DOI:10.14753/SE.2023.2900

SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

2900.

MINH NGOC TRAN

Krónikus betegségek gyermekkori prevenciója
című program

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DOI:10.14753/SE.2023.2900

INVESTIGATIONS OF SIGMA-1 RECEPTOR AS A NOVEL THERAPEUTIC TARGET FOR GLAUCOMA IN PRECLINICAL MODELS

PhD thesis

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Budapest
2023

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LIST OF ABBREVIATIONS

AH	aqueous humor
ANOVA	analysis of variance
AS	anterior segment
BL	baseline
CC	collector channel
CLAN	cross-linked actin network
CNS	central nervous system
CTGF	connective tissue growth factor
CTSK	cathepsin K
Dex	dexamethasone
DexAc	dexamethasone-21-acetate
DIC	differential interference contrast
ECM	extracellular matrix
ER	endoplasmic reticulum
EU	the European Union
FLU	fluvoxamine
GCs	glucocorticoids
HTM5	human trabecular meshwork
IOP	intraocular pressure
IW	inner wall
JCT	juxtacanalicular tissue
LDH	lactate dehydrogenase
MMP2	matrix metalloproteinase-2
MMP9	matrix metalloproteinase-9
MMPs	matrix metalloproteinases
MTT	Methyl-Thiazolyldiphenyl-Tetrazolium bromide
NE100	N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl]-ethylamine monohydrochloride

DOI:10.14753/SE.2023.2900

NO	nitric oxide
OHT	ocular hypertension
PDGF	platelet-derived growth factor
pMsTM	primary mouse trabecular meshwork
POAG	primary open-angle glaucoma
RGC	retinal ganglion cell
ROCK	Rho kinase
S1R	sigma-1 receptor
$S1R^{-/-}$	sigma-1 receptor knockout
SC	Schlemm's canal
TGFβ2	transforming growth factor-beta2
TIMPs	tissue inhibitors of metalloproteinases
TM	trabecular meshwork
WT	wild-type
αSMA	alpha smooth muscle actin

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1. INTRODUCTION

1.1. Glaucoma

Glaucoma is a chronic progressive degenerative optic neuropathy that is the leading cause of irreversible blindness and the second leading cause of blindness worldwide [1-5]. Glaucomatous optic neuropathies are associated with characteristic structural deformation at the optic nerve head and visual field defects, due to progressive retinal ganglion cell (RGC) death. The estimated total population of this sight-threatening eye disease in the world was about 76 million in 2020 and was predicted to increase to 111.8 million in 2040 [6]. The worldwide age-standardised prevalence of glaucoma in the population aged 40 years or older is about 3.54%, highest in Africa with 4.79% [6]. Primary open-angle glaucoma (POAG) is the topmost prominent form of glaucoma. Its global prevalence of about 3.1%, was six times more common than primary angle-closure glaucoma, which had a global prevalence of about 0.5% [6]. Its major risk factor is the elevated intraocular pressure (IOP) [7-17], even though other pathogenetic processes may also be involved in the development (Table 1). At present, the only approach affirmed to be effective in preserving visual function is lowering IOP [13]. Various treatment strategies - medical therapy, laser trabeculoplasty, and conventional surgery - aim to lower IOP, slow the disease progression, sustain visual field and prevent further vision loss. Despite these medical efforts, currently there is no cure for glaucoma.

Table 1. Major risk factors associated with POAG

Risk factors	References
Higher IOP	[7-9, 11, 12, 14-17]
Older age	[8, 9, 11, 12, 15-20]
Family history of glaucoma	[8, 9, 12, 15, 16]
African race or Latino/Hispanic ethnicity	[6, 8, 20]
Thinner central cornea	[12, 16, 17]
Lower ocular perfusion pressure	[16, 21, 22]
Diabetes mellitus	[8, 23, 24]
Myopia	[8, 11, 12]

POAG: primary open-angle glaucoma; IOP: intraocular pressure

1.1.1 Symptoms and diagnosis

The symptoms of glaucoma depend on the type and the stage of the disease [13]. In early stages, most of the cases the patients have no symptoms or warning signs. Gradually, blurred vision or loss of vision will happen in all types of glaucoma at the later stages. When the patients experience visual impairment, usually it starts with their side (peripheral) vision. Without treatment, eventually, glaucoma can cause blindness. Some other symptoms can exist, for example, in acute angle-closure glaucoma, severe headache, eye pain, nausea or vomiting, and eye redness could occur. Halos or colored rings around lights could also happen in acute angle-closure glaucoma or in pigmentary glaucoma.

The only way to diagnose glaucoma is to perform a complete eye exam. Besides the clinical characteristic manifestations (in advanced stages) of the disease, the following diagnostic methods can be used [13, 25]. On initial diagnosis, measurement of the IOP (applanation tonometry) is mandatory. The IOP is currently the unique modifiable risk factor for the onset and progression of glaucoma. Ophthalmoscopy is also a part of an ocular examination, allowing a view of the fundus of the eye. Glaucomatous changes in the optic nerve head, the retinal nerve fiber layer, and neuroretinal rim can also be confirmed with an optical coherence tomography or a slit lamp microscope. A gonioscopy helps to determine whether the iridocorneal angle is open or closed to classify open-angle or angle-closure glaucoma. A visual field test (perimetry) examines the missing areas of vision. If the diagnosis is made at the early stage, the early treatment can help to prevent or slow visual defects. Therefore, regular eye check-up is important, especially for elderly or people with higher risk for glaucoma (Table 1).

1.1.2 Pathogenesis

Elevated IOP is the key causative factor of the final optic nerve damage in glaucomatous pathogenicity.

IOP homeostasis is maintained by the equilibrium of secretion and drainage of aqueous humor (AH), the natural nutrient fluid for the tissues in anterior part of the eye. AH drains through two pathways, either the conventional (so-called trabecular meshwork route) or the alteration (so-called uveoscleral route) outflow pathway, both are located at the

DOI:10.14753/SE.2023.2900

iridocorneal angle. AH drainage occurs principally through the conventional pathway (Figure 1). The direct measurements in human eyes had suggested that more than 85% of total AH was drained by conventional route [26]. Moreover, the proportion of AH egress through the uveoscleral pathway even decreases further with age [27].

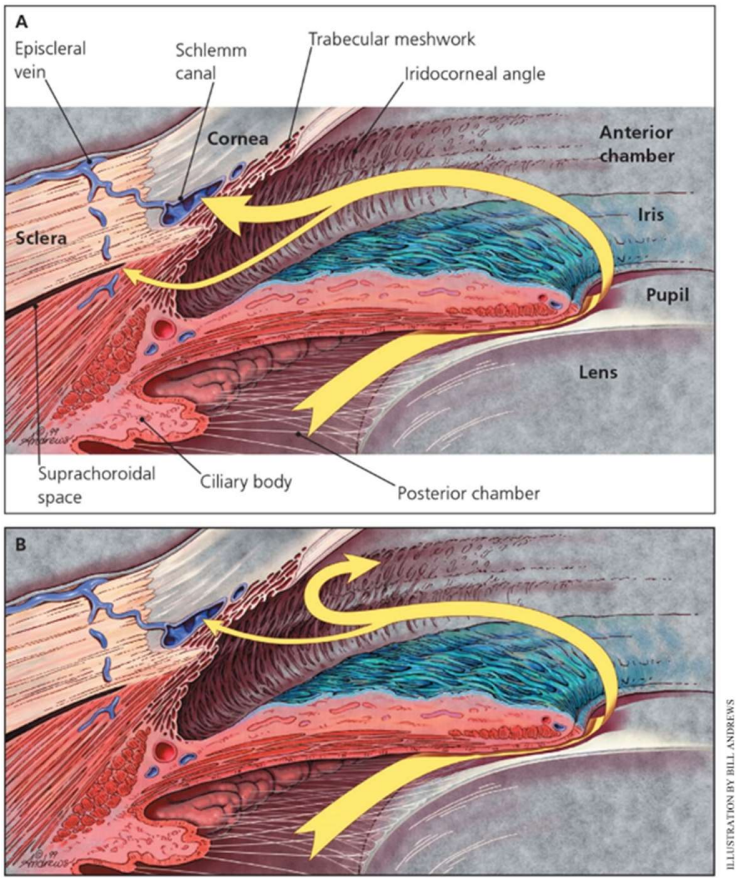

Figure 1. Normal (A) and abnormal (B) aqueous humor (AH) flow. Diagram displaying the iridocorneal angle formed between iris and cornea. After produced by the ciliary body, AH enters and circulates within the anterior chamber prior to exiting from the eye either through trabecular meshwork (conventional pathway) or uveoscleral outflow pathway. (**A**) The proportion of aqueous drainage through trabecular meshwork (large arrow) and uveoscleral routes (small arrow) and related anatomy. Each pathway is drained by the eye's venous circulation. (**B**) In primary open-angle glaucoma, these outflow pathways is diminished, resulting in elevated intraocular pressure. Image was reused with permission from Bill Andrews [28, 29].

Besides the imbalance of AH production and drainage, many other factors can result in IOP changes, including body posture, circadian rhythms, age, blood pressure, stress or pain levels, water consumption, and more [30-32].

i. Role of trabecular meshwork on IOP regulation

The trabecular meshwork (TM) which is located in the iridocorneal angle is the main egress route of AH from the anterior chamber. It occupies and forms the proximal portion of the conventional drainage. Most of the AH passes through the TM, traverses Schlemm's canal (SC), collector channels, and finally empties it into episcleral veins (Figure 2). In this passageway, outflow resistance is generated, controlled, and responsible for homeostatic IOP regulation. Impairment of the cellular properties and function in the conventional outflow pathway, and particularly the TM, results in increased outflow resistance that leads to IOP elevation [33-37], characteristic of most types of POAG. Despite elevated IOP can result from either increased formation of intraocular fluid from the ciliary body or increased outflow resistance, recent researches report that progressive fibrosis of the tissues on the main outflow, especially the TM, is responsible for most of increased outflow resistance and IOP elevation that results in glaucoma [38-40].

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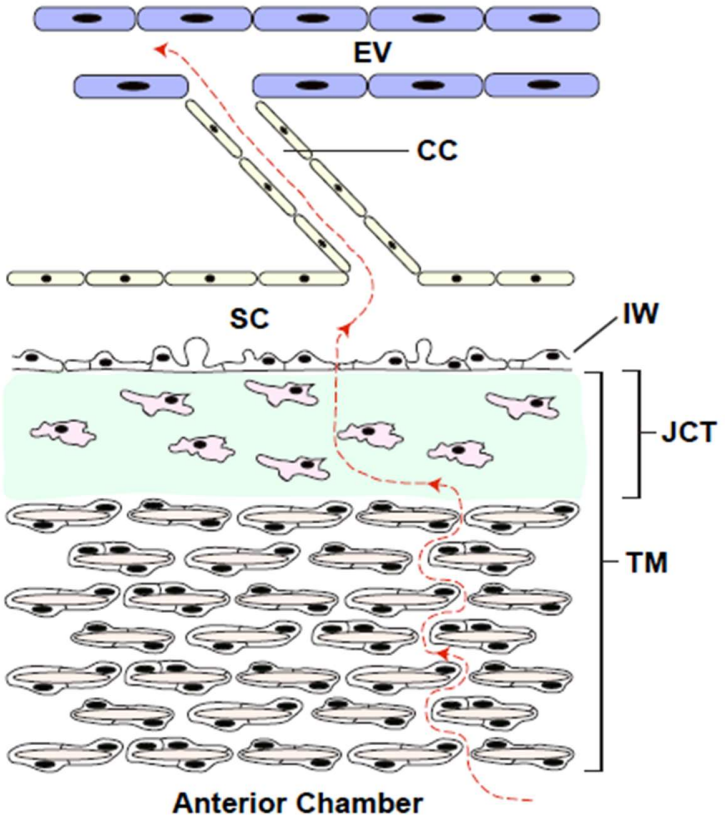

Figure 2. Schematic of the conventional aqueous humor (AH) outflow pathway (so-called the trabecular meshwork (TM) pathway). Arrows indicate the direction of aqueous flow. The AH moves from the anterior chamber into the TM, through the cells and extracellular matrix (green) of the juxtacanalicular tissue (JCT), into Schlemm's canal (SC) via the inner wall (IW), into collector channels (CC), and ultimately into episcleral veins (EV). The AH then joins the general venous circulation. Image was reused with permission from Swarup Sai Swaminathan [41].

TM is a complex connective tissue, anatomically divided into three layers. From inner to outermost, the first two layers, named the uveal meshwork and the corneoscleral meshwork, function as a self-cleaning biological filter [42]. The outermost layer, named the juxtacanalicular (JCT) TM, is a loose, amorphous region containing extracellular matrix (ECM) amongst a small number of cells supported by an incomplete basement membrane, consisting of TM cells (inner side) and the inner wall of SC (outer side) [42, 43]. The JCT and SC function to generate and modulate for outflow resistance [42, 43]. It has been

suggested that cells in JCT region respond to sustained IOP fluctuations by changing the balance of matrix metalloproteinases (MMPs, ECM degrading enzymes) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs), thus alter ECM quality and quantity, thereby resulting in modulation of AH outflow and IOP homeostasis [44-48].

Numerous studies have been carried out to explore the location in the iridocorneal angle having the greatest resistance and the role of ECM in outflow regulation. It has demonstrated that the JCT TM is a key regulation zone or the anatomic location of the majority of outflow resistance [34, 43, 49-51]. Further, it has been postulated that the amount of resistance of outflow and outflow facility correlates with the amount of ECM in the TM [44, 48, 52-55]. A variety of ECM elements have been determined within the TM, mostly similar to those found in other tissues. ECM turnover and remodeling occur constantly due to enzymes responsible for ECM degradation and biosynthesis. Thus, dysregulation in these enzyme activities results in ECM alteration that is likely related to the increase in IOP and has a critical role in POAG development [47, 48, 56-61].

Besides ECM, TM cells are also crucial on outflow regulation. Aberrant alterations in TM cytoskeleton have been associated with the modification on AH outflow facility and the development of ocular hypertension (OHT) [62-64]. The cytoskeleton, consisting of three types of protein assemblies (actin filaments, microtubules, and intermediate filaments), is essential for maintaining cell shape, controlling adherence to the ECM, cytokinesis, cell motility, phagocytic activity, and protein synthesis, to mention but a few [65-68]. The development of cross-linked actin networks (CLANs) and the increase in actin stiffness have been revealed to induce a more contraction and rigid morphology in TM cells, resulting in decreased AH outflow [33, 69-73]. TM cell rigidity and surrounding ECM accumulation together contribute to tissue stiffness and fibrosis, negatively correlating with AH outflow facility [69, 74, 75]. Fibrotic pathology in the TM tissue is associated with glaucoma [39, 40].

Collectively, an increased understanding of the molecular mechanisms for TM malfunction and fibrosis linked to elevated resistance to outflow in glaucoma is promising and beneficial for developing therapeutics for this relentlessly progressive disease.

ii. Pro-fibrotic factors

The key to the proper functioning of tissues in outflow pathways is the composition of AH, which supplies growth factors and a homeostatic environment. In glaucomatous AH, the levels of growth factors and inflammatory cytokines are important. Several studies have examined cytokine levels in the AH of POAG patients [76-85]. Of these, transforming growth factor-beta2 (TGFβ2) has been the most widely investigated in relation to POAG.

TGFβ2, the most abundant TGFβ isoform in the eye, is known to be strongly associated with the development of elevated IOP and POAG [56, 58, 86, 87]. Clinically, it is significantly increased in the AH of patients with POAG [76, 81-84]. Its levels in AH were also correlated with the severity of visual field defects in POAG patients [85]. *In vitro*, TGFβ2 is remarkedly increased in cultured glaucomatous TM cell lines compared to normal TM cell lines [88]. This pro-fibrotic cytokine has been reported to contribute to TM fibrosis. Firstly, TGFβ2 upregulates ECM protein synthesis and concurrently downregulates ECM turnover in the TM, thus causing aberrant ECM deposition that appears to be causatively involved in increased outflow resistance [57, 58, 87, 89-97]. In addition, substantial evidence exists revealing that TGFβ2 is also involved in the modulation of the actin cytoskeleton of the TM and its contractile properties [57, 90, 92, 98-100]. Furthermore, this growth factor also accelerates cell proliferation and migration of TM cells [90, 101].

TGFβ2 also plays a key role in glucocorticoids (GCs)-induced OHT in mice model [86]. Despite GCs-induced glaucoma is a type of secondary iatrogenic open-angle glaucoma, its clinical characteristics are similar to POAG in many ways, including elevated aqueous outflow resistance and morphological and biochemical alterations of the TM [102]. Thus, this glaucomatous model has been used widely in preclinical studies.

Another cytokine, platelet-derived growth factor (PDGF), is a well-documented factor that drives fibrosis in almost all organs [103]. PDGF has been involved in eye fibrosis, particularly in age-related macular degeneration or proliferative diabetic retinopathy [104, 105]. Along with TGFβ, PDGF is among the crucial mediators of fibrotic changes in the eye following glaucoma surgery [106, 107]. Blockage of PDGF inhibits fibrosis and therefore might be a potential therapeutic approach for pathological fibrotic diseases. There have been

clinical trials for fibrotic diseases of the lung, bone marrow, eye, and systemic fibrosis, using PDGF-targeted anti-fibrotic therapy [108-111]. However, the involvement of PDGF in TM fibrosis is still unclear.

1.1.2 Treatment

The goal of glaucoma treatment is to promote the well-being and quality of life of the patients by preserving their visual function. Currently, lowering IOP is the only therapeutic approach which is proven to be effective to slow the rate of visual impairment [13]. IOP can be lowered with medications, laser, or surgery. However, treatment must be individualized based on patients' basis.

A topical IOP lowering treatment is typically the first choice for glaucoma therapy. Since the introduction of antiglaucoma drugs in 1875, a wide variety of glaucoma medications have been available and there was a significant development in the last 30 years. However, it is necessary to individualize each patient's treatment regimen to get the maximum safety and benefit. Consequently, glaucoma remains a complicated disease and a challenge to treat. The current eye drops do not work in each case, although in general they are effective. To accomplish the need for additional glaucoma therapies, many studies have been conducted to develop drugs with novel mechanisms of action. Currently, the medications either decrease the inflow of AH (the production of AH at ciliary body) or increase the outflow through the two drainage pathways (Table 2). In a long time, the majority of glaucoma medications that facilitate AH outflow mainly targeted the uveoscleral drainage which is responsible for only 10 to 15% of total AH egress and even less with age [13, 26, 27]. In addition to this, it should be noticed that POAG prevalence considerably increases with age [13]. Thus, the other approach, targeting the trabecular pathway, the main route of AH outflow, seems an attractive therapeutic strategy. Recently, Rho kinase inhibitors (ROCK inhibitors) and nitric oxide (NO) have emerged as potential novel therapeutic compounds for IOP lowering in glaucoma via modification of trabecular outflow facility. Although some medications which target the trabecular outflow have been developed to lower IOP, there is a need for developing new medical therapies targeting this main AH route due to the refractory to these available drugs or significant adverse effects [13, 112-114].

Table 2. Medications used for POAG treatment [13, 115]

Classification	Mode of action	Side effects
Beta-blockers	Decrease AH production	Hyperemia, ocular irritation, bradycardia, bronchoconstriction, syncope
Carbonic anhydrase inhibitors	Decrease AH production	Hypersensitivity, dysgeusia, paresthesia, gastrointestinal distress, renal calculi
Alpha-adrenergic agonists	Decrease AH production /Increase uveoscleral outflow	Hyperemia, allergy, ocular irritation, anterior uveitis, lacrimation, fatigue
Prostaglandin analogues (PGA)	Increase uveoscleral outflow	Eyelash growth, eyelid darkening, periocular fat atrophy
Parasympathomimetic agents	Facilitate outflow via contraction of ciliary muscle and widening of the TM	Pseudomyopia, miosis, nyctalopia, brow ache, ocular irritation, retinal tear
Rho kinase inhibitors	Increase trabecular outflow	Hyperemia, subconjunctival hemorrhage, cornea verticillata, headaches
NO donors*	Increase trabecular outflow	Eyelash growth, eyelid darkening, periocular fat atrophy

**fixed combination with PGA; TM: trabecular meshwork*

NO, a critical signaling molecule, has emerged as a potential novel candidate for IOP lowering in glaucoma treatment. This simple, versatile gaseous biological messenger plays an important role in many cellular and physiological processes in the human body [116]. In the ocular anterior segment, NO is synthesized endogenously by NO synthases in the ciliary muscle and the outflow tissue [117], where it takes part in the regulation of IOP. It is thought to change IOP via alteration of conventional outflow facility [118-123].

TM tissue, possessing contractile properties, is responsive to NO, similar to vascular smooth muscle cells. It has been observed that NO plays a crucial role in regulating TM contractility, particularly relaxing the TM [124, 125]. TM relaxation may be involved in the increased AH dynamics [72, 73, 126]. Besides the effect on the IOP, NO system also plays a role in improving ocular blood flow and neuroprotection of RGCs [127, 128].

NO's function in the maintenance of physiological IOP makes it a promising target for IOP lowering therapeutic strategies. Latanoprostene bunod, a novel glaucoma drug, is an NO-donating prostaglandin F2α analog, with a unique dual action mechanism-latanoprost (a PGA) increases uveoscleral outflow and the NO donor increases trabecular meshwork outflow.

1.2. Sigma-1 receptor (S1R)

S1R is a ligand-operated chaperone, having a unique sequence of 223 amino acids with no mammalian homologues. S1R ligands can be agonists or antagonists, and, by definition, they can elicit different or opposing cellular responses. Fluvoxamine (FLU, a specific S1R agonist) and N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl]-ethylamine monohydrochloride (NE100, a selective S1R antagonist) were used in this study. S1R locates predominantly at the endoplasmic reticulum, however its expression is also detected in the plasma membrane, mitochondria, and the nucleus, depending on the cell and organ types [129-131]. It is expressed in numerous organs with broad spectrum of biological functions and as a promising target for treating many medical conditions. In addition to the well-documented neuroprotective effects on the central nervous system (CNS) [132-140], the role of S1R in various other diseases, including cancer, cardiovascular, pulmonary diseases, and COVID-19 have also been reported [141-152].

Our research group investigates the role of S1R in various organs [143, 153]. As an important note, we were the first investigators to demonstrate the protective effect of S1R in the kidney. S1R activation slows the progression on chronic renal impairment in diabetic kidney disease. It prevents hypoxic, inflammatory and fibrotic damages in the kidney [153]. It also shows renoprotective effect in renal transplantation and renal ischemic reperfusion injury [143]. Furthermore, our recent data demonstrate the protective effect of S1R in the lung (manuscript submitted).

Also, S1R has been identified in many tissues of the visual system, including cornea, lacrimal glands, iris-ciliary body, the lens, and retinal tissues [154-157]. Surprisingly enough, there is only one study that reported the S1R expression in human TM cells [158]. However, the subcellular localization of S1R in TM cells has been so far unanswered.

Regarding the functions of S1R in the eye, there have been numerous investigations showing its neuroprotective effects in the retina. Many S1R functions appear to modulate biological functions related to degenerative disease of the retina, including calcium regulation, control of oxidative stress, ion channel modulation, cell autophagy, cell apoptosis, anti-inflammation, and molecular chaperone actions [159-168].

A few studies reported the IOP lowering effect of S1R activation [154, 159, 169], but the mechanism of action remains unclear. It seems possible that this effect may be mediated by facilitating the conventional outflow of the AH through TM. To the best of our knowledge, there is a unique article which demonstrated that S1R agonist (+)- pentazocine protects TM cells from pressure-induced apoptosis [158]. There is no evidence about the effect of S1R on TM fibrosis which is associated with elevated IOP, a crucial role in pathogenesis of glaucoma.

Our group's patent directed at the use of S1R agonists in the treatment of progressive fibrosis of the kidney and lung has been approved in many countries (the US, EU, and others) [170]. Recently, it has been reported that S1R activation with its agonists PRE-084 or FLU mitigated renal, pulmonary, and cardiac fibrosis [141, 151, 171]. Therefore, it is postulated that S1R agonists could have similar effect in the eye. However, the anti-fibrotic effect of FLU on TM cells has not been described by others.

Our group has also published that FLU triggers the Akt-NO synthase signaling pathway, resulting in NO production in rat kidney, that contributes to the vasodilation of peritubular capillary, results in a better blood supply and a milder ischemic injury [143]. Based on this finding, it is rational to investigate the effect of FLU on NO release in TM cells.

The overall focus of this dissertation is to investigate the novel glaucoma treatments that target the conventional AH outflow route. Thus, this dissertation subjected to the anterior part of the eye, particularly the TM. The posterior part, including the retinal tissues, will not be discussed here.

DOI:10.14753/SE.2023.2900

2. OBJECTIVES

Our research has focused on the investigation of S1R as a novel therapeutic target for glaucoma. To conduct the experiments, our working group adopted several new methods and experimental models, refining and optimizing them. The most important established methods were the isolation of primary mouse trabecular meshwork (pMsTM) cells and the dexamethasone-21-acetate (DexAc)-induced ocular hypertensive model in mice.

In our preclinical experiments, we evaluated the *in vitro* effect of FLU using TGFβ2- or PDGF- induced TM models, and explored the IOP lowering effect of FLU eye drop *in vivo* in DexAc-induced mice model.

The following objectives have been set to fulfil the aims:

To assess the effect of S1R agonist FLU on HTM5 cells

1. Determine cellular localization of S1R in HTM5 cells
2. Study the effect of FLU on fibrotic changes of HTM5 cells
3. Evaluate the stimulating effect of FLU on NO release of HTM5 cells

To assess the effect of S1R activation on pMsTM cells

1. Detect the presence of S1R in pMsTM cells
2. Explore the protective effect of S1R on PDGF-induced pMsTM cells

To assess the IOP lowering effect of FLU on DexAc-induced ocular hypertensive mice model

1. Examine the irritating effect of FLU eye drop on the mice eyes
2. Investigate the protective effect of S1R on this glaucomatous model

DOI:10.14753/SE.2023.2900

3. METHODS

For the methods already included in my published articles, I only described them here briefly, for more details, cited publications are preferred [172, 173].

3.1. Assessment the effect of S1R agonist fluvoxamine on HTM5 and pMsTM cells

All methods used in this section were described in my publications [172, 173]. In brief, first, we characterized TM cells by the morphology and the induction of myocilin, alpha smooth muscle actin (αSMA), and fibronectin by dexamethasone (Dex) as described previously [174]. Next, the cells were treated with pro-fibrotic factors in the presence or absence of S1R agonist FLU (Sigma-Aldrich, Budapest, Hungary), and of S1R antagonist NE100 (Bioscience, Bristol, UK) in some cases. Fibrotic-like changes in HTM5 and NO level were investigated and the effect of FLU was shown [172, 173] (Figure 3, lower panel).

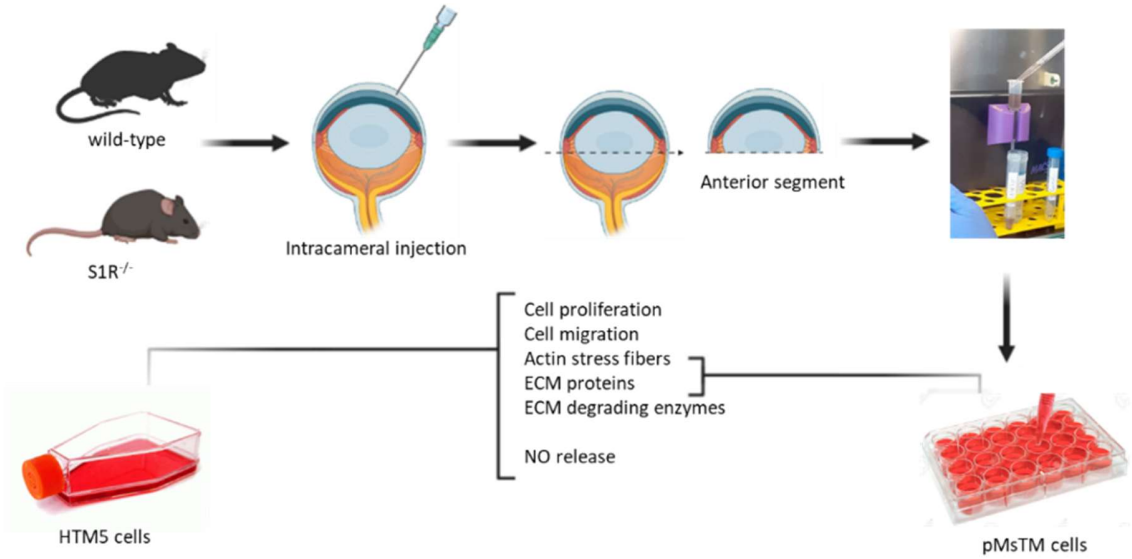

Figure 3. **Brief illustration of the experiments with HTM5 cells and pMsTM cells.** Upper panel showed the isolation of pMsTM cells from C57BL/6J wild-type and sigma-1 receptor knockout (S1R$^{-/-}$) mice.

Besides TM cell line, we isolated pMsTM cells from C57BL/6J and S1R knockout (S1R$^{-/-}$) mice on a pure C57BL/6J background with positive magnetic bead selection [175] (Figure 3, upper panel). The expression of S1R was confirmed by immunofluorescence and

the effect of S1R on PDGF-induced actin remodeling and ECM protein expression were examined in pMsTM cells [173].

3.2. Assessment the effect of S1R agonism on DexAc-induced ocular hypertension in mice model

3.2.1. Animals

Male and female C57BL/6J mice were purchased from Animalab (Budapest, Hungary). The first pair of $S1R^{-/-}$ mice were kindly gifted from Dr. Adrian Y.C.Wong (University of Ottawa, Japan). All animals were bred and housed in the animal facilities of the Pediatrics Center at Semmelweis University. The mice were kept in 12-hour light/12-hour dark cycle in constant temperature (22 ± 2°C), food and water were supplied *ad libitum*. All animal studies were conducted in accordance with the Animal Experiment Regulations of the Animal Welfare Committee of Semmelweis University (PE/EA/916-7/2020), Budapest, Hungary.

3.2.2. Examination the presence of S1R in the mouse TM region

The anterior segments (AS) were dissected from enucleated eyes and subjected to immunofluorescence staining with S1R antibody [172].

3.2.3. IOP measurement

Animals were gently handled to avoid stress, and anesthesia was administered with a 26-gauge needle. Mice were anesthetized intraperitoneally with a ketamine/xylazine mix (90mg/10mg/bwkg) and each mouse was monitored carefully to assess the state of anesthesia. IOP was measured as soon as the mice stopped moving using an Icare Tonolab (Figure 5B). Six to eight IOP measurements from each eye were averaged together at each time point. IOP was measured weekly at approximately the same time of the day under light conditions, before every dexamethasone injection.

3.2.4. DexAc-induced ocular hypertensive mice model

The animals were three to four months old at the first injection. Mice were anesthetized intraperitoneally with 90mg/10mg/bwkg of ketamine/xylazine and anesthetized topically with Oxybuprocaine hydrochloride 0.4% w/v (Novesine, OmniVision GmbH).

After measuring IOP, DexAc injection was performed periocularly as previously described [176]. Briefly, after the lower eyelid was retracted, 20 µL of DexAc 10 mg/mL or vehicle suspension was injected by a 32-gauge needle with a Hamilton glass microsyringe (25-µL volume, Hamilton Company, Reno, NV) under the conjunctival fornix of both eyes (both eyes with DexAc or both eyes with vehicle) in the process of 10 – 15 seconds. The formation of a subconjunctival bleb ensured successful injection. The duration of the experiments depends on the case (4-5 weeks).

3.2.5. FLU eye drop instillation

Eye irritation was tested before use. Eye drops were applied 7 µL per eye, twice daily on the conscious mice (Figure 5A) and the eyes were examined every second day on C57BL/6J mice by a slit lamp microscope (Figure 5C) and fluorescein test, which is the gold standard for corneal epithelial cells' damage detection. The well-tolerated formulation was used for further experiments.

To investigate the IOP lowering effect of FLU eye drop, after two weeks of DexAc injection, eye drop administration began and lasted for 14 days (Figure 4). Micro pipette was used to apply 7 µL of FLU 100 mM or vehicle eye drops topically to both eyes of each animal (one mouse received either FLU or vehicle eye drop to both eyes). Animals were hold for 1 minute after the instillation before returned to the cage. Eye drops were given twice daily.

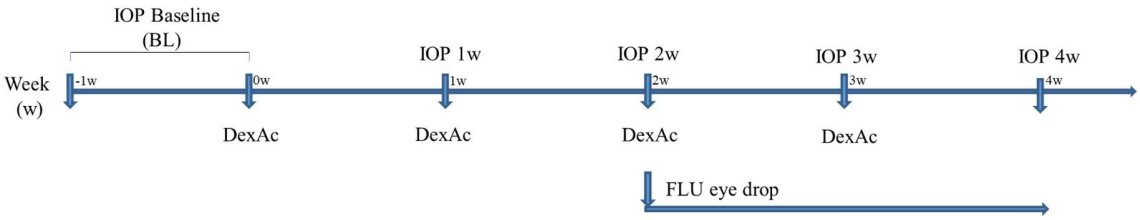

Figure 4. Treatment timeline. Timeline showing which date intraocular pressure (IOP) measurements were taken and when the first dexamethasone-21-acetate (DexAc) was injected into the eyes (week 0) or the first fluvoxamine (FLU) eye drop was instilled into the eyes (week 2).

DOI:10.14753/SE.2023.2900

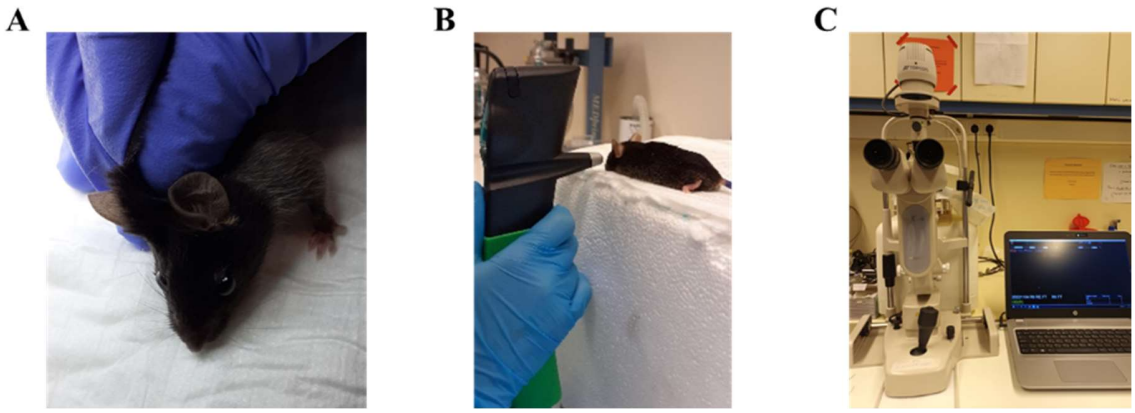

Figure 5. Eye drop instillation (A), non-invasive measurement of intraocular pressure of the mouse eye (B), and slit lamp microscope used in the project (C).

3.2.6. Statistical analysis

Individual sample sizes are specified in each figure legends. Statistical analyses were performed using Prism version 8.0 (GraphPad, USA). Differences among groups were evaluated *via* one-way or two-way analysis of variance (ANOVA), and two groups' comparisons were done using Student's *t-test.* Data were presented as mean ± SEM, or mean with error, depended on each analysis and described in each figure; $p < 0.05$ was considered as statistical significance.

DOI:10.14753/SE.2023.2900

4. RESULTS

4.1. S1R activation is protective in HTM5 cells

4.1.1. Characterization of TM cells

Before used in our experiments, TM cells were characterized as described previously [174]. Dex accelerated the expressions of myocilin, αSMA, and fibronectin (Figure 6) and the cultured cells exhibited basic and specific morphology [42, 174] (Figure 10A, Figure 11A).

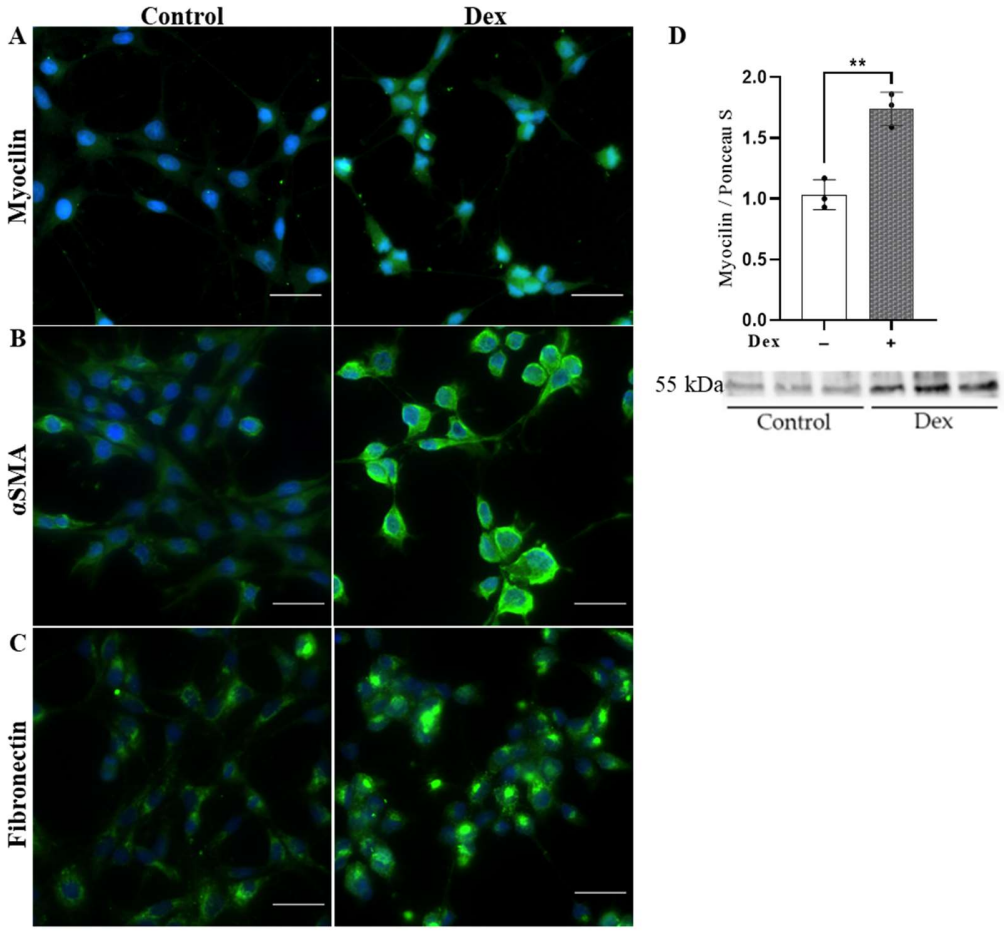

Figure 6. Immunocytochemistry and western blot of HTM5 cells [173]. HTM5 cells were treated with 100 nM dexamethasone (Dex) for 7 days. (**A**) Induction of myocilin in control and Dex-treated cells (Myocilin: green, nuclei: blue; magnification: 400X; scale bar: 20 µm). (**B**) Alpha smooth muscle actin (αSMA) or (**C**) fibronectin level in the TM cells treated with Dex compared to control

(αSMA: green, fibronectin: green, nuclei: blue; magnification: 400X; scale bar: 20 μm). (**D**) Representative western blot of myocilin (55 kDa). (Data: mean ± SEM. **p<0.01; n=3/group; Student's *t-test*).

4.1.2. S1R resides in the endoplasmic reticulum (ER) of HTM5 cells

In order to investigate the role of S1R stimulation on this cell line, the expression of S1R was elucidated. Although one study detected the expression of S1R in human TM cells [158], there is no data about its localization in TM cells. Up to date, this is the first research showing the cellular localization of S1R in HTM5 cells. Co-localization analyses revealed that S1R localizes mainly in the ER of HTM5 cells, as shown by the co-localization of S1R and the ER marker Grp94 (Figure 7) [173]. In addition, S1R can be found in the cytoplasm as well.

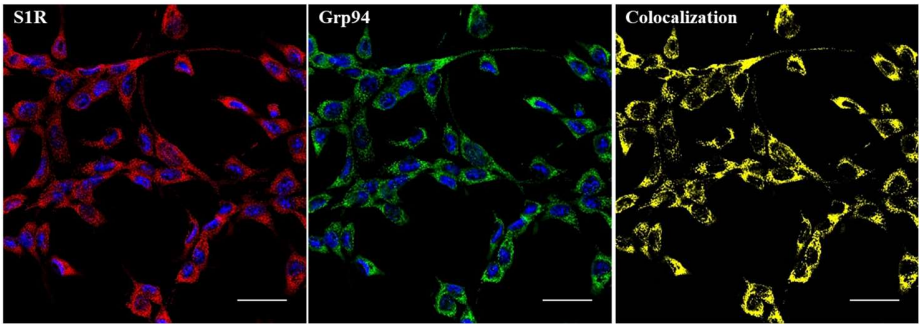

Figure 7. Cellular localization of sigma-1 receptor (S1R) in HTM5 cells [173]. Nikon Eclipse Ti2 microscope; magnification: 600X; scale bar: 20 μm; S1R: red; Grp94: green; nuclei: blue; colocalization of S1R and Grp94: yellow.

4.1.3. S1R activation mitigates cell proliferation and migration induced by pro-fibrotic factors

Two major fibrotic inducers (PDGF and TGFβ2) were used to activate HTM5 cells. In this first experiment, we targeted the effect of FLU on an aspect of fibrotic process, named overgrowth. Methyl-Thiazolyldiphenyl-Tetrazolium bromide (MTT) assay and Lactate Dehydrogenase (LDH) assay were applied to test cell viability and toxicity, respectively, in response to different FLU concentration for 24 hours. MTT assay was also used to determine the minimum effective concentration of PDGF and TGFβ2 on HTM5 cell growth.

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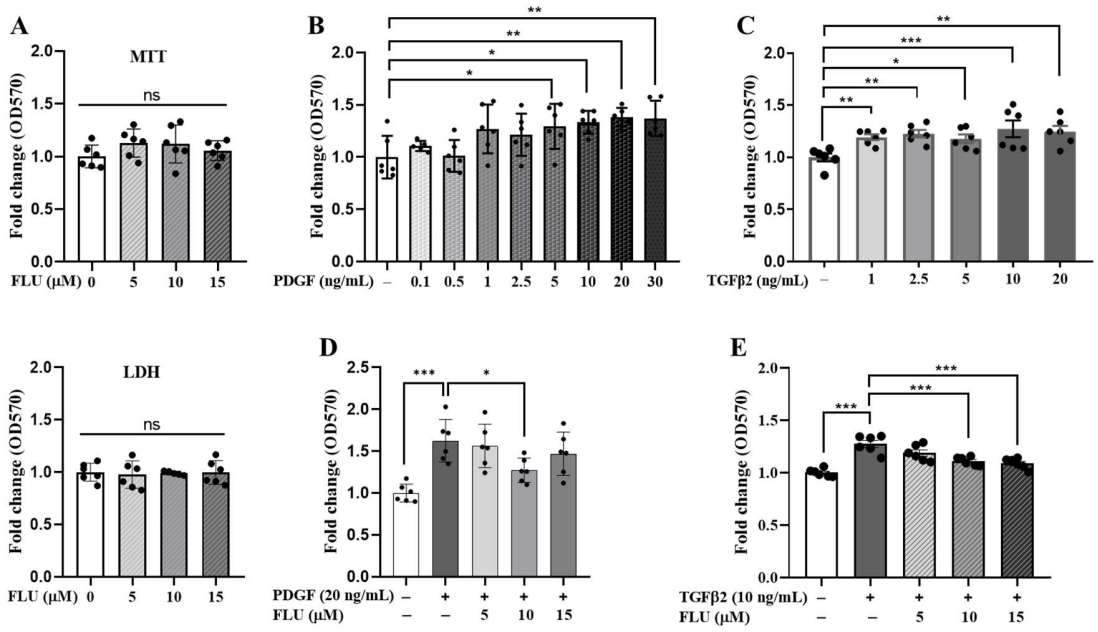

Figure 8. Cell proliferation and cell toxicity measurements for HTM5 cells [172, 173]. (**A**) Cell proliferation (upper panel, MTT assay) and cell toxicity (lower panel, LDH assay) after 24 hours of treatment with different concentrations of FLU (5, 10, 15 μM). (**B**) Cell proliferation with PDGF (0.1, 0.5, 1, 2.5, 5, 10, 15, 20, 30 ng/mL), or (**C**) with TGFβ2 (0, 1, 2.5, 5, 10, 20 ng/mL). (**D, E**) Effect of FLU on cell proliferation after induction with 20 ng/mL PDGF (**D**) or 10 ng/mL TGFβ2 (**E**). (Data: mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ns: non-significant; n=5-6/group; one-way ANOVA analysis).

As we expected, any of the FLU doses tested did not affect cell proliferation and did not exhibit any toxicity to HTM5 cells when applied without any other stimulus (Figure 8A). However, both PDGF and TGFβ2 modulation resulted in a dose-dependent cell proliferation, with a plateau at 20 ng/mL or a peak at 10 ng/mL, respectively (Figure 8B, Figure 8C). These two concentrations (20 ng/mL of PDGF or 10 ng/mL of TGFβ2) have been determined to use further. 10 μM FLU attenuated HTM5 cell proliferation induced by both cytokine stimuli (Figure 8D, Figure 8E).

The effect of FLU on PDGF-induced cell migration was investigated with a commonly used scratch assay. By periodic monitoring, every 24 hours, cell-free area was evaluated compared to 0 hour state, right after the scratch was made, and presented as % of

the original cell free area. The results revealed that PDGF induced cell migration, while FLU prevented this effect in time-dependent manner (Figure 9).

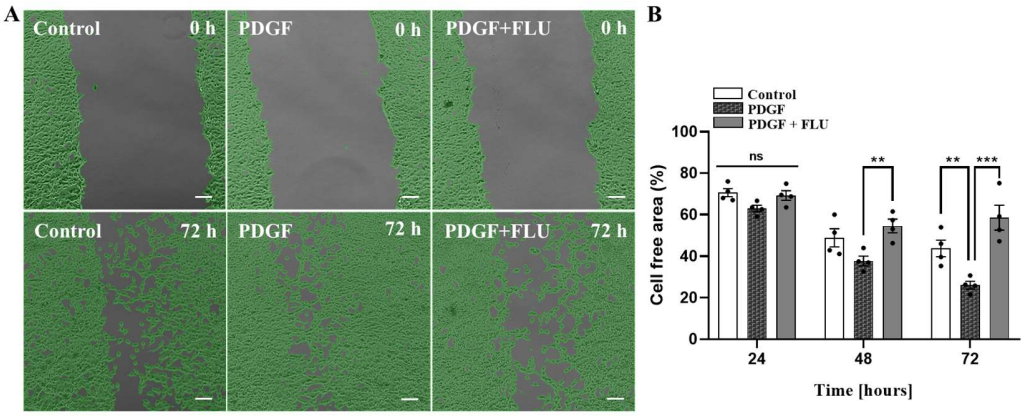

Figure 9. Cell motility of HTM5 cells [173]. HTM5 cells were activated with 20 ng/mL PDGF with or without 10 μM FLU. **(A)** FLU inhibits cell migration after 72 hours following PDGF stimuli (Nikon Eclipse Ti2 microscope; magnification: 100X; scale bar: 100 μm). **(B)** Time curve of cell migration**.** Data represented the cell-free area (%) compared to 0 hour (Data: mean ± SEM. **p<0.01, ***p<0.001, ns: non-significant; n=4/group; two-way ANOVA followed by Holm-Šidak multiple comparison test).

4.1.4. S1R stimulation attenuates actin cytoskeletal remodeling

As actin cytoskeleton is critical not only for maintaining the shape and structure of the cells, but also for a broad variety of functions like cell migration and division, and so on [66, 67]. In the previous results, we showed that prevents profibrotic factor-induces in cell motility and proliferation. Additionally, various changes have been described in glaucomatous eyes, actin stress fiber modification is among the most prominent alterations. They lead to the changes in TM stiffness and TM contractility, which in turn result in increased outflow resistance [33, 69, 71, 177]. Collectively, next, we assessed the effect of FLU on F-actin levels of HTM5 cells with PDGF or TGFβ2 induction, both widely known as potent fibrogenic cytokines.

Both growth factors increased the cell number and caused morphological changes characterized by more elongated shape and increased cell-cell connections of HTM5 cells, as shown in differential interference contrast (DIC) images. All these alterations were

prevented by co-treatment with FLU (Figure 10A and upper panel of Figure 11A). A S1R antagonist, NE100 was also applied, that suspended the effect of FLU (Figure 10A), thus confirming the involvement of S1R in this process.

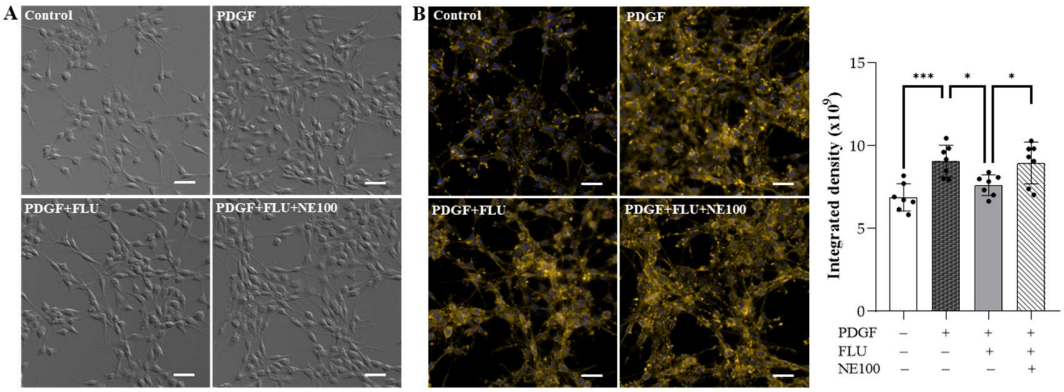

Figure 10. Cytoskeletal remodeling in HTM5 cells [173]. Cells were activated with 20 ng/mL PDGF in the presence or absence of 10 μM FLU with or without 3 μM NE100 for 24 hours. (A) FLU moderates the morphological changes in PDGF-induced HTM5 cells. (B) F-actin filaments and clump formation is ameliorated by FLU treatment. In both cases NE100 suspended the effect of FLU. (Nikon Eclipse Ti2 microscope; F-actin: yellow, nuclei: blue; magnification: 200X; scale bar: 20 μm; data: mean ± SEM; *p<0.05, ***p<0.001; n=6-7/group; ANOVA followed by Holm-Šidak multiple comparison test).

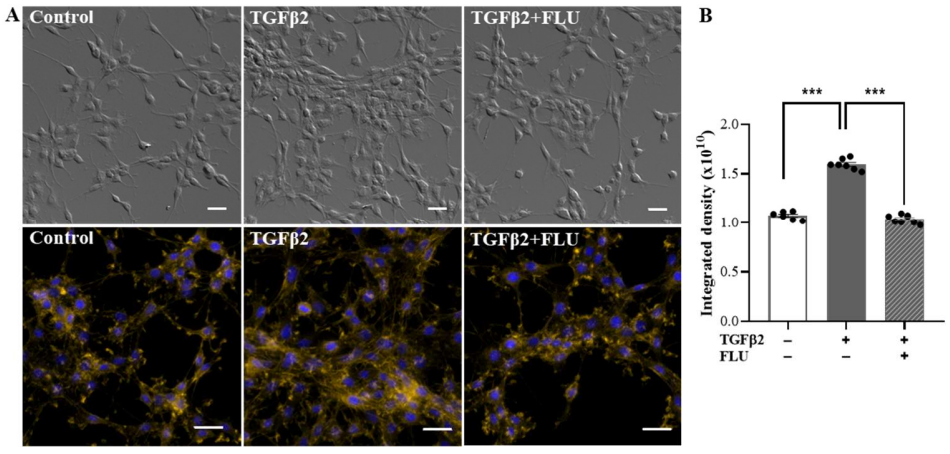

Figure 11. Morphological alterations and F-actin reorganization of HTM5 cells [172]. Cells were induced with 10 ng/mL TGFβ2 and treated with 10 μM FLU for 24 hours. (**A**, upper panel) Differential interference contrast (DIC) images of HTM5 cells (Magnification: 200X; scale bar: 20

μm). (**A**, lower panel) Representative images of cytoskeletal rearrangement. F-actin was visualized with phalloidin-Alexa Fluor 546 (F-actin: yellow, nuclei: blue; magnification: 400X; scale bar: 20 μm). (**B**) F-actin integrated density values represent quantification of fluorescence. (Data: mean ± SEM. ***p<0.001; n=6-8/group; ANOVA followed by Holm-Šidak multiple comparison test).

In parallel, visualization of F-actin with phalloidin staining and the fluorescence integrated density evaluation revealed a remarked elevated actin stress fiber level with both inducers. Similarly, treatment with FLU demonstrated a prominently less fluorescence and less actin clump and bundle formation (Figure 10B, Figure 11).

Interestingly, we also provide more evidence that reinforces the protective role of S1R. NE100, a S1R antagonist, reversed the effect of FLU on HTM5 cells as shown on DIC images (cell density and morphology) and on immunofluorescence staining (F-actin) (Figure 10).

In the next experiment, we investigated the effect of FLU on ECM deposition that is strongly associated with cytoskeletal remodeling and a major characteristic in fibrosis.

4.1.5. FLU ameliorates ECM elements level induced by the pro-fibrotic factors

Fibronectin, an important ECM element, considerably increased in response to both growth factors in HTM5 cells (Figure 12B, E). Other crucial ECM proteins, collagen IV and collagen 1a1 (col1a1) also increased in TGFβ2 and PDGF-treated HTM5 cells, respectively (Figure 12C, D). In line with literature, TGFβ2 increased the expression of CTGF, an important modulator of fibrosis, in HTM5 cells (Figure 12A). All these cytokines-induced elevated ECM protein levels were suppressed significantly by FLU, except for col1a1 (p value=0.3) (Figure 12).

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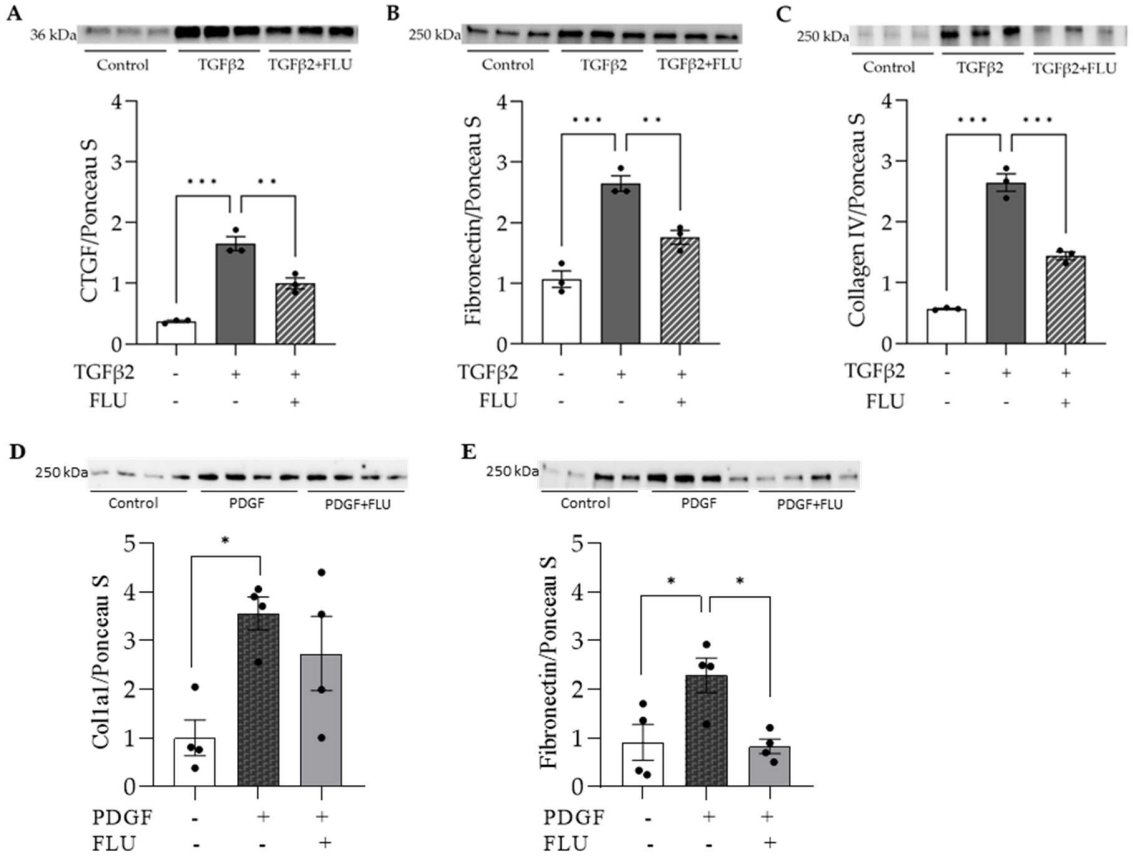

Figure 12. Extracellular matrix-related protein levels in HTM5 cells [172, 173]. Cells were induced by 10 ng/mL TGFβ2 or 20 ng/mL PDGF and treated with 10 μM FLU for 24 hours. (**A**) Representative western blot images of CTGF (36 kDa), (**B, E**) fibronectin (250 kDa), (**C**) collagen IV (250 kDa), and (**D**) collagen 1a1 (Col1a1) (250 kDa). Quantification of western blots presented as relative protein level normalized to the total protein of each sample (**lower panels**). (Data: mean ± SEM. *p<0.05, **p<0.01, ***p<0.001; n=3/group (A-C), n=4/group (D, E); ANOVA followed by Holm-Šidak multiple comparison test).

4.1.6. FLU activates ECM degrading enzymes

ECM deposition can result from the decreased ECM degrading proteolytic enzymes. One may speculate that FLU can exert its protective effect, at least in part, by modulating ECM degradation.

Cathepsin K (CTSK), a lysosomal proteinase, has been recently reported to reduce actin stress fibers and ECM elements and associated to IOP homeostasis [178].

Immunofluorescence images showed that FLU increases CTSK level in PDGF-treated HTM5 cells (Figure 13A).

Another enzyme that is known to be responsible for the degradation of ECM, is Matrix metalloproteinase-2 (MMP2), a member of the MMPs family. Representative western blot showed that TGFβ2 decreases MMP2 level, while FLU suspended TGFβ2-induced suppression of MMP2 (Figure 13B).

These effects could explain the F-actin decreasing potential of FLU.

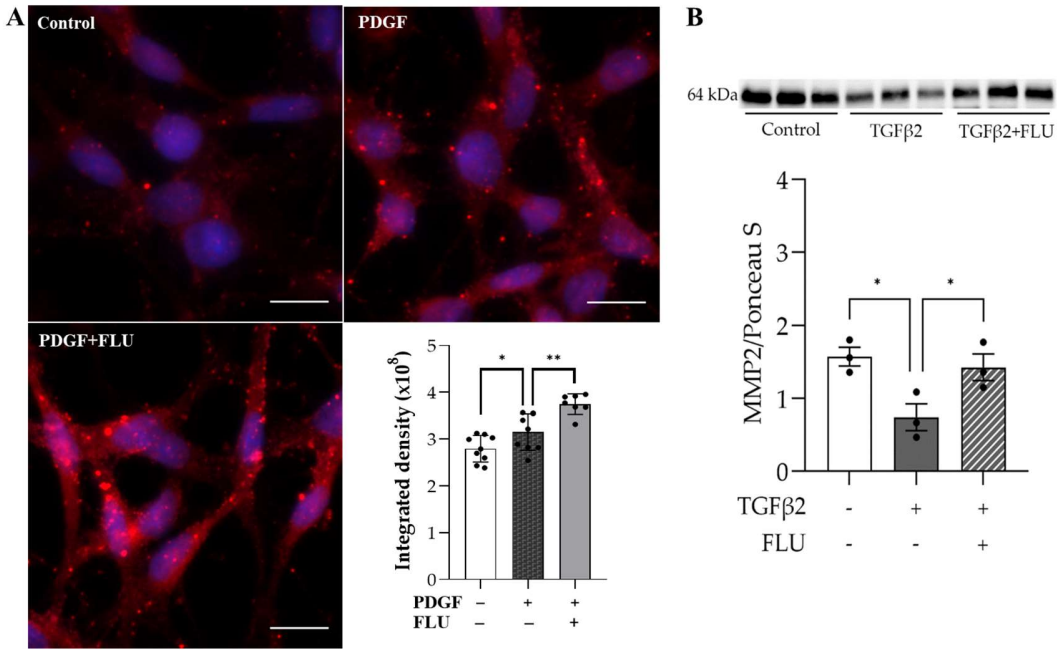

Figure 13. Extracellular matrix degrading enzyme levels in HTM5 cells. (**A**) Treatment of 10 µM FLU for 24 hours elevates cathepsin K level after PDGF induction (20 ng/mL, 24 hours) (Nikon Eclipse Ti2 microscope; cathepsin K: magenta, nuclei: blue; magnification: 600X; scale bar: 20 µm; data: mean ± SEM; *p<0.05, **p<0.01; n=7-9/group; ANOVA followed by Holm-Šidak multiple comparison test) [173]. (**B**) Western blot of MMP2 (64 kDa) after induction with 10 ng/mL TGFβ2 for 24 hours alone or in combination with 10 µM FLU (Data: mean ± SEM. *p<0.05; n=3/group; one-way ANOVA analysis) [172].

4.1.7. FLU increases NO release in HTM5 cells

One important compound that has been recently described to have an IOP-modulating effect is NO. It targets the conventional drainage tissues, as if TM, and restores TM function,

leading lower IOP [118-123]. Therefore, it was rational to explore the role of FLU on endogenous NO level of HTM5 cells. Fluorescent measurements revealed that FLU increases NO level in PDGF-treated HTM5 cells (Figure 14).

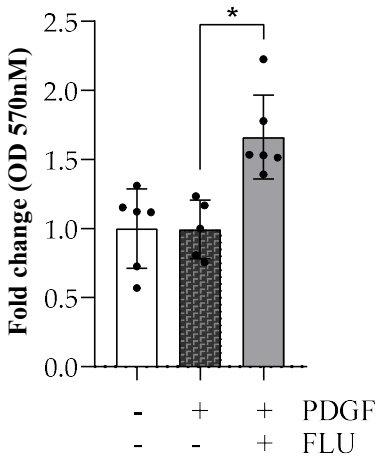

Figure 14. Effect of FLU on released nitric oxide (NO) level in HTM5 cells [173]. FLU elevates NO release in PDGF-induced cells (Data: mean ± SEM. *p<0.05; n=5-6/group; Kruskal-Wallis test followed by Dunn's multiple comparison test).

To confirm the protective role of S1R shown in HTM5 cells, as further step we conducted experiments on primary TM cells isolated from WT and S1R$^{-/-}$ mice.

4.2. S1R is protective in pMsTM cells

4.2.1. S1R detection in pMsTM cells

Based on the specific phagocytosis ability of TM cells, we isolated primary TM cells from C57BL/6J mice following magnetic bead intracameral injection [173, 175]. Primary cells were isolated successfully and were used for experiments after passages 4 or 5. The presence of S1R was confirmed also in these cells, as immunofluorescence images show below (Figure 15).

DOI:10.14753/SE.2023.2900

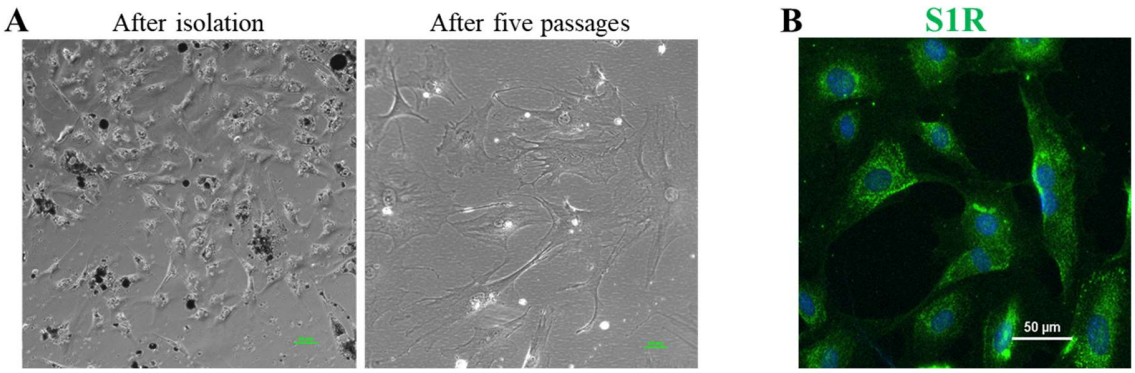

Figure 15. Morphology of pMsTM cells (A) and sigma-1 receptor (S1R) detection (B). (A) Bright field images of pMsTM cells. Magnetic beads are visible inside the cells (left panel) and they are diluted as the cells proliferate (right panel) (Magnification: 200X; scale bar: 50 µm). (**B**) S1R is detected on pMsTM cells (Nikon Eclipse Ti2 microscope; S1R: green, nuclei: blue; magnification: 200X; scale bar: 50 µm).

4.2.2. S1R activation protects pMsTM cells from PDGF-induced F-actin rearrangement

Similar to HTM5 cells, pMsTM cells also responded with increased F-actin to PDGF. This elevation exists in both WT and $S1R^{-/-}$ mice, more importantly, actin clumps and bundles were denser in $S1R^{-/-}$ cells, indicating a protective effect of S1R. As expected, FLU reduced PDGF-mediated F-actin enhancement on WT pMsTM cells, but FLU did not show any effect on $S1R^{-/-}$ cells (Figure 16). This shows the specificity of FLU and confirms the protective role of S1R in PDGF-induced actin reorganization.

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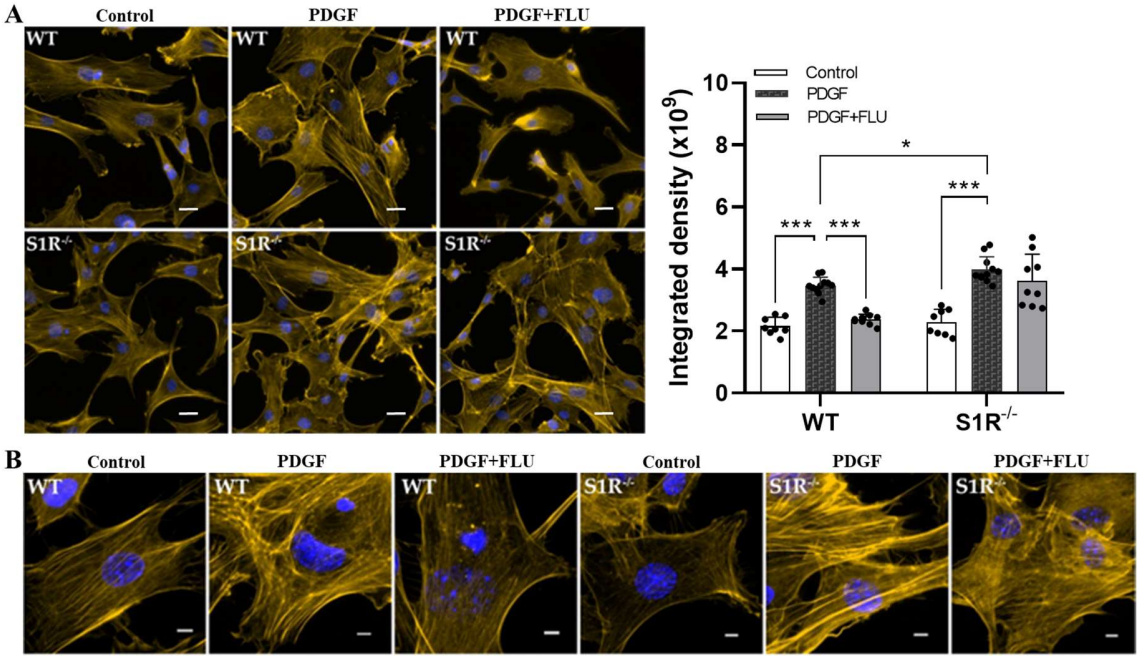

Figure 16. F-actin distribution in wild-type (WT) and sigma-1 receptor knockout (S1R$^{-/-}$) pMsTM cells [173]. The primary cells were induced with 20 ng/mL PDGF and treated with 10 µM FLU for 24 hours. (**A**) F-actin clump and filament formation accelerate in PDGF-induced pMsTM cells. FLU ameliorates cytoskeletal rearrangement in WT pMsTM (**upper panel**) but not in S1R$^{-/-}$ pMsTM cells (**lower panel**) (Nikon Eclipse Ti2 microscope; F-actin: yellow, nuclei: blue; magnification: 200X; scale bar: 20 µm; data: mean ± SEM; ***p<0.001; n=8-11/group; ANOVA followed by Holm-Šidak multiple comparison test). (**B**) Images with higher magnification for better visualization (magnification: 900X; scale bar: 5 µm).

4.2.3. S1R$^{-/-}$ pMsTM cells exhibit higher αSMA expression in response to pro-fibrotic factors

In addition to F-actin, αSMA, a key element in fibrosis, was also examined in WT and S1R$^{-/-}$ pMsTM cells. PDGF increased the level of αSMA in both kinds of cells. Furthermore, S1R loss led to a higher response compared to WT cells (Figure 17).

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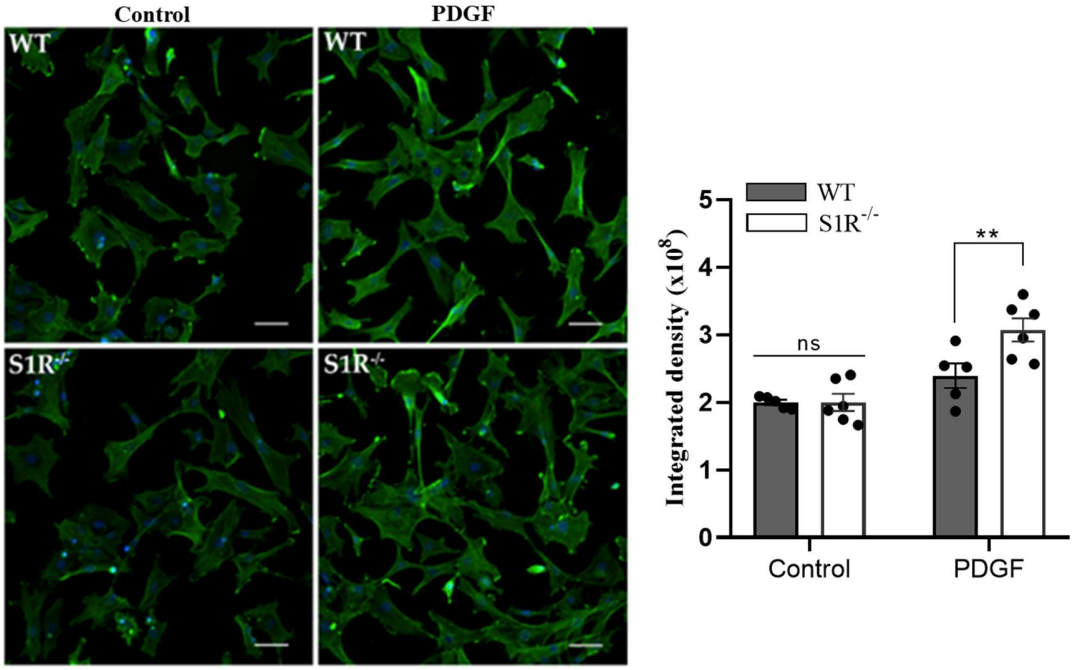

Figure 17. Alpha smooth muscle actin (αSMA) protein level in pMsTM cells [173]. Wild-type (WT) and sigma-1 receptor knockout (S1R $^{-/-}$) cells were induced with 20 ng/mL PDGF for 24 hours. (Nikon Eclipse Ti2 microscope; α-SMA: green, nuclei: blue; magnification: 100X; scale bar: 20 µm; data: mean ± SEM; **p<0.01; n=5-6/group; two-way ANOVA analysis).

All these findings in pMsTM cells, together with those in HTM5 cells reinforce the protective role of S1R in TM fibrosis, a major causative effect in outflow resistance and IOP elevation. Therefore, we next investigated IOP lowering effect of S1R on OHT in mice model *in vivo*.

4.3. S1R lowers IOP in DexAc-induced ocular hypertensive mice model

4.3.1. S1R is present in TM region of mouse anterior segment

Prior to investigation the effect of the agonist FLU eye drop and exploration if its molecular mechanism of action due to TM function regulation, we confirmed that S1R expresses in the mouse TM. Confocal immunofluorescence microscope images and western blot illustrated the abundant presence of S1R in TM region of WT C57BL/6J mice. As predicted, S1R has not been detected in the AS of S1R$^{-/-}$ mice (Figure 18).

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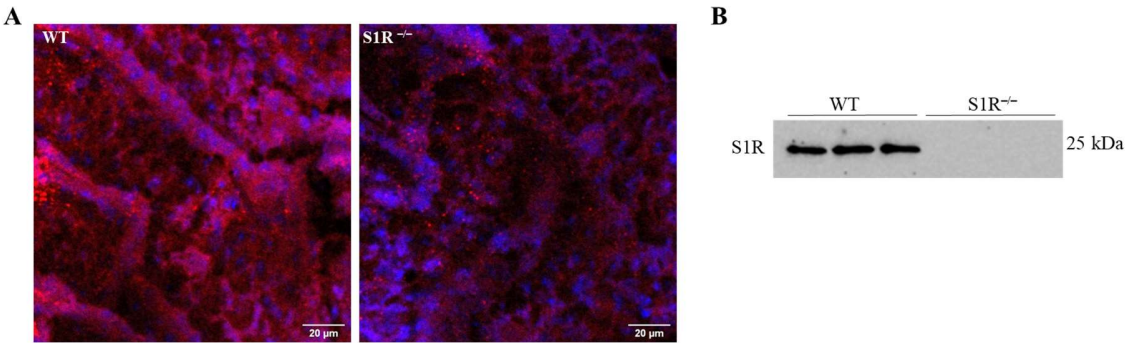

Figure 18. Confocal microscope images (A) and western blot (B) of the mouse anterior segment. Sigma-1 receptor (S1R) expression in the trabecular meshwork region of C57BL/6J mice [172]. WT: wild-type, $S1R^{-/-}$: S1R knockout (S1R: red, nuclei: blue; magnification: 200X; scale bar: 20 µm).

On the other hand, it is necessary to test the safety of FLU eye drop before using it in our model.

4.3.2. FLU eye drop is safe and does not cause irritative signs in mice eyes

The FLU active compound was formulated in various ways and applied 7 µL for each eye, twice daily. The corneal epithelial damage was investigated regularly (every other day) using fluorescein test and images were taken with a slit lamp. After many formulations that had caused damaged eyes (Figure 19B), our final formulation was well-tolerated and non-irritant. This was used in all further experiments (Figure 19C).

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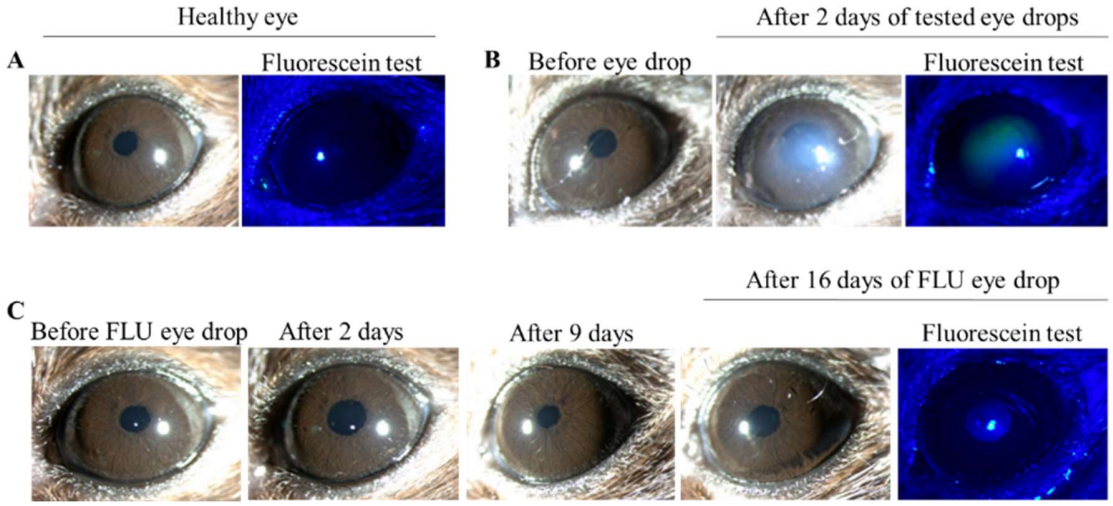


Figure 19. Slit lamp images in irritation tests of the eye drop on mice's eyes. (**A**) healthy eye with negative fluorescein test result. (**B**) damaged eye after 2 days indicated by central opaque cornea area that is also positive with Fluorescein test. (**C**) Slit lamp images of an eye which had been applied the well-tolerated eye drop (FLU) for 16 days. The eyes are totally clear during the period of the irritation test (16 days) and have negative results with fluorescein test.

4.3.3. FLU eye drop drops back the elevated IOP to baseline in WT mice

In WT mice, FLU eye drop lowered DexAc-induced OHT after 2 weeks of instillation (Figure 20). Obviously, at week 4, IOP in DexAc-injected group instilled with FLU eye drop was 16.77 ± 1.12 mmHg, significantly lower than DexAc-injected group instilled with vehicle eye drop (18.61 ± 1.91 mmHg) (-9.89%; $p<0.01$, n=12 mice/group) (Figure 20).

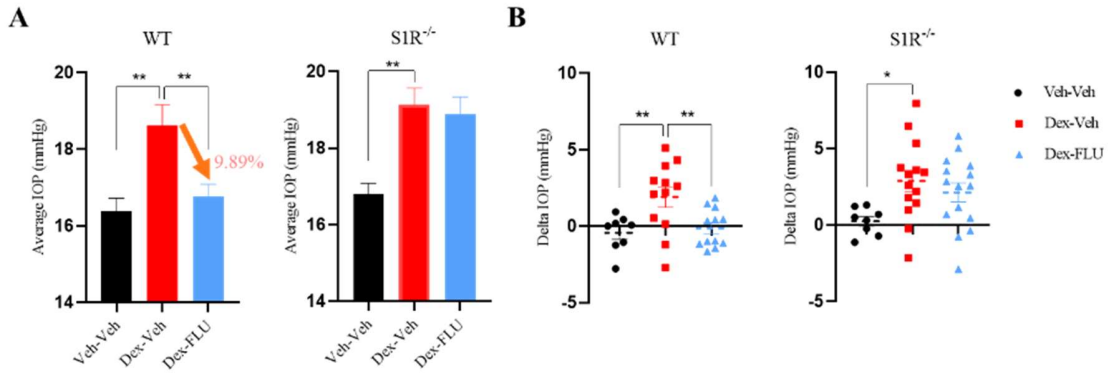
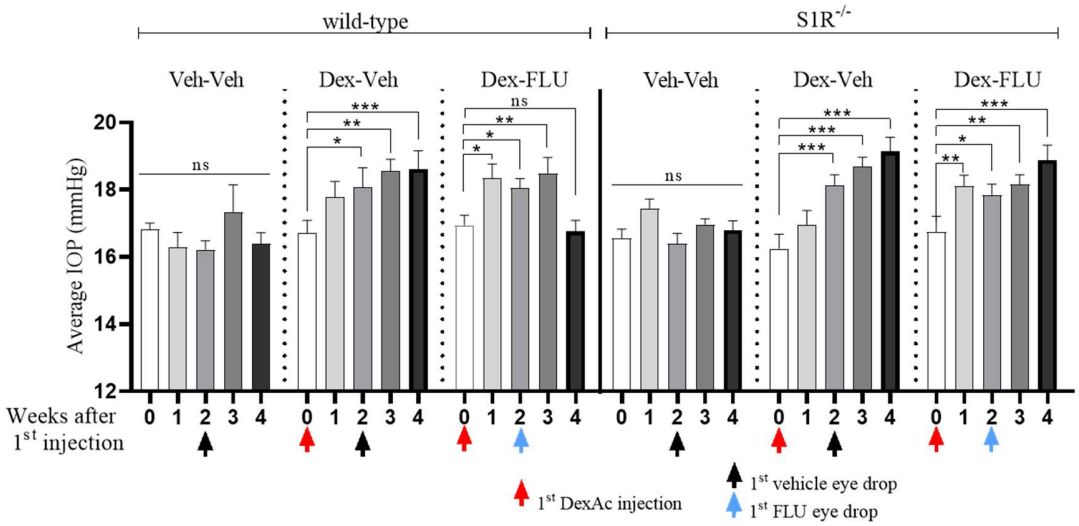


Figure 20. Intraocular pressure (IOP) at week 4 after two weeks of FLU eye drop. (**A**) Average IOP (Data: mean ± SEM) or (**B**) changes in IOP compared to baseline (Data: mean with error) (n=8-15 mice/group; *p<0.05, **p<0.01, one-way ANOVA followed with Fisher's LSD test or Kruskal-Wallis test followed with Dunn's test).

Comparatively, FLU eye drop did not decrease DexAc-induced OHT in S1R$^{-/-}$ mice (Figure 20). IOP of FLU-treated group at week 4 (18.88 ± 1.76 mmHg, n=15 mice/group) is even slightly higher than IOP at 2 week and before FLU instillation (17.84 ± 1.25 mmHg) (p=0.0746) (Figure 21).



Veh-Veh: *Vehicle injection + vehicle eye drop;* Dex-Veh: *DexAc injection + vehicle eye drop;* Dex-FLU: *DexAc injection + FLU eye drop*

Figure 21. Intraocular pressure (IOP) changes in wild-type (WT) and sigma-1 receptor knockout (S1R$^{-/-}$) mice in DexAc-induced ocular hypertensive model. Dexamethasone-21-acetate (DexAc) or vehicle suspension was injected periocularly once a week for 4 weeks and FLU or vehicle eye drop was instilled twice daily for 2 weeks to both eyes of each animal after 2 weeks of DexAc. IOP measurement was performed weekly before the injections. FLU eye drop lowers DexAc-induced elevated IOP in WT mice. Lack of S1R results in a sustained considerable increased IOP until the end of this experiment. (Data: mean ± SEM; n=8-15 mice/group; *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA followed with Fisher's LSD test).

We successfully set up DexAc-induced ocular hypertensive mice model. In DexAc-injected groups of WT, IOP increased from baseline 16.83 ± 1.19 to 18.07 ± 1.54 mmHg

(+7.37%; p<0.01, n=25 mice/group) after 1 week, and then to 18.61 ± 1.91 mmHg (+11.37% *vs* BL; p<0.01, n=12 mice) after 4 weeks. Similarly, in S1R$^{-/-}$ mice, IOP of DexAc-injected group increased from baseline 16.51 ± 1.69 to 17.55 ± 1.50 mmHg (+6.30%; p<0.05, n=29 mice/group) after 1 week and then to 19.13 ± 1.61 mmHg (+17.72%; p<0.001 *vs* BL, n=14 mice, Mann-Whitney test) after 4 weeks. IOP values fluctuated non-significantly in vehicle-injected groups of both WT and S1R$^{-/-}$ mice (Figure 21).

4.3.4. DexAc-induced OHT in S1R$^{-/-}$ mice lasts longer than in WT mice

DexAc was administered (WT and S1R$^{-/-}$ mice) weekly for 5 weeks. Figure 22 shows changes in IOP compared to baseline. In the WT group, IOP returned to baseline by 5 weeks of DexAc treatment while IOP of S1R$^{-/-}$ group did not drop back to the baseline.

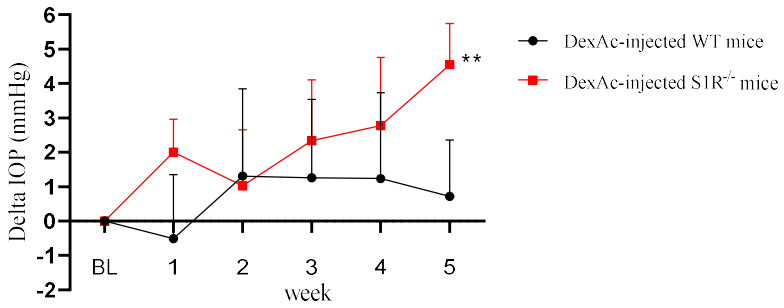


Figure 22. Changes of intraocular pressure (IOP) of wild-type (WT) and sigma-1 receptor knockout (S1R$^{-/-}$) mice during 5 weeks of dexamethasone-21-acetate (DexAc) treatment. Data were presented as delta IOP *vs* BL. In age-matched S1R$^{-/-}$ mice, increased IOP sustained and was still high by 5 week, otherwise, IOP started to reduce after 4 weeks and was close to the baseline values by 5 week in WT group. (Data: mean with error; n=3-4 mice/group; **p<0.01 compared with baseline (BL); two-way ANOVA followed with Fisher's LSD test).

DOI:10.14753/SE.2023.2900

5. DISCUSSION

S1R has been investigated extensively in the CNS and its neuroprotective role has been explored in numerous studies [132-140, 179]. In the eye, S1R also showed its retinal neuroprotection with a large body of evidence [159-168]. However, only one study is available regarding the function of S1R in the TM, revealing its role in pressure-induced apoptosis of TM cells [158]. In line with this article, we identified the presence of S1R in HTM5 cells (Figure 7) [173]. More importantly, by colocalization analysis, we showed, for the first time, that S1R resides in the ER membrane prominently (Figure 7) [173], and for a lesser extent it can be found in the cytoplasm as well. *In vivo*, since 1999 when the first publication reported IOP lowering effect of S1R agonist (+)-pentazocine in rabbit [154], little work has been done to study the role of S1R on IOP regulation [159, 169]. Moreover, the molecular mechanism of this IOP lowering effect was not elucidated at all. Our research is the first that intensely investigates S1R abundance and function in the ocular AS, particularly in the TM.

In this project, we explored the role of S1R agonist FLU on IOP regulation and its potential mechanism of action.

For investigating the IOP lowering effect of FLU, the focus of this project was to establish GCs-mediated OHT in mice model using DexAc which was proved to increase IOP significantly and mimicked many aspects of GCs-induced OHT [176]. GCs are prescribed widely to treat a variety of inflammatory eye diseases. However, prolonged therapy of GCs is linked to many ocular adverse effects, including GCs-induced OHT with similar clinical presentation to POAG. These similarities make GCs-induced OHT useful for *in vivo* glaucoma-related studies providing better insights into the pathogenesis of glaucoma. Furthermore, a more straightforward and more potent method to study the protective functions of S1R is the use of $S1R^{-/-}$ mice. Taken together, the availability of $S1R^{-/-}$ mice in our laboratory makes this GCs-induced high IOP the most suitable model to be used in our study. Regarding medical treatment for glaucoma, there are desired features of an ideal IOP lowering agent, including high effectivity, minimum undesirable side effects, and convenient dosing. On the safety side, our eye drop did not cause any irritation (Figure 19). Although

twice daily is an acceptable dosing, we hope that in the future a more convenient dosing can be developed by improving the drug delivery. Here, our IOP results provide evidence of the effectiveness of the eye drop (Figure 20, Figure 21).

In our animal study, the topical FLU demonstrated an ocular hypotensive efficacy in DexAc-induce OHT. Indeed, this effect occurred only in the presence of S1R (Figure 20, Figure 21). These findings provide evidence the involvement of S1R in IOP homeostasis.

Interestingly, in a pilot study with longer following period, WT mice did not exhibit a prolonged elevation in IOP in response to DexAc treatment, showing their IOPs returned to baseline levels after 5 weeks of treatment (Figure 22). This phenomenon was also reported previously [180] and suggests that there is a compensatory mechanism which can prevent the secondary GCs response. Of note, in our experiment, lack of S1R made the increased IOP lasted longer than in WT mice, providing more evidence to protective role of S1R.

To explore the possible physio-pathological mechanism of IOP regulating effect of S1R, in *in vitro* experiments, we studied the role of S1R activation with FLU in TM cells, which have been shown that their malfunctions lead to IOP dysregulation. We found that FLU restored functions of HTM5 and pMsTM cells after fibrotic induction.

TM fibrosis was reported to be associated with glaucoma [39, 40]. FLU is a specific S1R agonist and its protective effect on fibrosis of some other organs was reported [141, 143, 144, 151]. In this study, we investigated the protective role of FLU against fibrogenic stimuli-induced fibrotic changes in TM cells. To the best of our knowledge, ours is the first study to report the role of FLU in the glaucomatous fibrotic-like changes of TM cells.

TGFβ2 plays a crucial role in GCs-induced OHT [86]. TGFβ2, a predominant isoform identified in the eye, accounts for the greatest fibrosis driving. Similarly, PDGF, another cytokine that is also a well-known tissue fibrosis inducer, promotes cell proliferation, migration, and other ECM-related alterations [103, 106, 181, 182]. PDGF and TGFβ are also among the key mediators in ocular fibrosis following glaucoma filtration surgery [106, 107]. Numerous studies have been done to examine the effect of TGFβ2 on ECM remodeling and TM fibrosis [57, 58, 90-92, 98, 183-186]. In line with the literature, we confirmed that TGFβ2 stimulation substantially caused morphological changes, F-actin enhancement and

rearrangement, increased cell proliferation and accumulation of ECM components (fibronectin and collagen) in HTM5 cells (Figure 8C, Figure 11, Figure 12B-C). However, little investigations on PDGF effects on TM cells, especially on TM fibrosis, have been done. In this study, we found similar changes in proliferation, morphology, actin stress fibers, and ECM elements in HTM5 cells followed PDGF induction (Figure 8B, Figure 10, Figure 12D-E). Moreover, we also detected that PDGF increased F-actin and αSMA levels in pMsTM cells as well (Figure 16, Figure 17).

The mechanisms regulating egress of AH and IOP are heavily dependent on physiological changes in the cytoskeleton and ECM surrounding TM cells [187]. The impairments on TM structure and function, and ECM dysregulation contribute to cause upregulation of IOP [56, 87, 188-190]. Subsequently, the effect of FLU on inhibition of these changes could benefit in glaucoma treatment.

Here we demonstrated that S1R agonist FLU prevented all of these fibrotic-like alterations of HTM5 cells caused by TGFβ2 or PDGF (Figure 8_panel D-E, Figure 10, Figure 11, Figure 12). We also showed that FLU regulated PDGF-induced cell motility in HTM5 cells (Figure 9). In pMsTM cells, FLU decreased PDGF-induced actin arrangement (Figure 16), as such in HTM5 cells. The role of FLU in attenuating these changes, especially the downregulation of fibronectin and collagen, the main ECM elements, is essential to maintain normal TM outflow resistance.

Multiple possibilities need to be under consideration beyond these protective roles of S1R activation implicated in IOP homeostasis. Despite describing all mechanisms of the protective effect of S1R agonism is beyond the scope of this study, our results in *in vitro* studies partly contribute to implications in *in vivo* experiment. Firstly, FLU treatment considerably resulted in the decreased level of F-actin, a major component of the cell's cytoskeleton, and the inhibition of actin clump and stress fiber formation. Thus, cellular processes and properties, such as cell division, cell movement, and cell shape, in all that F-actin plays a key role [66, 67], are directly targeted by FLU, in agreement with our findings as shown in this study (Figure 8, Figure 9). It is also noteworthy that S1R antagonist NE100 abrogated the effect of FLU on F-actin architecture of HTM5 cells (Figure 10). This finding

consolidated the important role of S1R in TM cells. In cancers, FLU has been found to inhibit actin polymerization and cell migration of glioblastoma multiforme cells and glioma stem cells [152], suppress proliferation and migration of colon tumor cells [150]. FLU also inhibits the proliferation and migration of cardiac and pulmonary fibroblasts, contributing to ameliorate fibrosis in the heart and the lung [141, 151]. In agreement with these findings, our investigation is the first study showing the inhibition of FLU on cell proliferation and migration of TM cells. It has been indicated that genes involved in cell proliferation correlate with the formation of collagen [191], a key component of ECM. In this study, we demonstrated that not only cell proliferation but also the levels of collagens and fibronectin, another crucial ECM element, increase with cytokine inductions (Figure 12). Collagen and fibronectin deposition is the hallmark of fibrosis. In the TM, ECM deposition characterizes the TM fibrosis, increases tissue rigidity, resulting in elevated outflow resistance and high IOP [35, 48, 69, 192]. FLU efficiently prevents the elevation of these proteins (Figure 12).

Secondly, FLU acts on CTGF activity (Figure 12A) is also a possibility. CTGF, a downstream cytokine of TGFβ2, is well known to induce the remodeling and deposition of ECM [193-195], while its inhibition could diminish fibrotic processes [193, 194]. We presume that the increased collagen IV and fibronectin expression could be a consequence of CTGF stimulation. Therefore, its downregulation by FLU can lead to the reduced level of these fibrotic proteins, and by this way, FLU could mitigate tissue stiffness. However, further investigation is needed to fully elucidate their potential interactions.

Targeting ECM decomposition is another way how FLU might be protective. The deposition of ECM could be the consequence of the decrease of ECM degrading enzyme activity (MMPs and some other proteases). TGFβ2 treatment was reported to inhibit MMP2 expression or activity in human TM cell cultures [92, 95]. We showed that FLU prevented TGFβ2-induced MMP2 reduction (Figure 13B), therefore it could maintain ECM degrading function of the enzyme and prevent ECM deposition by consequential removal of ECM. Beside MMPs, a well-documented group of ECM degrading enzymes, cathepsins have also recently been reported to be involved in the remodeling and degradation of the ECM in TM [178, 196]. Increased CTSK was showed to reduce actin stress fibers and ECM component levels [178, 197]. Another novelty we found here is that FLU significantly increased CTSK

level in HTM5 cells (Figure 13A). This increase of CTSK can in turn decrease actin stress fibers and accelerate the breakdown of ECM, resulting in decreased ECM accumulation. We speculate that the effect of FLU on decreasing PDGF-induced F-actin (Figure 10) and fibronectin (Figure 12B) upregulation may result from the increased CTSK. However, the marginal reduction of colla1 could be attributed by the small number of cases. On the other hand, in a research conducted in porcine eyes it was revealed that inhibition of CTSK significantly elevated IOP [178], thus it seems possible that FLU-mediated CTSK activation results in decreased IOP. Regarding the interaction between CTSK and MMPs, it was reported that CTSK activates MMP9 [198]. Thus, the role of CTSK in ECM remodeling is likely through MMP9 activation as well. Altogether, the effect of FLU on the increase MMP2 and CTSK presumably contributes to facilitate aqueous outflow and lower IOP.

Aside from providing evidence of the anti-fibrotic effect of FLU on TM cells, our work presents the novel discovery of the stimulation effect of FLU on NO release in human TM cells. NO is another potent mediator of IOP by facilitating TM outflow [118-123] and relaxing the TM [124, 125]. In the posterior eye structures, NO also exhibited protective effects in optic nerve and ocular blood vessels [119, 123, 128, 199, 200]. Interestingly, we showed that FLU increases released NO level in HTM5 cells (Figure 14). This increase likely contributes to the observed IOP lowering effect of FLU in our animal model.

More importantly, the protective role of S1R was obviously confirmed by our knockout studies. *In vitro*, the expression of αSMA in pMsTM cells induced by PDGF was significantly more robust in the lack of S1R (Figure 17). Furthermore, FLU could ameliorate PDGF-induced actin cytoskeletal rearrangement only in WT pMsTM cells (Figure 16). *In vivo*, FLU also exhibited its IOP regulation only in WT mice (Figure 20, Figure 21). Thus, these observations suggest a direct beneficial effect of S1R activation.

In summary, we provided evidence and illustrated deeper insights into the protective role of the multifunctional chaperone protein, S1R, in IOP homeostatic regulation. Its IOP lowering effect is likely a consequence of accelerated AH outflow facility through anti-TM fibrosis and released NO stimulation (Figure 23).

DOI:10.14753/SE.2023.2900

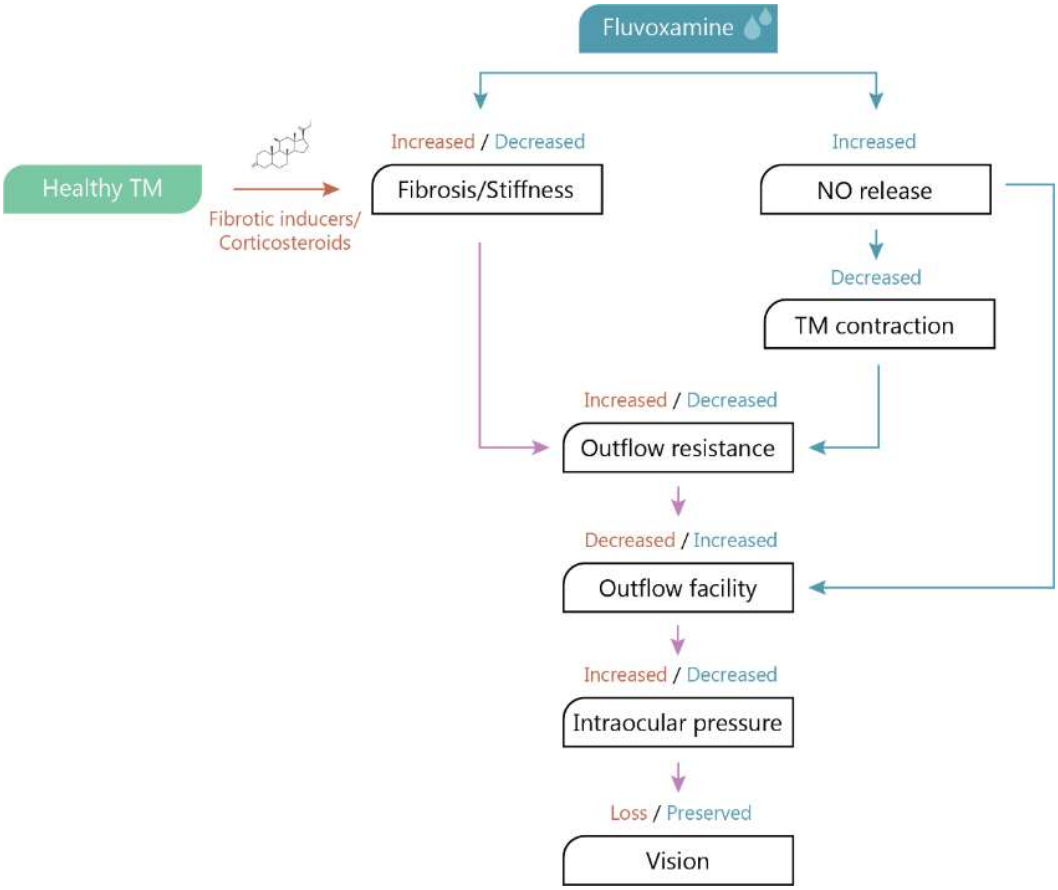


Figure 23. Schematic representation of a model of fibrotic disease in the conventional outflow responsible for ocular hypertension and the potential protective mechanisms of fluvoxamine (FLU). In this study, we triggered this process by fibrotic factors (TGFβ2, PDGF) or corticosteroids administration and restored trabecular meshwork (TM) function by FLU. The flow in red: pathological processes in glaucoma; blue: protective effects of FLU. NO: nitric oxide.

Besides our data result from experiments in the cell lines and primary cells, for exploring the mechanism of IOP lowing effect of FLU, further similar experiments performed in the samples harvested from experimental animals are required.

Though, our findings reveal a novel attractive strategy in glaucoma treatment. This is the first study explored the role of S1R in fibrosis inhibition and NO stimulation in TM cells. FLU is able to reduce the fibrotic stimuli-induced cellular changes in TM cells, which

resemble the cellular events of the glaucomatous TM. The anti-fibrotic effect of S1R is not only beneficial on IOP maintenance but also benefits in prevention of fibrosis following glaucoma filtration surgery. Additional benefits of S1R would be demonstrated as followed. S1R also showed retinal neuroprotection [159-168, 201]. Moreover, FLU alleviated ER stress in neuronal cells [136], and another S1R agonist aniline derivative compound (Comp-AD) also reduced the ER stress response following ischemic stroke in male mice [202]. S1R also benefits in inflammatory conditions [203].

Thus, targeting S1R is a potential approach to develop a novel therapeutic medication for glaucoma. Furthermore, it is worth to investigate its effect on other fibrosis-associated eye diseases.

DOI:10.14753/SE.2023.2900

6. CONCLUSIONS

1. In human TM cells, S1R resides mainly in the ER.
2. S1R activation with FLU protects HTM5 cells against TGFβ2- or PDGF-induced fibrotic changes. FLU decreases F-actin, cell proliferation, migration and pro-fibrotic elements such as CTGF, fibronectin, and collagen IV.
3. FLU may facilitate ECM degradation through the elevation of cathepsin K and MMP2.
4. S1R agonist FLU accelerates NO release in HTM5 cells.
5. S1R is protective in PDGF-mediated fibrotic alterations in pMsTM cells.
6. A well-tolerated, FLU- containing, new eye drop has been developed.
7. The FLU eye drop lowers DexAc-induced high IOP in WT mice.
8. S1R is protective in DexAc-induced glaucoma model, since lack of S1R results in a longer lasting and slightly higher IOP increase.

DOI:10.14753/SE.2023.2900

7. SUMMARY

Primary open-angle glaucoma is a chronic progressive degenerative optic neuropathy that is the second leading cause of blindness worldwide. This sight-threatening ocular disease affected about 68 million people in 2020 and poses a huge burden on society globally. It remains a complicated disease and a challenge to treat due to the refractory to the current medications or significant adverse effects. The only modifiable risk factor is increased intraocular pressure (IOP). Trabecular meshwork (TM) is the main aqueous egress route and its fibrosis is associated with elevated outflow resistance and IOP increase.

The sigma-1 receptor (S1R) is a multifunctional chaperone protein and a promising target for treating many medical conditions. Its neuroprotection has been widely documented in the central nervous system and the retina. We previously demonstrated its anti-fibrotic effects on the kidney and the lung. In this dissertation, we explore its role in the eye, particularly the TM.

We confirmed the presence of S1R and for the first time, showed that it resides mostly in the endoplasmic reticulum of TM cells. Our results elucidated that S1R is involved in outflow facility regulation. We revealed that eye drop formulated with S1R agonist fluvoxamine reduces dexamethasone-induced ocular hypertension and this effect does not occur in S1R knockout mice. *In vitro*, we as the first in the literature showed that S1R protects human and primary mouse TM cells against pro-fibrotic stimuli-induced fibrotic-like alterations. Indeed, S1R knockout cells showed more pronounced fibrotic changes, and these changes resisted fluvoxamine treatment. Another interesting novelty finding is that fluvoxamine accelerates nitric oxide release of human TM cells, this stimulation is likely to facilitate the outflow pathway.

We propose that the anti-fibrotic protective effect and released nitric oxide increase of S1R activation in TM cells contribute to its role in increasing aqueous outflow facility and lowering IOP. Based on our data, S1R agonism could emerge as a new and effective treatment for glaucoma.

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9. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

9.1. Publications related to the PhD thesis

1. **Tran MN**, Medveczki T, Besztercei B, Torok G, Szabo AJ, Gasull X, Kovacs I, Fekete A, Hodrea J. (2023) Sigma-1 Receptor Activation Is Protective against TGFβ2-Induced Extracellular Matrix Changes in Human Trabecular Meshwork Cells. Life, 13: 1581. **IF=3.2**

2. Hodrea J, **Tran MN**, Besztercei B, Medveczki T, Szabo AJ, Őrfi L, Kovacs I, Fekete A. (2023) Sigma-1 Receptor Agonist Fluvoxamine Ameliorates Fibrotic Response of Trabecular Meshwork Cells. Int J Mol Sci, 24: 11646. **IF=5.6**

9.2. Publications not related to the PhD thesis

1. Mai NT, Hoa NT, Nga TV, Linh le D, Chau TT, Sinh DX, Phu NH, Chuong LV, Diep TS, Campbell J, Nghia HD, **Minh TN**, Chau NV, de Jong MD, Chinh NT, Hien TT, Farrar J, and Schultsz C. (2008) *Streptococcus suis* meningitis in adults in Vietnam. Clin Infect Dis, 46: 659-667. **IF=8.266**

DOI:10.14753/SE.2023.2900

10. ACKNOWLEDGEMENTS

First, I would like to thank my supervisor, Dr. Judit Hodrea, for her patience, unflinching support, persistent guidance, and inspiring discussions throughout my PhD study. I am also profoundly grateful to the leader of our group, Dr. Andrea Fekete, who is a role model in my life and scientific work. Her wise suggestions, tireless dedication, and full support are vital to our research group. I would like to express my heartfelt gratitude to both of them for the opportunity to embark on this challenging and interesting project. Without their help, I would still be preparing for publication and could not complete this dissertation in time.

Additionally, I express my sincere gratitude to Professor Attila Szabó for his approval of my application for a PhD position in his institute, Pediatric Center, Semmelweis University.

Further, special thanks to Ms. Krisztina Tölgyesi-Lovász, and the Doctoral School for their support in the period of my study, particularly during preparing my dissertation and completing it as soon as possible.

Moreover, I am very grateful to our senior researchers, Dr. Dóra Balogh, Dr. Ádám Hosszú and our ophthalmologist consultant, Dr. Illés Kovács, for their valuable experience and advice. I also kindly acknowledge my colleagues Tímea Medveczki, Tamás Lakat, Ákos Tóth, Éva Fórizs, and Adar Saeed at MTA-SE Lendület "Momentum" Diabetes Research Group for their useful help and support during my study. Thanks for Dr. Ágnes Molnár and Dr. Lilla Lénárt for their assistance in the beginning of my studies. Many thanks to Maria Bernath for her help in breeding the mice, her friendliness, and her unforgettable delicious cakes. I also would like to thank all my other colleagues in the research laboratory for a very friendly atmosphere. Thank you, Eszter Lévai, Csenge Pajtók, I treasure our friendship. I would like to thank Balázs Besztercei (Institute of Clinical Experimental Research) and Dr. György Török (Department of Biophysics and Radiation Biology), for their contribution.

I am also very grateful to our collaborators in the project, without their contribution, we could not proceed as far as we are.

Finally, a big thank you to my family for all their consistent support, encouragement, and love. Thank you, my love, Nep and Na, I will make up for the lost time.