew, NY, USA) before and 30-60-120 and 240 minutes after administration of compounds in the acute model. In the chronic OHT model, IOP was assessed at baseline and starting from the stabilization of hypertension every day for 12 days before drug dosing.

3.1.3 Retinal artery ischemia/reperfusion model was obtained by subtenon injection (twice a week for 6 weeks) of 200 µl of 250 nM ET-1 (Fluka, Israel) in water, using a lacrimal cannula under anaesthesia produced by xylazine plus ketamine as previously described. The ET-1 was injected twice a week for six weeks. During the experimental protocol, several functional assessments such as IOP, ophthalmic artery resistance index (OA-RI) and electroretinogram (ERG) were conducted. The IOP was measured every week after 36 hours of wash-out. The hemodynamic profile and retinal function were measured at basal levels, 2 weeks after ET-1 injections and at the end of experimental protocol. The animals were treated with vehicle or ST-1989 twice a day for four weeks (Figure 10 panels A-B).

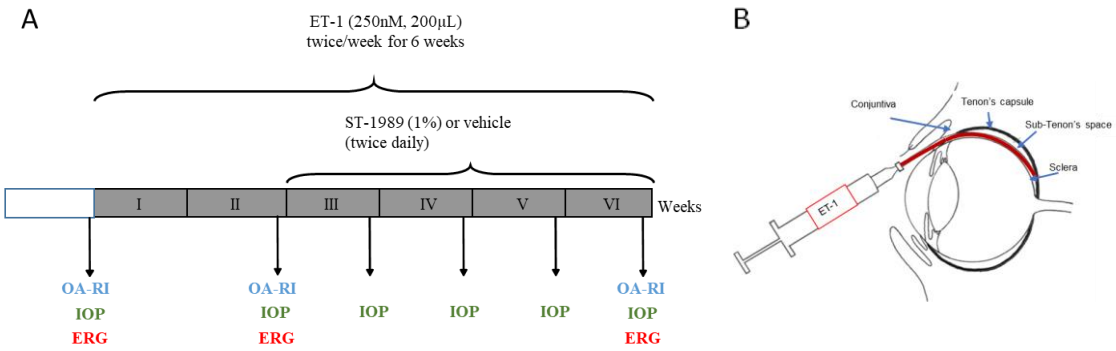
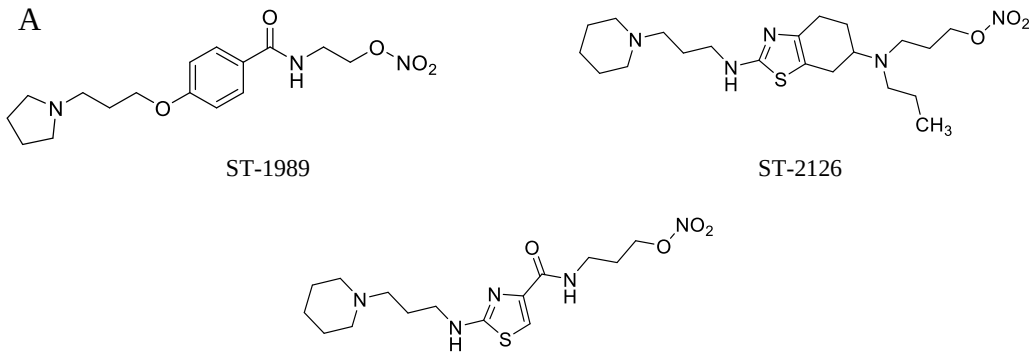


Figure 10: A) Retinal artery ischemia/reperfusion (I/R) model and time-course of the functional assessments. The ET-1 was injected into the subtenon capsule of the eye twice a week for six weeks. The treatments were administrated twice a day starting from week 2 until the end of the experimental protocol. Functional analyses were performed at basal condition, after two weeks of ET-1 injections and at the end of week 6. The IOP was measured every week to understand the pressure lowering profile trend.
B) Schematic representation of ET-1 injection below the Tenon capsule.

3.2 Compounds

Figure 11 (panel A) represents the formulas of hybrid compounds kindly provided by Professor Holger Stark (Department of Medicinal Chemistry, University of Düsseldorf, Germany). These are histamine H_3R antagonist-NO donor hybrid compounds with a high affinity for histamine H_3 receptors (Figure 11, panel B). A dimethyl sulfoxide (DMSO) stock solution of each compound was prepared. Immediately before use, an aliquot has been collected and dissolved in saline (NaCl 0.9%). The animals were randomized in independent experimental sessions with a washout period of at least 1 week. A drop of 0.2% (4 mg/ml) oxybuprocaine hydrochloride was injected into the eyes immediately prior to each measurement. All compounds were topically administered as eye drops at 1% w/v concentration, and a vehicle solution composed of 0.9% NaCl, 1% DMSO was used as a control.



B

Code (mw)	H_3R K_i [nM]
ST-1989 (337.38)	6
ST-2126 (439.62)	136
ST-2130 (371.46)	34

Figure 11: Formulas of histamine H_3R antagonist-NO donor hybrid compounds and their receptor affinity.

3.3 Functional assessments

3.3.1 Intraocular pressure (IOP) determination

All the IOP measurements were performed by applanation tonometry using a pneumatonometer (Model 30™, Reichert Technologies, Depew, NY, USA). It is an easy-to-use instrument that provides fast and accurate tonometry and optional tonography functions. The probe tip, which floats on an air bearing, is gently touched to the anesthetized cornea and a precisely regulated flow of filtered air applies force to the tip. A small (5 mm diameter) fenestrated membrane permits the air to flow through vents in the tip until it conforms to the shape of the cornea. Increasing pressure is continually applied to the cornea until the force being applied is equal to the pressure in the anterior chamber. When these forces are in balance a pneumatic sensor records the intraocular pressure. The pneumatonometer was used both in acute and chronic OHT model. One drop 0.2% oxybuprocaine hydrochloride diluted 1:1 with saline, was instilled in each eye immediately before each set of pressure measurement. To better understand the IOP-reducing effects of hybrid compounds in both animal models, delta IOP (ΔIOP) was calculated with the following formula: IOPpost-IOPTx where the former represents the maximum IOP reached, and the latter is the IOP at different time points after compounds administration. This ΔIOP was then used to calculate ΔΔIOP, a useful parameter for understanding the activity profile of different molecules in relation to vehicle.

In the ischemia/reperfusion model, the IOP was measured on Monday (36h free of treatment) before morning administration of compounds. IOP changes from baseline were calculated as follows: IOPTx-IOPT0 where IOPTx and IOPT0 are, respectively, the IOP at the time of interest and at baseline.

3.3.2 Ophthalmic artery hemodynamics

Measurements were taken using an Echo Color Doppler (Philips Ultrasound HD7XE; Philips, Milan Italy), before ET-1 treatment (basal, time 0), and weekly thereafter on Mondays until the end of the study. Pourcelot resistance index for OA-RI was calculated using the following formula: OA-PSV–OA-EDV/OA-PSV where OA-PSV and OA-EDV are the ophthalmic artery peak systolic velocity and ophthalmic artery end diastolic velocity, respectively. This Index is useful because provides a dimension of local perfusion impairment.

3.3.3 Electroretinogram (ERG) analysis

The electroretinogram (ERG) is a diagnostic test that measures the electrical activity of the retina in response to a light stimulus. The ERG arises from currents generated directly by retinal neurons in combination with contributions from retinal glia. It is an objective measure of retinal function that can be recorded non-invasively under physiological conditions. ERGs are often recorded using a thin fiber electrode that is placed in contact with the cornea or an electrode that is embedded within a corneal contact lens. These electrodes permit the electrical activity generated by the retina to be recorded at the corneal surface. The ERG can be elicited by diffuse flashes stimuli. The International Society for Clinical Electrophysiology of Vision (ISCEV) has introduced standards for the different forms of ERG recordings. The ERG has important clinical utility, in that it provides diagnostic information concerning a variety of inherited and acquired retinal disorders. Moreover, the ERG can be used to monitor disease progression and evaluate retinal toxicity due to various drugs or retained intraocular foreign bodies.

In my experimental protocol the eyes were dilated by topical application of tropicamide 1% and, when needed, adapted to darkness for at least 2 h before standard ERGs recording of both eyes using contact lens corneal electrodes so to have sufficiently stable and amplified recordings. The ERG signals were recorded using Retimax Advanced (CSO, Florence, Italy) according to the current ISCEV indications, as previously reported [222]. To better understand the extent of retinal damage and the effects of the compound in ameliorating this damage, three different recordings were made, specifically the dark-adapted 0.01 ERG (rod response), the light-adapted 3.0 ERG (cone response), and the dark-adapted 3.0 ERG (combined rod-cone response). The intensity of the flashes varied according to the test performed:

a) DARK-ADAPTED 0.01 ERG: from 0.01 photopic cd.s.m^{-2} to 0.025 scotopic cd.s.m^{-2} with an interval between flashes of 2s.

b) LIGHT-ADAPTED 3.0 ERG: from 3.0 photopic cd.s.m^{-2} to 7.5 scotopic cd.s.m^{-2} with an interval between flashes of 0.5s and a light adaptation strength of 30 cd.s.m^{-2}

c) DARK-ADAPTED 3.0 ERG: from 3.0 photopic cd.s.m^{-2} to 7.5 scotopic cd.s.m^{-2} with an interval between flashes of 10s.

In all cases ERG recordings lasted 250ms.

Measurements were taken before ET-1 first dose (basal), at the end of week 2 (before vehicle, or ST-1989-first day-first dose) and at the end of week 6 (36 h after vehicle, or ST-1989-last day-last dose).

3.4 Tissue Sampling

At the end of chronic OHT and I/R model the animals were euthanized with an overdose of anaesthetic (Pentothal sodium 0.15 g/kg, i.v. bolus), AH, CB and retina samples were collected and stored at -80°C to perform biochemical assay. For each treatment, the whole eye was harvested and fixed with 4% paraformaldehyde in phosphate-buffered saline for morphological and histopathological evaluations.

3.5 Biochemical determinations

3.5.1 NO production

To measure the accumulation of nitrite (i.e., NO_2^-), a stable end product of NO metabolism, the AH was collected 60 minutes after hybrid compounds administration. The amount of nitrite was determined spectrophotometrically by the Griess reaction as previously described [223]. In brief, the samples were allowed to react with Griess reagent (1% sulfanilamide and 0.1% N-[1-naphtyl] ethylendiamine in 5% phosphoric acid) to form a stable chromophore which absorbed at 465 nm wavelength. The optical density was measured with a Bio-Rad 550 micro plate reader. Nitrite concentrations in the supernatants were calculated by comparison with standard concentrations of $NaNO_2$ dissolved in Tyrode solution. The values are expressed as nanomoles of nitrite per milligram of protein. The protein concentrations were determined according to Bradford [224].

3.5.2 cGMP levels

To confirm the release of NO from hybrid compounds, cGMP levels were assessed. I used an experimental protocol previous developed in our lab for a company. Briefly, the animals were pre-treated with vehicle or soluble guanylate cyclase stimulator to enhance signal-to-noise ratio, after 30 minutes vehicle or hybrid compounds were administered and then the AH were collected at different time-points. After collection, we added to our samples isobutyl-methil-xantine (or avanafil) a PDE-5 inhibitor, which is the enzyme responsible for cGMP hydrolysis.

The samples were processed according to the protocols specified in the cGMP ELISA kit (Catalog Number KA3389; Abnova Corporation, CA, USA). Specifically, 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 [221].

3.5.3 Inflammatory response, IL-6, and TNF-alpha quantifications

Retina and CB tissues were homogenized in 2 mL of 10 mM phosphate buffer, pH 7.4 and then centrifuged at 10,000 g per 30 min at 4° C. To quantify both cytokines were used two RayBiotech IL-6 and TNF-α ELISA Kit (Prodotti Gianni, Milan, Italy) following the instructions provided by the manufacturer. Briefly, 100 µl of samples were added into appropriate wells and incubated for 2.5 hours at room temperature. After washing, 100 µl of biotinylated antibody were added into the wells and incubated for 1 hour. The samples were incubated for 45 minutes with 100 µl of a Streptavidin solution and 100 µl of TMB One-Step Substrate reagent. Finally, 50 µl of Stop Solution were added. The absorbance was measured at 450 nm and expressed as pg/ml sample.

3.5.4 Oxidative stress evaluation

3.5.4.1 Reduced glutathione (GSH) content

Retina and CB tissues were homogenized, and centrifuges as previously described, then centrifuged at 10,000 g per 30 min at 4° C and a fixed volume of supernatant was diluted with phosphate-EDTA buffer to a final volume of 2 ml and processed to determine the levels of reduced glutathione (GSSH). The content of GSSH was calculated versus a standard curve and expressed as pmol/µg protein [225].

3.5.4.2 Manganese Superoxide Dismutase (MnSOD) activity

Retina and CB tissue samples were homogenized and centrifuged as above reported and centrifuged at 100xg for10 min. Manganese superoxide dismutase (MnSOD) activity was determined in a fixed volume of the supernatant using a method described previously [226]. The standard curve was performed in a sample free system containing increasing concentration of MnSOD. The amount of protein required to inhibit the rate of NBT reduction by 50% was defined as one U of enzyme activity. The values are expressed as mU per µg protein.

3.5.4.3 Determination of 8-Hydroxy-2-deoxyguanosine (8-OHdG)

Frozen AH samples were thawed at room temperature, and cell DNA isolation was performed as previously described [227] with minor modifications. Briefly, samples, added with 1 ml of 10 mmol/l Tris-HCl buffer, pH 8, containing 10 mmol/l EDTA, 10 mmol/l NaCl, and 0.5% SDS, incubated for 1 h at 37°C with 20 µg/ml RNase 1 (Sigma-Aldrich, Saint Louis, MO, USA) and overnight at 37°C in the presence of 100 µg/ml proteinase K (Sigma-Aldrich). The mixture was extracted with chloroform/isoamyl alcohol (10/2 v/v). DNA was precipitated from the aqueous phase with 0.2 volumes of 10 mmol/l ammonium acetate, solubilized in 200 µl of 20 mmol/l acetate buffer, pH 5.3, and denatured at 90°C for 3 min. The extract was then supplemented with 10 IU of P1 nuclease (Sigma-Aldrich) in 10 µl and incubated for 1 h at 37°C with 5 IU of alkaline phosphatase (Sigma-Aldrich) in 0.4 mol/l phosphate buffer, pH 8.8. The mixture was filtered by an Amicon Micropure-EZ filter (Merck-Millipore), and 50 µl of each sample was used for 8-hydroxy-2-deoxyguanosine (8-OHdG) determination using an ELISA kit (JalCA, Shizuoka, Japan), following the instructions provided by the manufacturer. The absorbance of the chromogenic product was measured at 450 nm. The results were calculated from a standard curve based on 8-OHdG solution. The values are expressed as pg 8-OHdG/µg total DNA. Data were reported as mean values (± S.E.M.) of individual average measures of the different animals per group, for each assay. Significance of differences among the groups was evaluated by one-way ANOVA followed by Bonferroni post hoc test for multiple comparisons.

3.5.5 Evaluation of apoptotic process

3.5.5.1 Measurement of caspase-3 activity

The enzymatic activity of caspase-3 was determined using the Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC; Bachem AG, Bubendorf, Switzerland) fluorescent substrate [228]. Samples of tissues were homogenized with 10 mmol/L HEPES (pH7.4) containing 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate, 42 mmol/L KCl, 5 mmol/L MgCl2, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, and 1 μg/mL pepstatin A. The homogenates were then centrifuged at 10,000 g for 10 min. Supernatants containing 250 μg of total protein were incubated with 40 μM of the caspase-3 substrate AC-DEVD-AMC for 60 min. at 37°C. Substrate cleavage was determined fluorimetrically (Spectrofluo JY3 D; Jobin Yvon, Paris, France) at 380 nm excitation and 460 nm emission wave lengths. Data are expressed as mU/μg proteins. One unit of enzyme activity is defined as the amount of enzyme required to liberate 40 μmol of Ac-DEVD-AMC upon 60 min. at 37°C. The determinations were done in quintuplicate.

3.5.5.2 Terminal deoxynucleotidyl Transferase (TdT) dUTP Nick End-Labeling (TUNEL) assay

The TUNEL assay is used to detect DNA fragmentation, such as in apoptosis. It uses terminal deoxynucleotidyl transferase (TdT) to catalyse the incorporation of deoxynucleotides at the free 3'-hydroxyl ends of fragmented DNA. The deoxynucleotides are then labelled in a variety of ways for detection of the degree of DNA fragmentation. To determine DNA fragmentation the paraffin-embedded eye samples were cut into 5μm thick histological sections, deparaffinised, and rehydrated, then tissues were incubated with proteinase K solution for 5 min at room temperature and refixed with formaldehyde. After an appropriated washing, sections were before incubated with DNA labelling solution for 60 min at 37ºC and then with antibody solution for 30 min at room temp. Finally, sections were counterstained with 7-AAD/RNase A solution and analysed with confocal microscope [229]. All sections were stained in a single session to minimize artefactual inconsistencies during the staining process. A confocal microscope equipped with objectives with different magnifications and connected to a digital camera was used to record photomicrographs of the histological slides in a random fashion, with computer-aided densitometry to quantitatively assess the stained sections. Optical density (OD) and surface area were measured using the free-share ImageJ 1.33 image analysis program.

3.6 Morphological evaluation of RGC layer

Histological sections, 5 μm thick, were cut from the paraffin-embedded eye samples, deparaffinised, and stained with haematoxylin-eosin, one of the most widely used staining techniques for making morphological assessments. It involves progressive staining using two staining solutions: haematoxylin, a basic dye that stains the nuclei violet, and eosin, an acidic dye that gives the cytoplasm a pink coloration. All sections were stained in a single session to minimize artefactual differences in the staining. Photomicrographs of the histological slides were randomly taken with a digital camera connected to a light microscope. Quantitative assessment of the stained sections was performed by computer-aided count on the optical field using image analysis program ImageJ (NIH, Bethesda, MD). Values are means ± SEM of individual rabbit (five images each) from the different experimental groups.

3.7 Statistical Analysis

For each assay, data were reported as mean values (± S.E.M.) of individual average measures of the different animals per group. The significance of differences among the groups was assessed by one-way ANOVA or two-way ANOVA for multiple comparisons followed by Bonferroni post-test. Calculations were made with Prism 6.1 statistical software (GraphPad Software Inc., San Diego, CA, USA). A probability value (p) of <0.05 was considered significant.

4. Results

4.1 The establishment of hybrid compounds best dose

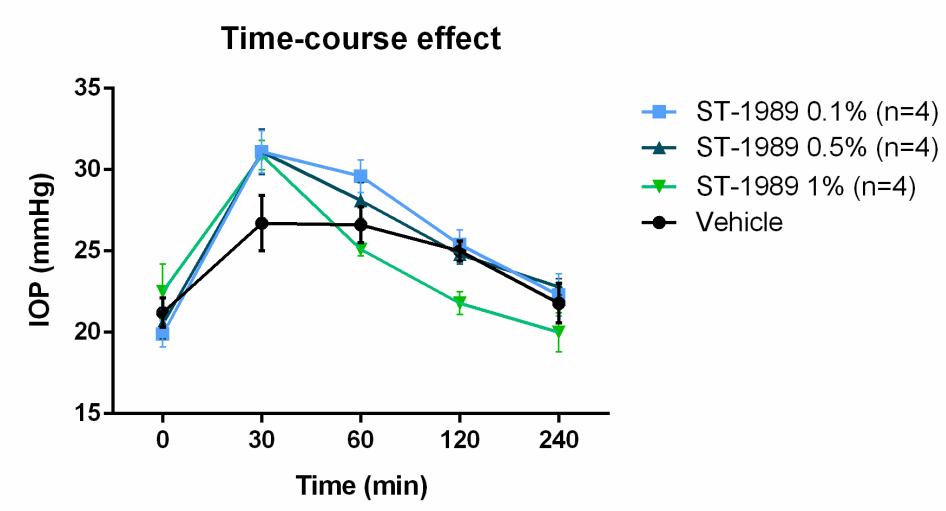
The determination of the best dose of histamine H_3R antagonist-NO donor hybrid compounds was carried out in transient OHT model. I performed three different experimental sets testing compounds at three different concentrations: 0.1%, 0.5% and 1%. Each compound was correlated to the vehicle to normalize the results. Two different evaluations were performed: the IOP lowering profile and the ΔΔIOP, the latter parameter is extremely useful to understand the peak of action of compounds at different timepoints, correlating the obtained values to the vehicle lowering profile.

None of the compounds examined caused any adverse effects. In fact, before each experiment, the tolerability of the study drugs was evaluated with the Draize test [230]. The animals did not exhibit evidence of ocular side-effects, even after repeated administrations, in fact no ocular irritation, redness, or itching was observed.

4.1.1 Time course effect of ST-1989

In this experimental set, IOP rose from 21±0.5 mmHg at baseline to 30±1.0 mmHg after hypertonic saline injection. ST-1989 has proven to be effective in reducing IOP in a dose-dependent and statistically significant manner, with $p<0.0001$ for ST-1989 1% at 120'; $p<0.001$ with ST-1989 1% at 60' and 240' and $p<0.05$ with ST-1989 0.5% at 120' *versus* vehicle (Figure 12, panels A-B).

A



B

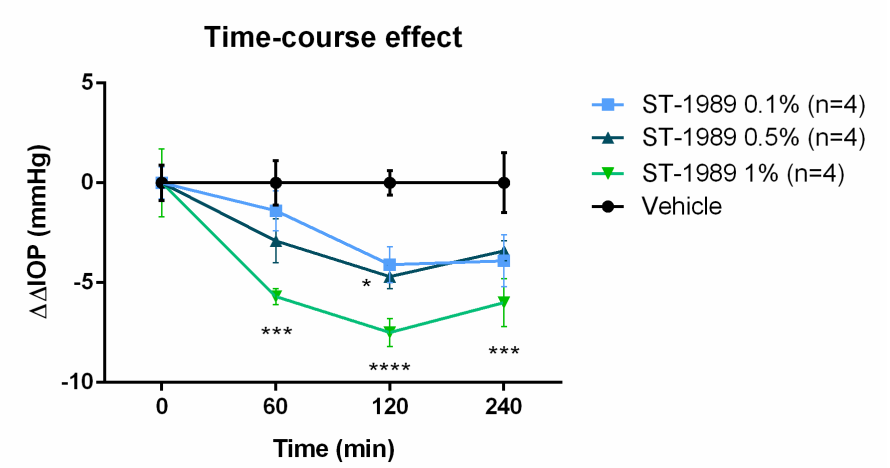
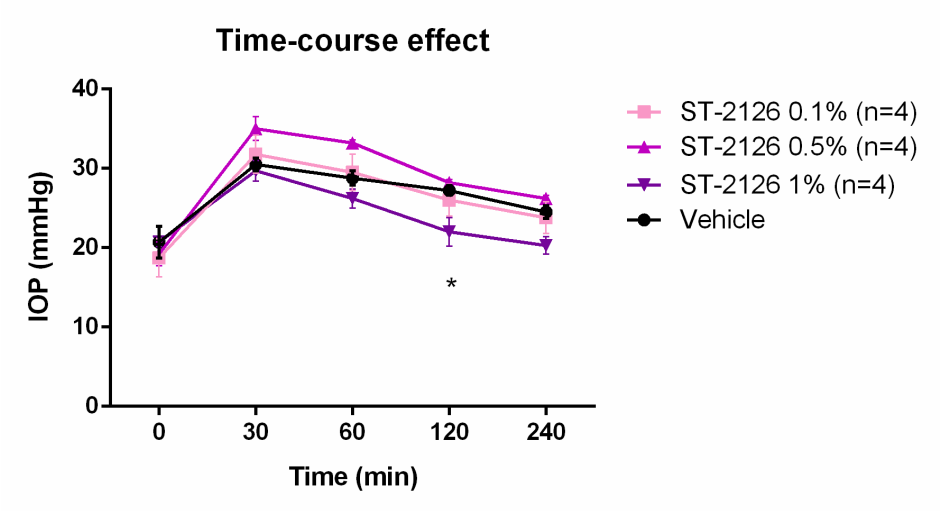


Figure 12: ST-1989 time course effect: A) IOP lowering profile; B) ΔΔ IOP time course. Data are expressed as mean ± SEM (n=4). ****p<0.0001 ST-1989 1% at 120'; ***p<0.001 ST-1989 1% at 60' and 240' and *p<0.05 ST-1989 0.5% at 120' *vs* vehicle. Two-way ANOVA followed by Bonferroni *post-hoc* test.

4.1.2 Time course effect of ST-2126

In this experimental set the IOP rose from 19.8±0.5 mmHg at baseline to 31.8±1.2 mmHg after hypertonic saline injection. Compound ST-2126 reduced dose-dependently the IOP but only the 1% concentration was statistically significant with $p<0.05$ at 120' *versus* vehicle (Figure 13 panels A-B).

A



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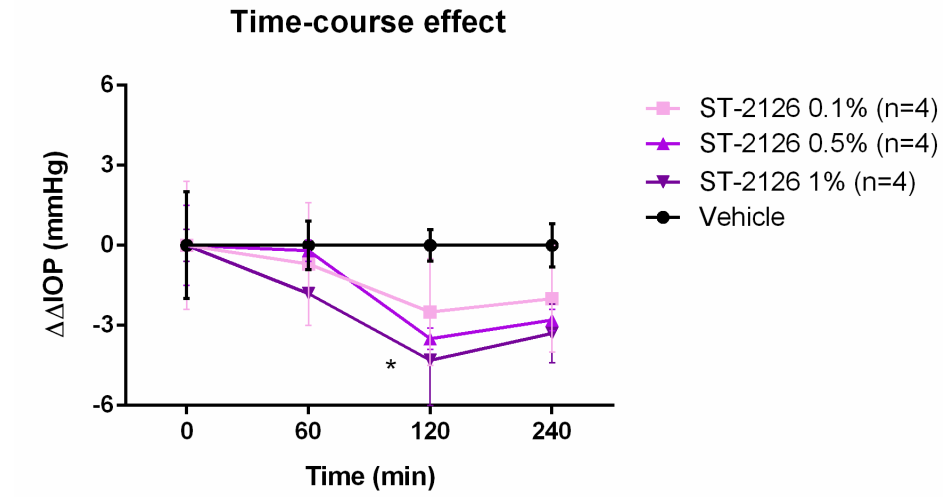
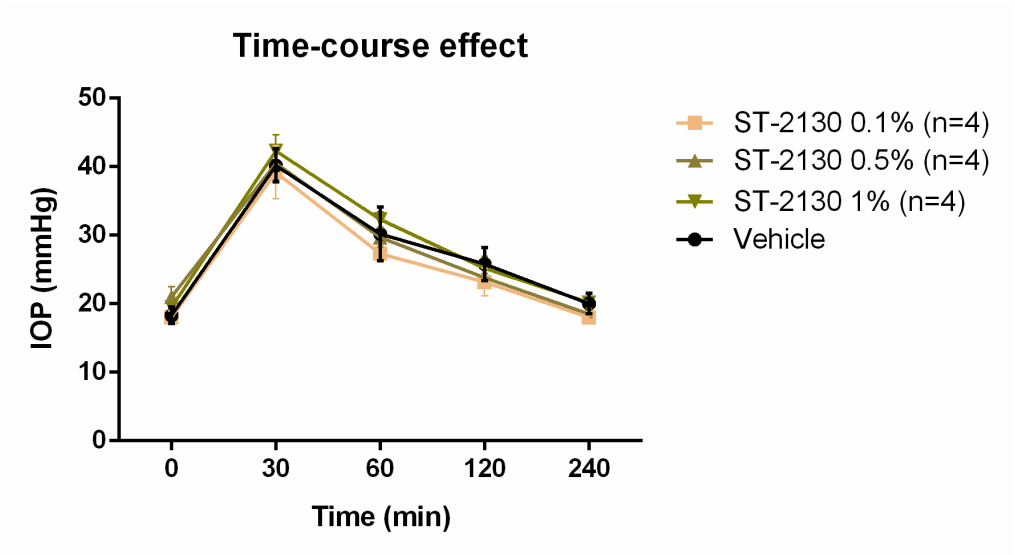


Figure 13: ST-2126 time course effect: A) IOP lowering profile; B) ΔΔ IOP time course. Data are expressed as mean ± SEM (n=4). *$p<0.05$ ST-2126 1% at 120' *vs* vehicle. Two-way ANOVA followed by Bonferroni *post-hoc* test.

4.1.3 Time course effect of ST-2130

In this experimental set the IOP rose from 19.2±0.7 mmHg at baseline to 40.5±0.6 mmHg after hypertonic saline injection. Compound ST-2130 has a slight effect on IOP even when tested at the highest concentration, 1%, but none of the tested doses were statistically significant (Figure 14 panels A-B).

A



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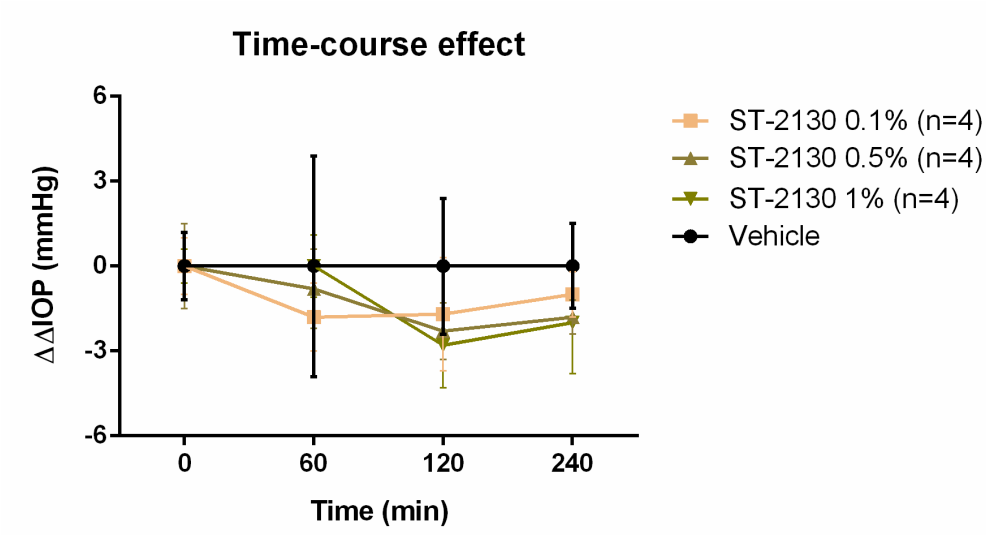
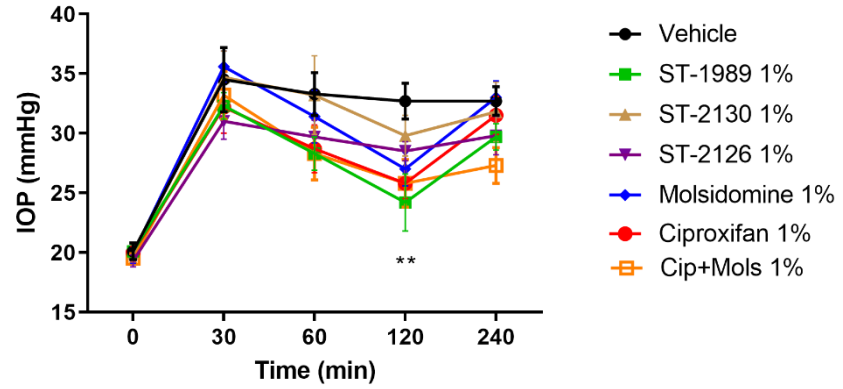


Figure 14: ST-2130 time course effect: A) IOP lowering profile; B) ΔΔ IOP time course. Data are expressed as mean ± SEM (n=4). Two-way ANOVA followed by Bonferroni *post-hoc* test.

4.2 Effects of hybrid compounds in a transient ocular hypertension model

In this experimental set the hybrid compounds were compared with reference drugs such as ciproxifan and molsidomine both administered alone and in combination. IOP rose from 19.8±0.1 mmHg at baseline to 33.3±0.6 mmHg after hypertonic saline injection. As shown in Figure 15 (panels A-B), compound ST-1989 maintained its hypotensive effect 60- and 120-minutes post dosing but only the effect at 120 minutes resulted statistically significant ($p<0.01$ *versus* vehicle). Ciproxifan and ciproxifan plus molsidomine were also effective in reducing IOP at 120' with $p<0.05$ *versus* vehicle suggesting that the hypotensive effect of hybrid molecule could be the result of action of both histaminergic and nitrergic systems.

A



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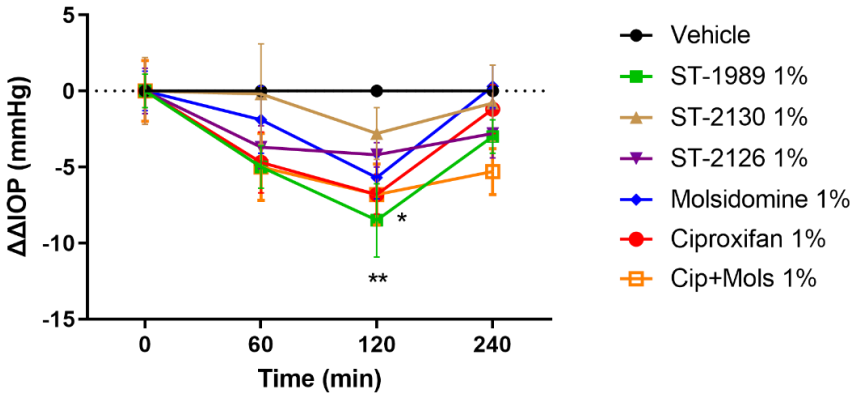


Figure 15: Effects of hybrid compounds in a transient ocular hypertension model. A) IOP lowering profile; B) ΔΔ IOP time course. Data are expressed as mean±SEM (n=6); **p<0.01 ST-1989; *p< 0.05 Ciproxifan and Cip+Mols *vs* vehicle. Two-way ANOVA followed by Bonferroni post hoc test.

4.3 Determination of nitrite (NO_2^-) release in the AH of glaucomatous rabbits

To investigate the action of hybrid compounds in metabolizing and releasing NO, I evaluated the capability of these molecules to accumulate nitrites (NO_2^-) in the AH of glaucomatous rabbits 60 min after administration of the compounds. In this experimental set, molsidomine, as I expected, showed the greatest accumulation of NO_2^- with $p<0.0001$ *versus* vehicle, ciproxifan plus molsidomine also increased NO release in a statistically significant manner with $p<0.01$ *versus* vehicle. Interestingly, among hybrid compounds only ST-1989 showed statistically significant increase in NO_2^- availability with $p<0.05$ *versus* vehicle, whereas in the other molecules no significant effects were observed (Figure 16).

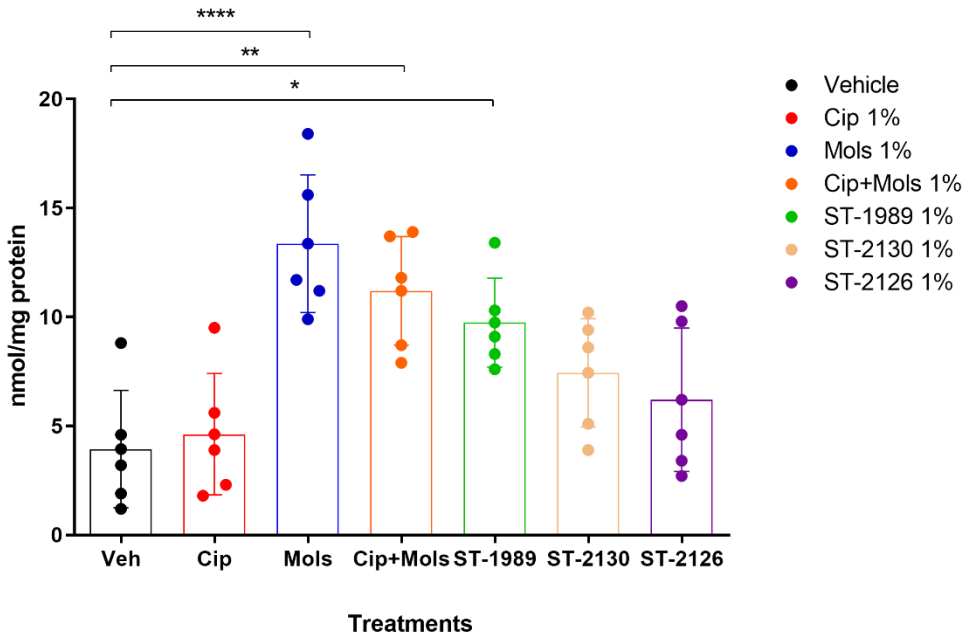


Figure 16: Nitrites accumulation in aqueous humor of glaucomatous rabbits 60' post drug dosing. Data are expressed as mean±SEM (n=6); ****p< 0.0001 Mols; **p<0.01 Cip+Mols and *p<0.05 ST-1989 *vs* vehicle. Two-way ANOVA followed by Bonferroni post hoc test.

4.4 Quantification of cGMP levels in AH of glaucomatous rabbits

To confirm NO release by hybrid compounds I assessed cGMP levels in the AH of rabbits treated with the molecules at different timepoints. It is well known that this nucleotide is the second messenger of NO-GC pathway, and its determination is useful to understand the mechanism of action of NO donors in promoting AH outflow into the conventional pathway. As showed in Figure 17, molsidomine, also in this analysis, showed a statistically significant increase in cGMP levels at both 60' and 120' post dosing. Notably, among the hybrid compounds, ST-1989 increased cGMP levels after 120' in a statistically significant manner.

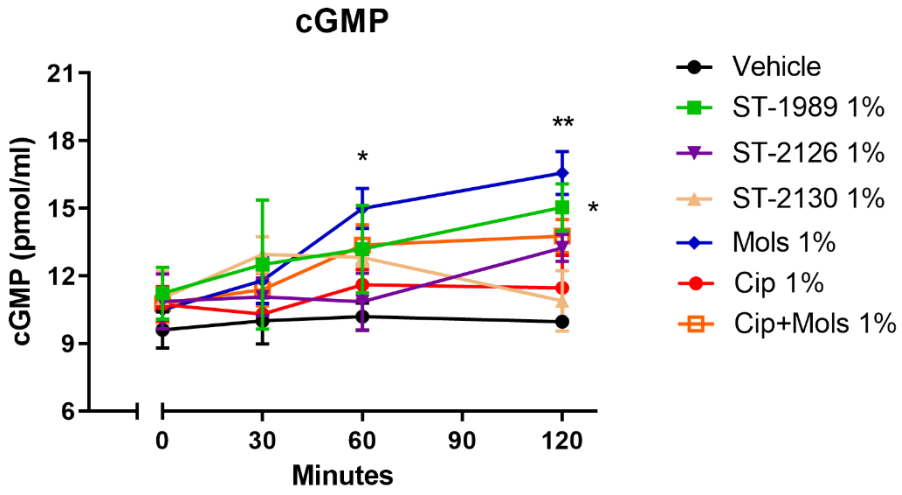
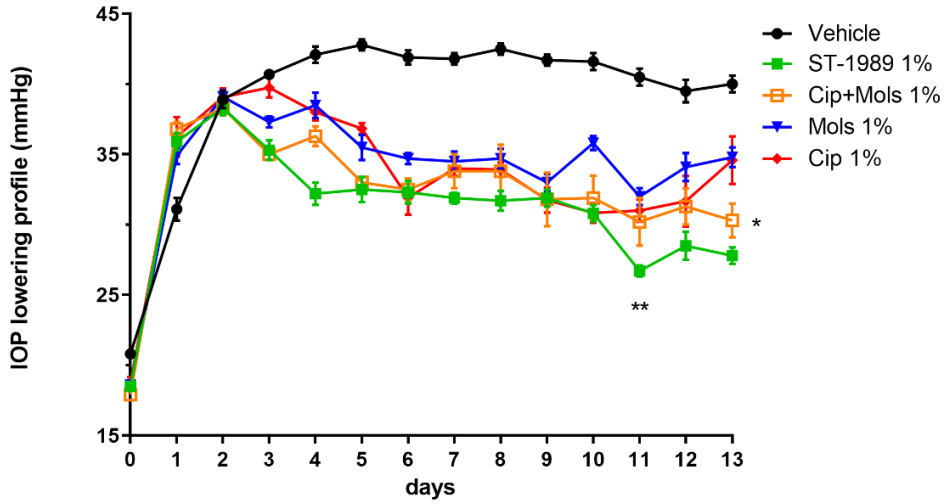


Figure 17: Determination of cGMP levels in AH of treated animals at different timepoints. Data are expressed as mean±SEM (n=6). **p<0.01 Mols at 120'; *p< 0.05 Mols and ST-1989 at 60' and 120' respectively *vs* vehicle. Two-way ANOVA followed by Bonferroni post hoc test.

4.5 Effects of hybrid compound ST-1989 in a chronic ocular hypertension model

Based on the promising results obtained in the transient OHT, I selected the most responsive hybrid compound i.e., ST-1989, and decided to study its long-term effect, so I validated the chronic OHT model, comparing ST-1989 with the same reference drugs, always administered alone and in combination. In this experimental set the IOP rose from 18.9±0.5 mmHg at baseline to 38.7±0.2 mmHg two days after carbomer injection and remained stable for two weeks in vehicle-treated animals. As shown in Figure 18 (panels A-B), compound ST-1989 showed a statistically significant reduction of IOP after 11 daily repeated doses with an increasing hypotensive effect over time. Ciproxifan and ciproxifan plus molsidomine reduced the IOP in a statistically significant manner only at day 10 and 13 respectively. These results suggest an enhancing effect of the histaminergic and nitrergic systems in reducing IOP.

A



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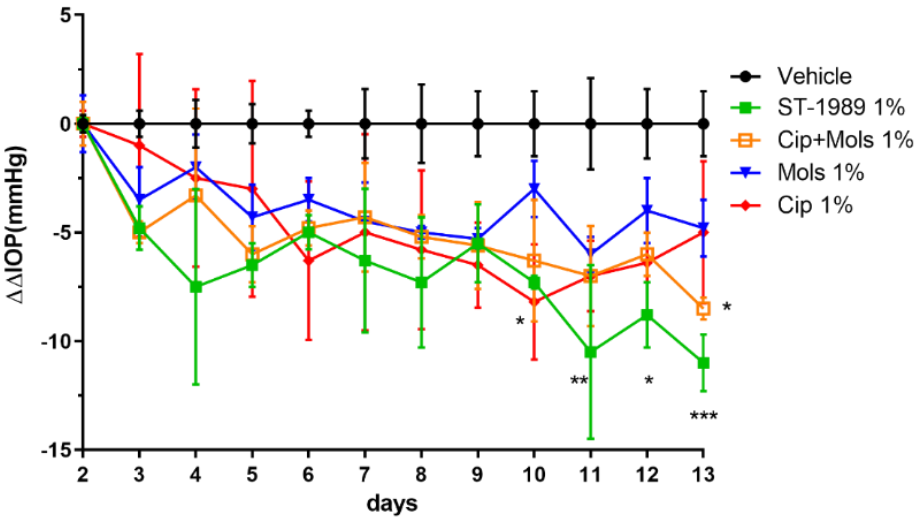


Figure 18: Effects of hybrid compounds in a chronic ocular hypertension model. A) IOP lowering profile; B) ΔΔ IOP time course. Data are expressed as mean±SEM (n=6); day 10, 12 and 13 *p<0.05 Cip, ST-1989 and Cip+Mols *vs* vehicle; day 11 **p<0.01 ST-1989 *vs* vehicle and day 13 ***p<0.001 ST-1989 *vs* vehicle. Two-way ANOVA followed by Bonferroni post hoc test.

4.6 Effects of hybrid compound ST-1989 in controlling inflammation

After studying the hypotensive action in chronic OHT model, I aimed to investigate the efficacy of compound ST-1989 in controlling the inflammatory response. For this purpose, I quantified two important cytokines such as IL-6 and TNF-α, using a specific ELISA kit, in retina and CB samples. IL-6 is a regulatory cytokine which is known to possess both pro- and anti-inflammatory actions depending on the organ in which it is expressed; in fact, it has been demonstrated that in neuropathic pain, this cytokine was able to up-regulate IL-10 improving the pathological condition [189] .

As reported in Figure 19 (panels A-B), it is possible to observe a statistically significant increase of IL-6 levels in ST-1989 treated-eyes (ST-1989 IL-6 CB $p<0.05$ and IL-6 retina $p<0.0001$), as well as in molsidomine- and molsidomine plus ciproxifan-treated eyes in comparison to vehicle group (Mols IL-6 CB $p<0.01$, IL-6 retina $p<0.001$ and Cip+Mols IL-6 CB $p<0.01$, IL-6 retina $p<0.0001$ respectively). This increase is directly correlated with the significant reduction of TNF-α (ST-1989 TNF-α CB $p<0.01$, TNF-α retina $p<0.0001$; Mols TNF-α CB $p<0.01$, TNF-α retina $p<0.0001$ and Cip+Mols TNF-α CB $p<0.01$ respectively). This effect is mainly due to the NO present in the structure. In fact, analyzing the action of treatments, it is noteworthy that the lowest levels of TNF-α, are found in tissues obtained from animals treated with ST-1989, and molsidomine alone and in association with ciproxifan. Tissues collected from animals treated only with ciproxifan show higher levels of TNF-α.

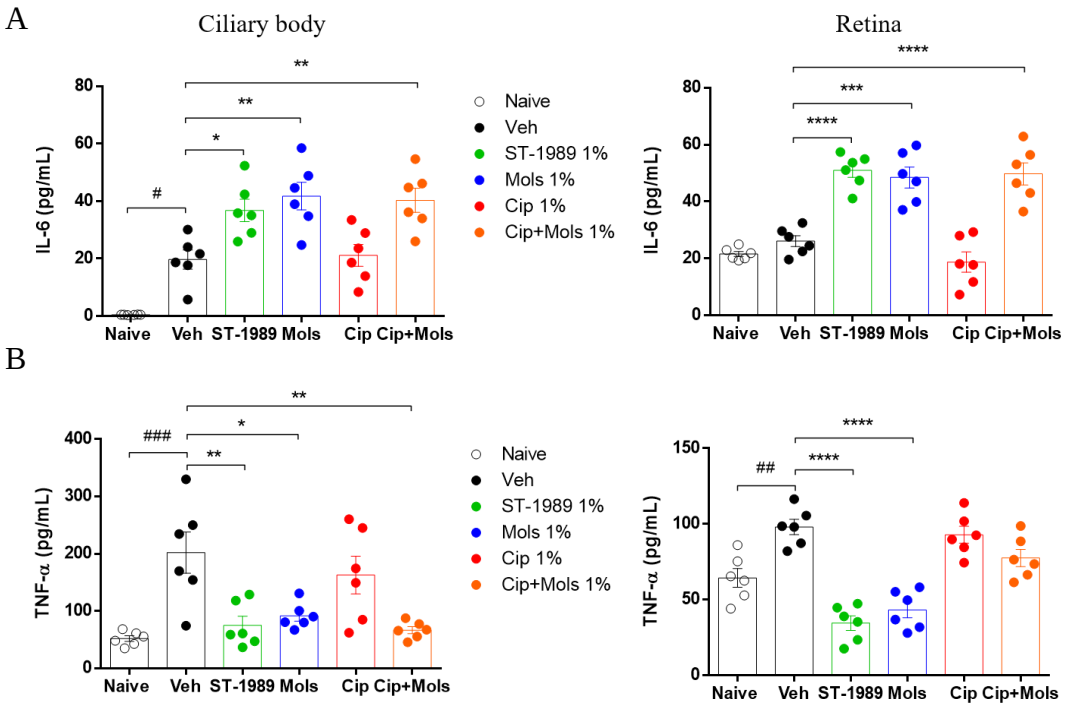
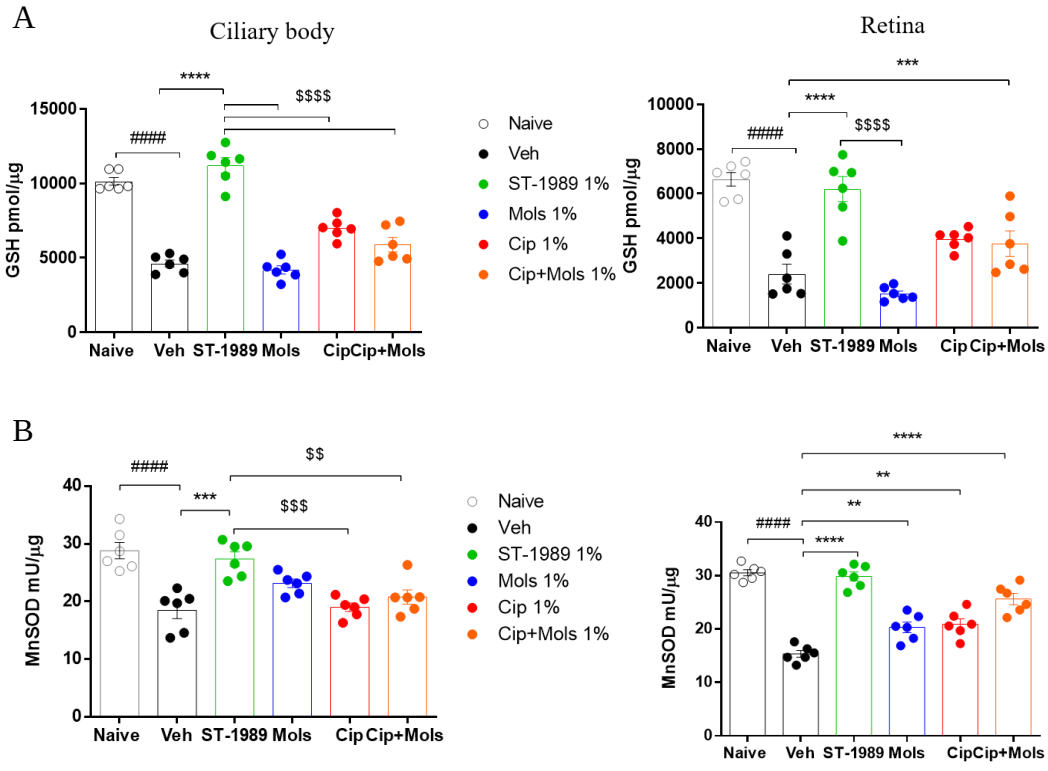


Figure 19: Quantification of inflammatory cytokines in retina and ciliary body samples. A) Determination of IL-6 levels; B) determination of TNF-α levels. Data are expressed as mean±SEM (n=6); ###p<0.001, ## p<0.01 and #p<0.05 *vs* Naive; ****p<0.0001, ***p<0.001, **p<0.01 and *p<0.05 *vs* Vehicle. One-way ANOVA followed by Bonferroni *post hoc* test.

4.7 Effects of hybrid compound ST-1989 in controlling the oxidative stress parameters

To better understand the mechanism of action of compound ST-1989 and evaluate its possible role in counteracting oxidative stress, I analyzed the content of reduced glutathione (GSH) and the activity of Manganese Superoxide dismutase (MnSOD), two of the most important scavengers of reactive oxygen species.

Moreover, in AH samples I evaluated 8-OH2dG, an oxidative stress marker of DNA. It is important to note that both in CB and retina samples, the treatment with compound ST-1989 was able to increase GSH content, as well as MnSOD activity in comparison to vehicle (GSH CB and GSH retina: $p<0.0001$; MnSOD CB and MnSOD retina: $p<0.001$ and $p<0.0001$ respectively). Interestingly the action of this compound is statistically significant not only in relation to vehicle but also when compared with reference drugs administered alone and in combination with $p<0.01$, $p<0.001$ and $p<0.0001$ (Figure 20, panels A-B). Remarkably, the chronic treatment with ST-1989 was also able to significantly reduce the oxidation of DNA with $p<0.0001$, confirming the potent anti-oxidative action of this compound (Figure 20 panel C). This is a novel and significant result because it is well known that increased oxidative stress contributes substantially to the retinal damage.



C

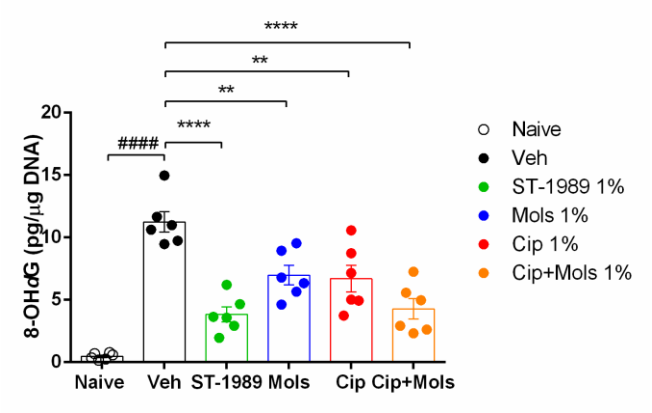


Figure 20: Evaluation of oxidative stress parameters A) Reduced glutathione (GSH); B) Manganese superoxide dismutase (MnSOD) quantification in retina and ciliary body and C) 8-hydroxy 2 deoxyguanosine (8-OH2dG) in aqueous humour. ####p<0.0001 *vs* Naive; ****p<0.0001, ***p<0.001 and **p<0.01, and *vs* Vehicle; $^{\$\$\$\$}$p<0.0001, $^{\$\$\$}$p<0.001 and $^{\$\$}$p<0.001 and *vs* ST-1989. Data are expressed as mean±SEM (n=6). One-way ANOVA followed by Bonferroni *post hoc* test.

4.8 Effects of compound ST-1989 on the apoptotic process

4.8.1 Evaluation of Caspase 3 activity

Caspase 3, an important early marker of apoptosis, is a protease able to activate pro-caspases 2, 6, 7 and 9, and induce chromatin condensation and DNA fragmentation.

As shown in Figure 21, caspase 3 activity increase in both CB and retina of rabbits undergoing chronic OHT (vehicle group), while chronic treatment with compound ST-1989 was able to reduce this marker in a statistically significant manner with $p<0.0001$ *versus* vehicle. Notably, also molsidomine alone and in combination with ciproxifan statistically reduced caspase 3 activity in comparison to vehicle (Mols CB $p<0.01$, Mols retina $p<0.05$ and Cip+Mols CB $p<0.01$ Cip+Mols retina $p<0.001$ respectively). These data confirm the hypothesis that NO plays a key role in the mechanism of action of this new molecule.

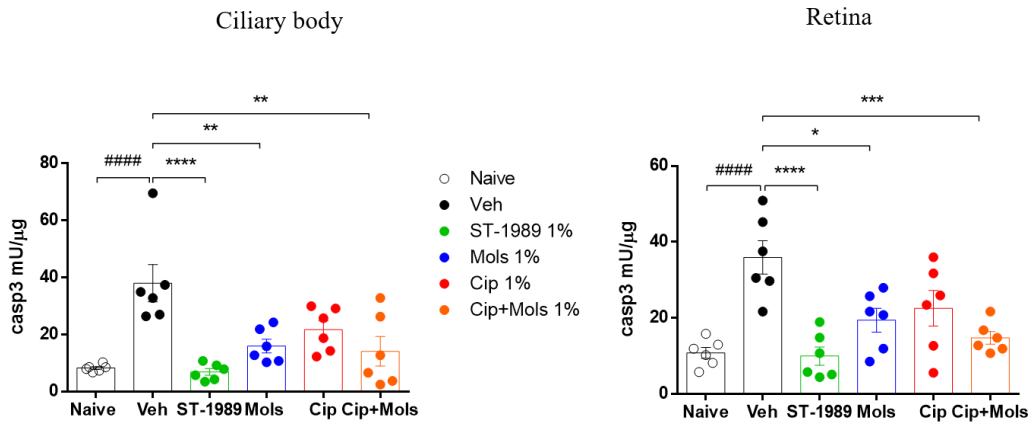


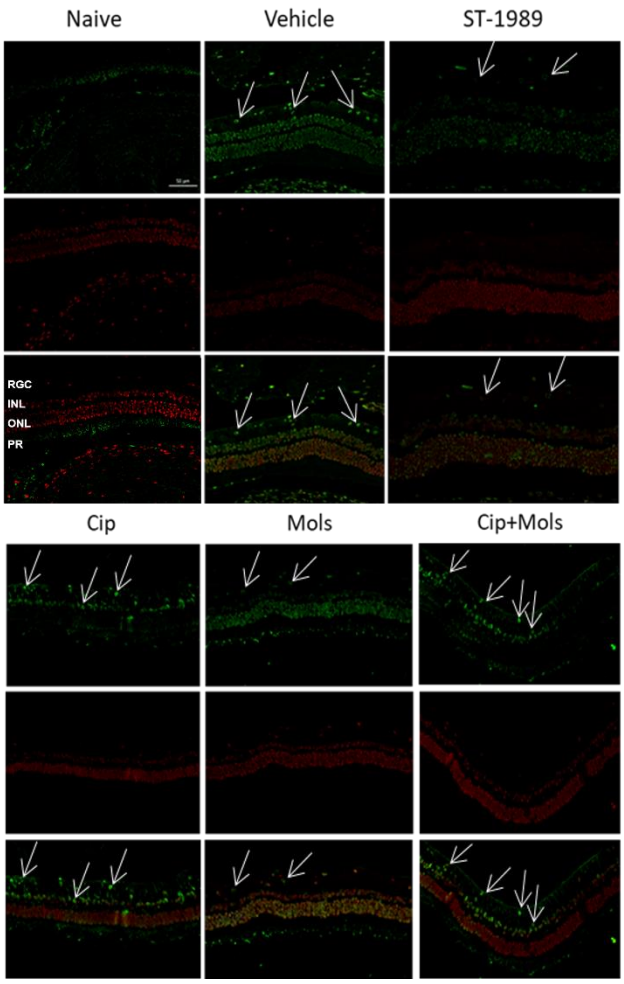
Figure 21: Quantification of Caspase 3 activity in ciliary body and retina. Data are expressed as mean±SEM (n=6). ####$p<0.0001$ *vs* Naive; ****$p<0.0001$, ***$p<0.001$, **$p<0.01$ and *$p<0.05$ *vs* Vehicle.; One-way ANOVA followed by Bonferroni *post hoc* test.

4.8.2 Terminal deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay

This is an assay for localization of apoptotic DNA fragmentation *in situ*. The method relies on the template-independent identification of blunt ends of double-stranded DNA breaks by the terminal deoxynucleotidyl transferase (TdT). The enzyme catalyzes the addition of labeled dUTPs to a 3'-hydroxyl termini of DNA ends, which can be visualized using immunohistochemical techniques.

The pictures showed in Figure 22 panel A, clearly indicate that the chronic treatment with compound ST-1989 significantly prevented RGCs death in comparison to vehicle group ($p<0.0001$). Interestingly, both molsidomine and ciproxifan, administered alone and in combination, showed a better preservation of ganglion cells but to a lesser extent than compound ST-1989 ($p<0.0001$ Mols; $p<0.001$ Cip+Mols and $p<0.01$ Cip *versus* vehicle), which is also evident from the graph depicting the fluorescence intensity of RGCs in apoptosis (Figure 22 panel B). This finding indicates that both systems, histaminergic and nitrergic, have a synergistic action in preventing depletion of RGCs, an action enhanced in the hybrid compound compared with co-administration of the individual compounds ($p<0.01$ ST-1989 *versus* Cip+Mols).

A



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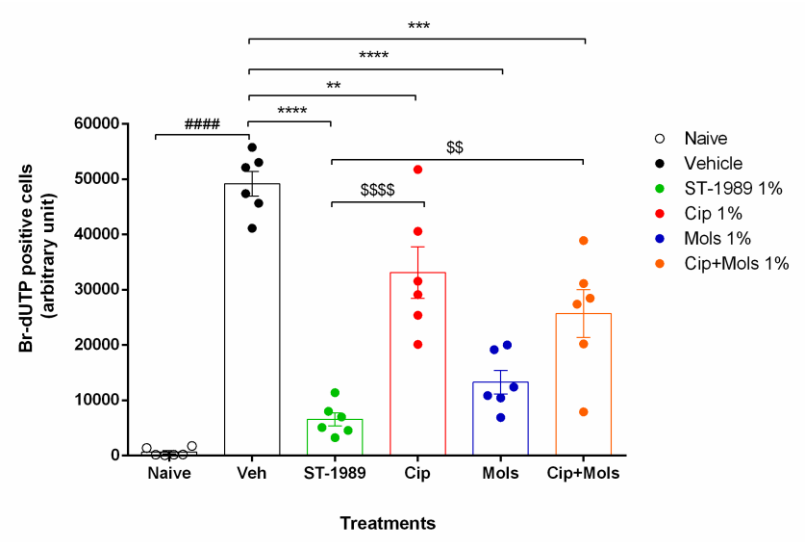

Figure 22: TUNEL assay. A) Retina sections labelled by immunofluorescence staining with BrdU- labelled (green) and 7-AAD/RNase A (red) to counterstain the nuclei (magnification 20x). Images show apoptotic retinal ganglion cells (RGCs) indicating retinal damage. B) Fluorescence intensity of apoptotic RGCs. Densitometric data are reported as relative optical density (OD) in each experimental group. Data are mean ± S.E.M. (n=6). ####p<0.0001 *vs* Naive; ****p<0.0001, ***p<0.001 and **p<0.01 *vs* Vehicle; $$$p<0.001 Cip and $p<0.05 Cip+Mols *vs* ST-1989. One-way ANOVA followed by Bonferroni *post hoc* test.

4.9 Effects of compound ST-1989 in a model of ischemia/reperfusion

4.9.1 Effects of compound ST-1989 in regulating the IOP

Average baseline IOP prior to ET-1 dosing was 20.3±1.0 mmHg and 20.9±0.4 mmHg respectively in animals later randomized for vehicle or ST-1989 treatments. Twice weekly dosing with ET-1 slightly raised average IOP to reach 22.0±1.7 and 23.1±0.9 mmHg for vehicle and ST-1989, respectively (Figure 23). ST-1989 daily dosing (1%, 30µL/eye) progressively counteracted ET-1-induced changes as documented by the decrease in IOP observed in drug-free conditions (36 h after last dose) to reach 18.9±0.6 mmHg on week 6 with $p<0.05$. IOPs in vehicle-treated animals increased over the same period (at week 6, 25.8±0.5 mmHg).

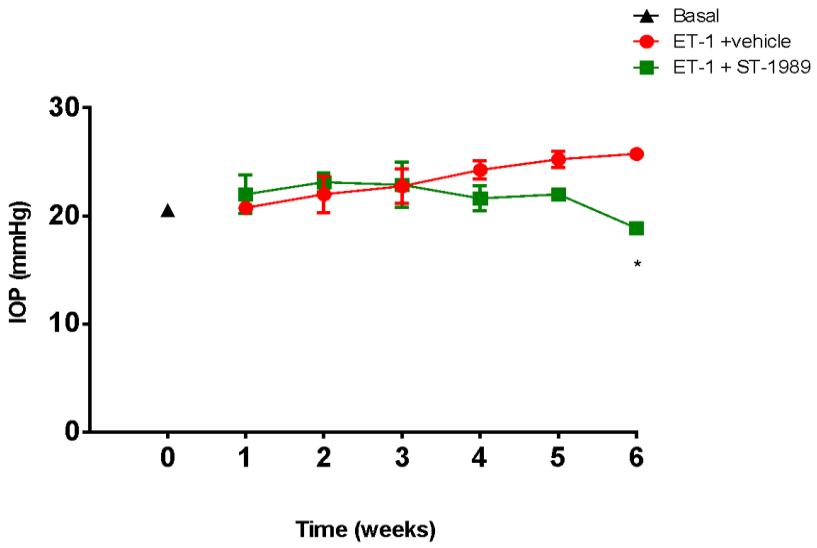

Figure 23: IOP lowering profile in a retinal I/R model. Data are expressed as mean±SEM (n=4); *p< 0.05 ST-1989 *vs* vehicle. Two-way ANOVA followed by Bonferroni *post hoc* test.

4.9.2 Effects of compound ST-1989 in regulating ocular perfusion in ischemia/reperfusion model

Ophthalmic artery peak systolic velocity and end diastolic velocity were measured over time. Data were then computed and used to calculate the respective Ophthalmic Artery-Resistance Index (OA-RI). The OA-RI prior to ET-1 dosing was 0.38±0.05 and 0.37±0.05 respectively in animals later randomized for vehicle or ST-1989 treatments (Figure 24). Twice weekly dosing with ET-1 weeks equally raised OA-RI in both experimental groups (0.53 ± 0.04 and 0.47 ± 0.02 for vehicle and ST-1989, respectively). Interestingly, while vehicle treatment resulted in a further increase over the following weeks (0.53 ± 0.03 and 0.56 ± 0.04 on week 4 and 6, respectively), OA-RI taken under drug-free conditions in ST-1989-treated animals decreased significantly (0.38 ± 0.03, with $p<0.05$ and 0.37 ± 0.04, with $p<0.01$ on week 4 and 6, respectively).

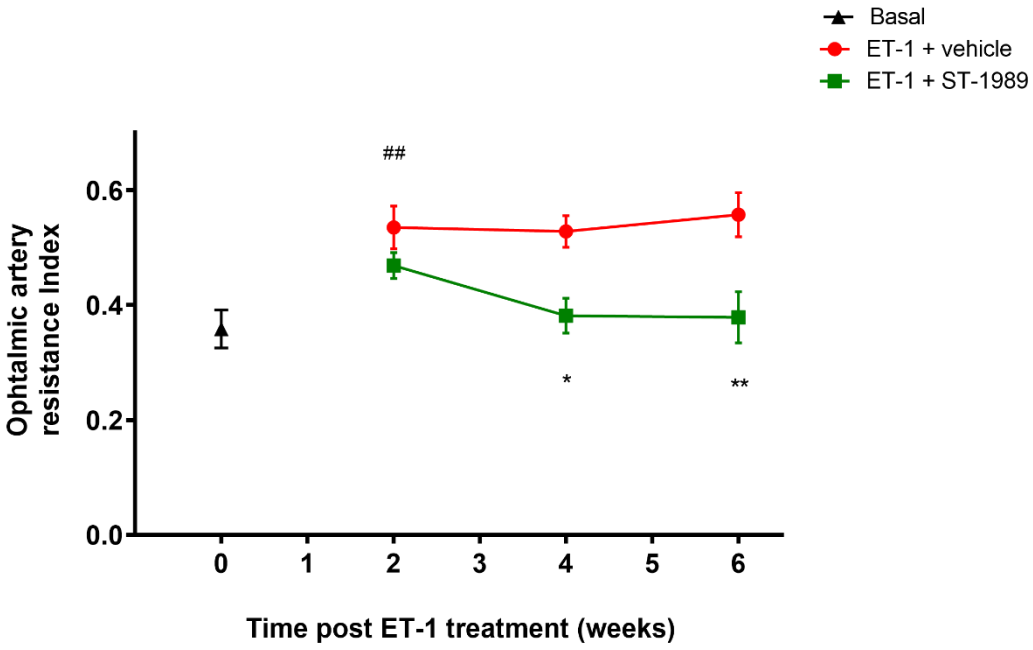

Figure 24: OA-RI. ## $p<0.01$ vehicle and ST-1989 *vs* Basal; **$p<0.01$ and *$p<0.05$ ST-1989 *vs* vehicle. Data are expressed as mean±SEM (n=4) Two-way ANOVA followed by Bonferroni post hoc test

4.9.3 Electroretinogram (ERG) changes after ST-1989 repeated dosing following ET-1-induced ischemia/reperfusion model

Before describing the results, it is important to explain how ERG works. Briefly, when the light pulse impacts the retina, the electroretinogram records the membrane potential of photoreceptors, and thus their activity, by generating a tracing (Figure 25). In this tracing we can observe: an "a-wave" or negative wave, representing the response of photoreceptors to the light stimulus, and a "b-wave", or positive wave, representing the response of cells deputed to light signal transmission. Amplitude, measured between the a- and b-wave peaks, is considered an index of retinal function.

The amplitude values of baseline scotopic rods response (ERG 0.01) were similar in animals later randomized for vehicle or ST-1989 treatment (42.5±4.5 and 38.6±2.0 respectively). ERG amplitude declined substantially after 6-weeks of twice weekly ET-1 treatment (29.3±3.5). Interestingly, dosing the animals for 4 consecutive weeks with ST-1989 abolished significantly ET-1-induced ERG amplitude decline with $p<0.05$ *versus* vehicle (42.8±4.7) (Figure 26 panel A).

The mean ERG amplitude after light-adapted, photopic cones stimulation (ERG 3.0) is reported in Figure 26 panel B. As in the previous measures described above, baseline amplitude was similar in eyes later randomized for vehicle or ST-1989 (92.1±4.5 and 94.9±2.2 respectively). Eyes receiving vehicle concomitantly with ET-1 twice weekly had at week 6 a lower response (46.4±7.4). Repeated dosing with ST-1989 significantly counteracted ET-1 effects with $p<0.01$ *versus* vehicle (68.1±4.3).

Dark adapted, scotopic ERG 3.0 amplitude representative of the combined rod/cone activity is shown in Figure 26, panel C. The response of both photoreceptors did not differ in eyes later randomized for vehicle or ST-1989 (141.9±5.9 and 103.8±4.5 respectively). Responses declined substantially in ET-1-treated eyes at week 6 in eyes receiving vehicle (80.7±14). ST-1989 treatment counteracted the detrimental effects of ET-1 in a statistically significant way with $p<0.05$ (119.2±3.9).

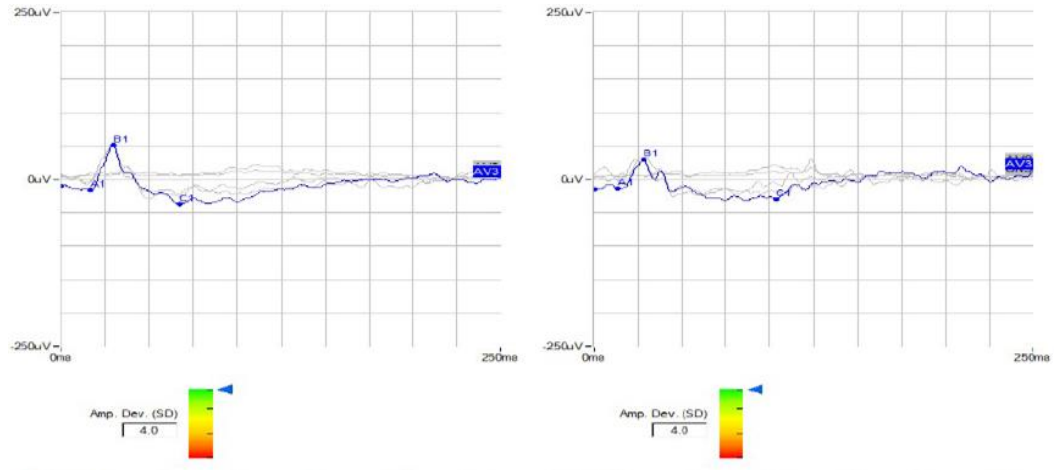

Figure 25: Example of ERG track representing the response of photoreceptors to a light stimulus recorded in both eyes simultaneously.

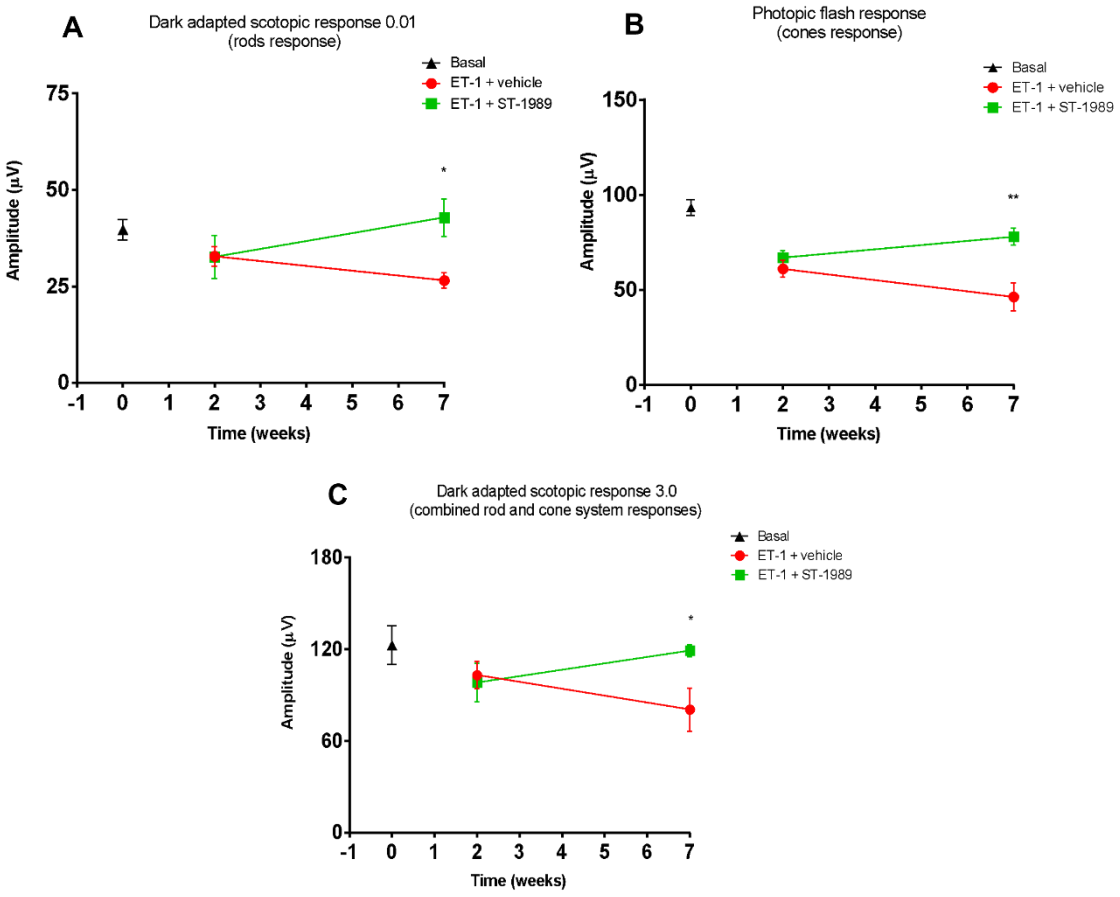

Figure 26: Graphs of amplitudes calculated between the first and the second peak generated by the response of photoreceptors to different light stimuli. A) DARK scotopic 0.01 ERG, rods response; B) LIGHT photopic 3.0 ERG, cones response. C) DARK scotopic 3.0 ERG, combined response of rods and cones Data are expressed as mean±SEM (n=4). **p<0.01 and *p<0.05 *vs* vehicle. Two-way ANOVA followed by Bonferroni post hoc test.

4.9.4 Morphological changes after ST-1989 repeated dosing following ET-1-induced ischemia/reperfusion model

The hematoxylin-eosin staining of retinal tissue is represented in Figure 27. Panel A shows the normal architecture of the retina starting from RGCs (top) to the sclera (bottom). In panel B, vehicle-treated animals show RGC depletion and altered tissue architecture (black arrows); in fact, both the choroid and sclera appear laxer. Conversely, in ST-1989-treated eyes the architecture of the whole tissue appears morphologically intact and the RGC layer appear restored. This suggests a potential ability of compound to restore the morphological condition of retinal tissue following an ischemia/reperfusion injury.

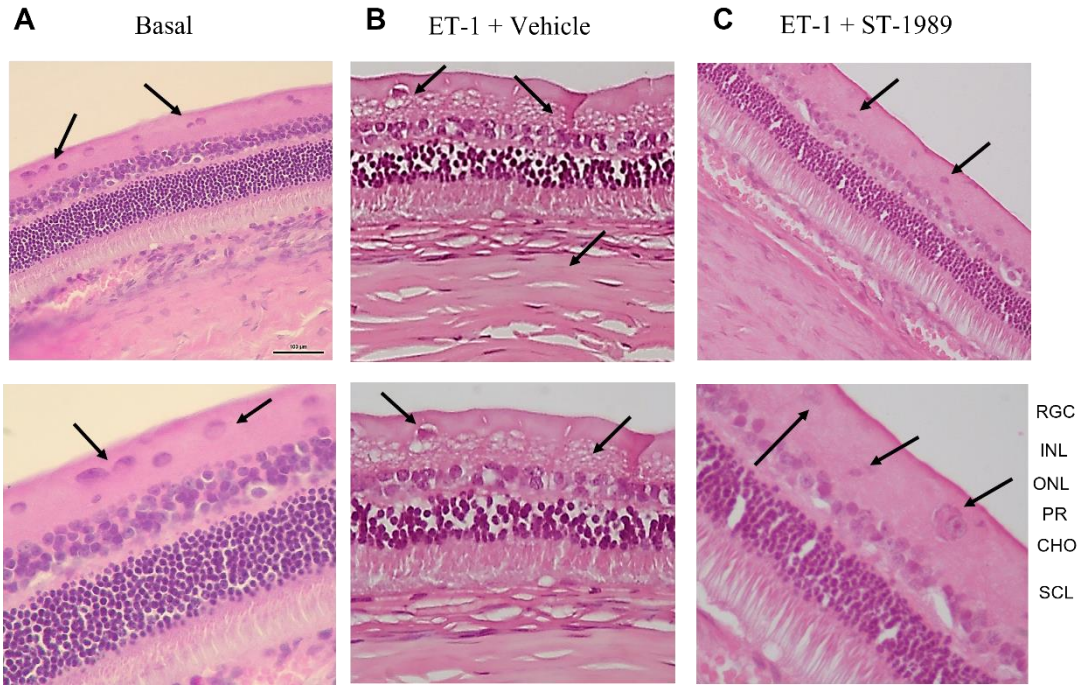

Figure 27: Representative hematoxylin-eosin micrographs. Morphological changes of retinal ganglion cell (RGC) layer after ET-1-induced ischemia/reperfusion injury in eyes treated with ST-1989 and vehicle. Black arrows indicate the RGC layer under: A) control conditions and after 4 weeks of treatment with B) Vehicle or C) compound ST-1989.

5. Discussion

The results reported in this thesis clearly indicate that both histaminergic and nitrergic system have an important role in the regulation of IOP and in retinal neurodegeneration.

Histamine plays a role in the regulation of circadian oscillations along with the circadian pacemaker, that is, the suprachiasmatic nucleus of the hypothalamus; moreover, it is known that histaminergic tone influences the circadian rhythm of IOP regulation and causes ciliary muscle contraction, and its dysfunction is expected to play a central role in glaucoma [231]. There are several studies confirming the important role of the histaminergic system in the regulation of the IOP; in fact, Gowtham and colleagues[232] observed a five-fold increase in histamine levels in the AH of POAG patients, regardless of its plasma concentration, indicating a possible involvement of the histaminergic system at the ocular level. Furthermore, Nowak and Nawrocki [233] evaluated histamine levels in various ocular tissues (iris, CB, choroid, retina, sclera, and optic nerve) from enucleated human eyes from patients with endophthalmitis, perforated wounds of the cornea or sclera or both, and uncontrolled glaucoma. The study revealed that inflammation (endophthalmitis) increases tissue histamine levels up to five- to ten-fold, followed by glaucoma in comparison to values obtained in eyes enucleated for penetrating trauma. This observation indicates the involvement of an inflammatory process in glaucoma causing an increase in the tissue levels of histamine.

It is known that histamine receptors are expressed at the ocular level in both the neuronal and non-neuronal compartments, and treatments with histamine H_3R antagonists reduce ocular hypertension with acute and long-lasting effects [234].

Moreover, in our paper [205], we demonstrated that the hypotensive effect became evident after several days of treatment indicating a mechanism of action involving other mediators, such as an activation of endogenous histamine release and the stimulation of the histaminergic H_1R at the endothelial level and the subsequent production of other mediators such as NO [199]. In fact, histamine causes an increase of intracellular calcium concentrations, which activate eNOS stimulating the formation of NO, the latter, diffusing into the muscle tissue activates soluble GC, resulting in the formation of cGMP, finally leading to a vasodilator effect (Figure 28).

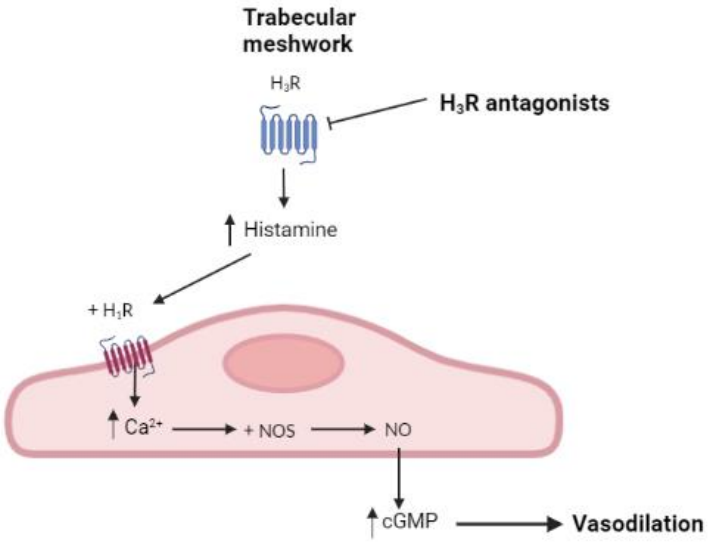

Figure 28: Mechanism of action by which histamine acts in trabecular meshwork and endothelial cells.

It is known that NO is an important mediator of homeostatic processes in the eye, such as regulation of AH dynamics, retinal neurotransmission and phototransduction. [235]. It plays a key role in vasodilation and, at high concentrations, cytotoxicity, due to its gaseous nature through which it readily diffuses into ocular membranes [236]. At the retinal level, NO is implicated in retinal transmission [237], RGC protection from infection [238] and endothelial vasodilation [239]. As we previously mentioned NO lowers IOP via increase of AH drainage from TM/Schlemm's canal [240].

It is well documented that a deficit of NO/sGC pathway is directly associated with an increase in IOP. In fact, decreased levels of NO and cGMP in the AH of glaucomatous patients as been reconducted with an alteration of this pathway [241,242].

Several therapies are available for the treatment of glaucoma; recently, NO donors alone or bound to another molecule, are under clinical study [240]. Latanoprostene Bunod (LBN) 0.024% (Bausch&Lomb, USA), a latanoprost acid (LA) linked to a NO-donating moiety, is the first NO-releasing prostaglandin analog that has been submitted for marketing authorization in the United States in 2017 [243,244]. This molecule is designed to improve permeability of the primary outflow pathway through TM relaxation [93,245], and demonstrated excellent hypotensive action, equal and/or superior to conventional drugs [246]. The validation of drugs with new therapeutic targets, such as hybrid compounds, is a topic of crucial importance, not only for eye diseases. In fact, one of the main purposes is to increase patient compliance by reducing side effects.

The results here reported, clearly indicate that the topical treatment with a histamine H_3R antagonist-NO donor hybrid compound is effective in reducing IOP in both acute and chronic ocular hypertension model (Figures 15 and 18). This action is due to both the blockade of the histaminergic H_3 receptor and the subsequent endogenous secretion of histamine, which interacting with the histaminergic H_1 receptor would activate phospholipase C and the phosphatidylinositol pathway, increasing intracellular Ca^{2+} and activating eNOS, but also to NO secretion, as evidenced by the increase in nitrites/nitrates in the AH (Figure 16). Nitric oxide released from the hybrid molecule and also endogenously secreted, activates GC/cGMP pathway induces vasodilation, and finally IOP reduction.

Knowing that oxidative stress is one of the key factors in the development of glaucoma and in RGC loss, I evaluated the concentrations of two most important antioxidants such as reduced glutathione (GSH) and the Manganese superoxide dismutase (MnSOD), both scavengers are significantly reduced in the hypertensive eyes. These results are supported by the observation of Schuster and colleagues reporting that higher levels of oxidation products were detected in the AH of OAG patients [247]. It is important to note that in ST-1989 treated-eyes the levels of both antioxidants are increased in comparison to vehicle groups (Figure 20), demonstrating that this compound significantly reduces the oxidative stress.

There is evidence that local para-inflammation plays a regulatory role in outflow facility through the TM. TM cells secrete various growth factors and cytokines in response to mechanical stretching [248,249]. Some of these cytokines have been shown to promote AH outflow facility in human and animal models, suggesting an autocrine feedback loop aimed at restoring normal IOP values in response to the mechanical stress produced by IOP elevations [125]. IL-6 partially increased outflow facility in perfused human organ cultures, through modulation of endothelial permeability and induction of MMPs, resulting in ECM degradation [250]. Moreover, in a model of human glaucomatous ONHs, astrocytes also expressed high levels of TNF-α, which parallels the progression of retina degeneration from glaucomatous donors. These eyes showed signs of chronic oxidative stress, as revealed by the increased levels of oxidative by-products immunolabeling compared to control donors [251]. The data obtained with this research are perfectly in agreement with previous findings [125,251], indeed it is possible that chronic treatment with the hybrid compound ST-1989 stimulates IL-6 production by triggering a “physiological” para-inflammatory response, through which up-regulate IL-

10 production [189] and down-regulation of TNF-α occurs. This action could contribute to ameliorating the pathological condition as demonstrated also by the reduction of apoptosis markers.

Another important point highlighted by our work [205] is that treatment with histamine H_3R antagonists is able to prevent RGC leakage by improving vascular performance of the central ophthalmic artery, indicating a role of the histaminergic system in maintaining ocular vascular tone. Yan and colleagues have shown that H_3Rs promote cerebral ischemia-reperfusion (I/R) injury in several experimental models and that H_3R antagonists have a preventive role [252].

It is well known that a vascular dysfunction at the ONH and in retina level can lead to ischemia that contributes to RGC degeneration [253,254]. In the vascular theory, glaucoma is seen as a consequence of insufficient ocular blood supply. This can result from either increased IOP or reduced ocular blood flow. In NTG, which occurs without overt elevations in IOP, vascular dysfunction may be a primary driver of disease progression through an increase of oxidative stress and inflammation at the level of the retina and ONH [255]. Mild and repetitive hypoxic events due to small fluctuations in IOP may lead to an unstable oxygen supply, generating chronic, low-grade I/R injury that does not differ in the progression of the disease from sustained hypoxic insults resulting from acute elevations in IOP [256,257].

Actually, the primary targets of current drug therapy are the rate of formation and resistance to AH outflow, having these as the goal to reduce IOP. However, another extremely important aspect to consider is the integrity of the ocular vascular system. In fact, being able to maintain adequate perfusion, or implement vascular performance in the posterior pole of the eye, would be a good therapeutic strategy for NTG or for hypertensive glaucoma caused mostly by ocular vascular dysfunction.

In 1985 it was found that the endothelium of blood vessels also secretes a vasoconstrictor substance, which was given the name endothelium-derived constrictor factor [258]. This substance was subsequently isolated and named endothelin. Endothelin-1 is the most potent known vasoconstrictor capable of inducing I/R injury in the optic nerve and retina. In addition, ET-1 mimics many situations observed in glaucomatous patients, including increased IOP, hemodynamic, and retinal changes; therefore, my validation of retinal ischemia induced by repeated injections of ET-1 below the Tenon's capsule in a rabbit model, has been considered one of the most suitable models to evaluate the effect of new molecules on retina protection [225].

The results confirmed our previous observation that retrobulbar administration of ET-1 twice per week resulted in a progressively increase in IOP, a profound dysregulation of ocular perfusion evidenced by the OA-RI increase, and a progressive decline of retinal function, involving both rods and cones [259]. The response of photoreceptors, recorded after several light stimuli, decreases over time, with effect maximum 6 weeks after initial administration. The treatment for 4 weeks with the compound ST-1989 twice daily progressively reduces the ET-1-induced increase in IOP (Figure 23). In addition, compound ST-1989 reduce I/R-induced retinal changes and specifically, ST-1989-treated eyes show a better response of both rods and cones than vehicle-treated eyes (Figure 26), a concept also highlighted by the restoration of the RGC layer, as shown in Figure 27.

In conclusion, the results showed in this thesis, strongly support the hypothesis that histaminergic and nitrergic systems cooperate in the regulation of IOP and monitoring RGC integrity.

Moreover, this research clearly points out that hybrid compound ST-1989 is extremely effective in reducing IOP, ameliorating ocular vascular performance and in preventing RGC loss. This could be a completely novel strategy to combat glaucomatous blindness and RGC depletion also for other diseases involving a retinal damage.

Currently, in collaboration with the European Laboratory for Non-Linear Spectroscopy (LENS), we are developing a method to make the retina optically transparent in order to perform a 3-dimensional reconstruction of the whole retina. The tissue can be immunostained with specific antibody and analysed in detail. This new technique could be very interesting to understand better the extent and the specific site of the damage.

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