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Discovery of novel inhibitors for the treatment of glaucoma

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Abstract

Introduction—Glaucoma is a neurodegenerative disease with heterogeneous causes that result in retinal ganglionic cell death (RGC). The discovery of ocular anti-hypertensives has shifted glaucoma therapy, largely, from surgery to medical intervention. Indeed, several intraocular pressure (IOP) lowering drugs, with different mechanisms of action and RGC protective property, have been developed.

Areas covered—In this review, the authors discuss the main new class of kinase inhibitors used as glaucoma treatments, which lower IOP by enhancing drainage and/or lowering production of aqueous humor. The authors include novel inhibitors under preclinical evaluation and investigation for their anti-glaucoma treatment. Additionally, the authors look at treatments that are in clinics now and which may be available in the near future.

Expert opinion—Treatment of glaucoma remains challenging because the exact cause is yet to be delineated. Neuroprotection to the optic nerve head is undisputable. The novel ROCK inhibitors have the capacity to lower IOP and provide optic nerve and RGC protection. In particular, the S-isomer of roscovitine has the capacity to lower IOP and provide neuroprotection. Combinations of selected drugs, which can provide maximal and sustained IOP lowering effects as well as neuroprotection, are paramount to the prevention of glaucoma progression. In the near future, microRNA intervention may be considered as a potential therapeutic target.

1. INTRODUCTION

Glaucoma is a multifactorial ocular disease characterized by progressive degeneration of retinal ganglion cells (neuropathy) and irreversible loss of visual field leading to blindness [1,2]. It is the second leading cause of blindness worldwide that disproportionately affect women and Asians. Approximately 2.7 million individuals in the United States are diagnosed with glaucoma [3,4]. Even now, etiology of glaucoma is poorly understood and appears to be an enigma. However, some of the risk factors contributing for glaucoma have been identified which include age, family history, elevated intraocular pressure (IOP), existing optic nerve damage, reduced corneal hysteresis, myopia, diabetes and

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pseudoexfoliation [5]. In glaucoma, optic nerve degeneration starts at the periphery and advances to center resulting in a scooped out appearance. Aqueous humor is produced by secretion of ciliary body processes which is drained through trabecular meshwork pathway and small portion (10%) by uveoscleral pathway [6–8]. A balance between aqueous humor inflow and outflow determines the IOP levels. Excessive inflow or obstruction in drainage of aqueous humor through iridocorneal angle (juxtacanalicular region or trabecular meshwork/Canal of Schlemm) leads to elevation in IOP which may cause optic nerve damage. The exact relationship between elevated IOP and glaucoma is incompletely understood.

Glaucoma is broadly classified into two main categories, “open-angle” and “closed-angled” depending on the iridocorneal angle. Other types of glaucoma include normal tension, congenital (ocular drainage canals do not develop) and secondary glaucoma. Open angle glaucoma is characterized by clogging of drainage canal with no physical changes in iridocorneal angle. Whereas, closed angle (angle closer) is characterized by narrow angle between iris and cornea through which fluid escapes via trabecular meshwork and causes occlusion of aqueous humor drainage canal. In both types of glaucoma aqueous humor drainage is obstructed resulting in upregulation of IOP. In normal or low tension glaucoma patients have normal IOP but still develop optic nerve damage leading to vision loss. Secondary glaucoma develops as a result of ocular insult or trauma. Early detection of glaucoma may help to lower the risk of visual impairment and related morbidity. Several strategies have been developed to detect glaucoma at early stages initiating treatment [9]. A classical treatment strategy is directed towards lowering ocular hypertension, since increase in IOP is considered as a major risk factor. It initiates the development of primary open angle and normal tension glaucoma [10]. Currently, topical prostaglandins, β-blockers, carbonic anhydrase inhibitors or combinations are prescribed as initial medical therapy for IOP management. Under severe ocular hypertension conditions where treatment with topical medications does not lower IOP, then clinician’s go on to laser surgery and finally perform trabeculectomy if IOP is not adequately regulated. In fact those drugs which lower IOP through one or more mechanisms of action may treat glaucoma. Pilocarpine was the first drug indicated to lower ocular hypertension. With the discovery and advent of new ocular hypotensive inhibitors, the rate of glaucoma drainage surgeries has been drastically reduced. Currently, anterior chamber IOP lowering agents such as prostaglandin analogues, β-Adrenergic antagonists, carbonic anhydrase inhibitors (CAI) which act either by increasing aqueous humor outflow *via* the uveoscleral pathway or reduce the production of aqueous humor are commonly recommended.

In the following sections this review provides readers with an overview of discovery and applications of inhibitors that reduce IOP in the anterior chamber and/or provide neuroprotection to retinal ganglionic cells (RGC).

2. GLAUCOMA INHIBITORS

2.1. Carbonic anhydrase inhibitors

Carbonic anhydrases are metalloenzymes ubiquitously expressed in the body. These enzymes are responsible for bicarbonate secretion in the anterior uvea of the eye [11].

Carbonic anhydrase inhibitors (CAI) inhibit the ciliary-process enzyme (sulphonamide susceptible isozyme CA II) in the non-pigmented epithelial cells and reduce rate of bicarbonate and aqueous humour secretion resulting in IOP reduction [12]. Also, CAIs are known to improve blood flow in retina and optic nerve. This class of drugs includes dorzolamide (Trusopt™, Merck, USA), brinzolamide (Azopt™, Alcon, USA) acetazolamide, and methazolamide. Dorzolamide is the first topical CAI that demonstrated similar magnitude of efficacy to that of timolol alone or combination [13,14]. Topical glaucoma treatment with dorzolamide is equally effective and better tolerated compared to systemic administration [15]. Dorzolamide demonstrated a statistically significant IOP reduction when used as an adjunct therapy with latanoprost [16]. Brinzolamide is a lipophilic drug that was introduced later. A viscous ophthalmic suspension of brinzolamide (1.0%) allows extended contact time with ocular surface. It is more comfortable and patient compliant than dorzolamide (2.0%) [17]. Common side effects associated with topical dorzolamide and brinzolamide include local irritation, stinging, skin rash, redness, pruritus, blurred vision and corneal decompensation [18]. Initially several CAI derivatives were synthesized to improve solubility, ocular tissue permeation and to overcome such adverse effects [19]. These derivatives demonstrated high reactivity with thiol groups of cysteine and glutathione which may lead to severe ocular side effects. Therefore, aromatic substitution reactions with aromatic/heterocyclic sulfonamides have been made and several derivatives were synthesized by conjugating a tail (2, 3, 5, 6-tetrafluorobenzoyl, 2, 3, 5, 6-tetrafluophenylsulfonyl, and pentafluorophenylureido) to CAIs [20]. Among the newly synthesized CAI derivatives, three compounds demonstrated better inhibitory activity against the carbonic anhydrase isoforms (I, II and IV) when compared to commercially available CAIs. *In vivo* IOP lowering effect of these fluorinated compounds demonstrated a potent and prolonged IOP reduction in ocular hypertensive rabbits relative to dorzolamide (2.0%) (Fig 1) [20]. Similarly, several new derivatives were synthesized and examined for their inhibitory activity against CA II isoenzymes. Nitric oxide donating sulfonamides, xanthates, and pyrazole derivatives have been synthesized which show improved antiglaucoma effect *in vivo* by CA II isoenzyme A inhibition (Fig 2). All nitric oxide (NO) donating sulfonamide compounds demonstrated IOP lowering effects in rabbits by inhibiting this enzyme [21]. NO participates in regulating IOP in glaucoma and also exerts anti-apoptotic and anti-inflammatory effects. These NO derivatives may improve blood supply to optic nerve artery by regulating systolic and diastolic velocities [22]. A combination of CAII isoenzyme inhibition and NO-donating property in one compound may be a more effective in glaucoma treatment strategy. With an addition of bromine to phenyl ring, the derivative becomes electro negative and produces excellent inhibitory activity against CA II isoenzyme [21]. The compounds are inhibitors of CAI-II isoenzyme and the range of inhibition is similar to sulfonamides (acetazolamide and dorzolamide). Release of NO in soluble guanylyl cyclase signaling pathway may lead to increase in local cyclic guanosine monophosphate (cGMP) levels. Such elevation may be presumably beneficial for aqueous humor homeostasis. Further, these derivatives may be explored to generate higher CA II isoenzyme inhibitory activity while retaining NO donating property. Xanthates possess an optimal hydrophilic/lipophilic balance which may aid in effective inhibition of CA II isoenzyme, *in vitro* [23]. Several xanthate derivatives were developed. These compounds demonstrated a low IC_{50} for CA II isoenzyme. In another study, novel pyrazole derivatives

of 5-amino-1,3,4-thiadiazole-2-sulfonamide were prepared [24]. These compounds demonstrate potent inhibitors of CAII isoenzyme hydratase and esterase activities. These compounds are highly effective relative to parent compound, acetazolamide. The new derivatives (sulfonamides, xanthates and pyrazole) exhibited high CA II inhibitory activity (Ki) as summarized in Fig 2.

2.2 Acetylcholinesterase inhibitors

Acetylcholinesterase inhibitors with minimal/no ocular or systemic adverse effects have been explored. Organophosphates such as diisopropyl fluorophosphates (DFP, DIFP, diisopropyl phosphorofluoridate) and trichlorton are administered as oily eye drops to induce miosis in the eye and lower IOP. But, the application of these agents is limited due to severe ocular side effects associated with acetylcholinesterase inhibition and possible delayed induction of peripheral neuropathy [25]. Three different molecular forms of acetylcholinestrase have been identified in human ciliary body. Rivastigmine (SDZ ENA 713) is though a non-selective acetylcholinestrase inhibitor which selectively inhibits the globular monomer enzymatic (G1 subtype) form of acetylcholinestrase. Topical drop application to pigmented normotensive rabbit eye demonstrated IOP lowering effect in a dose dependent manner [26]. High dose of topical rivastigmine was well-tolerated with no sign of toxicity. It produced rapid, within 1 h, IOP reduction. The mechanism of action for rivastigmine is not well delineated. Since constriction in pupil is observed which may lead to a hypothesis that rivastigmine may induce ciliary body constriction allowing more aqueous humor outflow.

2.3. Angiotensin Converting Enzyme inhibitors

Danser and Wagner reported the presence of local renin-angiotensin regulation in the eye [27,28]. Renin-angiotensin system is known to regulate systemic blood pressure by controlling electrolyte balance, body fluid volume and vascular remodeling [29,30]. Angiotensin converting enzyme (ACE) inhibitors as therapeutic agents was initially selected for the treatment of hypertension. But later it was used for additional clinical indications such as glaucoma [31]. ACE inhibitors (ramiprilat, enalaprilat, fosinopril and perindopril) recently received attention as a new class of drugs for glaucoma treatment. Ocular hypotensive effect of ramiprilat, enalaprilat and fosinopril by inhibiting ACE (kininase-II) were shown in acute and chronic hypertension rabbits [32]. Perindopril produced similar results [33]. Although, ACE inhibitors produce ocular hypotensive effect, these agents simultaneously inhibit cholinesterase. However, the exact mechanism of IOP lowering by this class of drugs is yet to be delineated.

2.4. Cellular kinase inhibitors

Kinase inhibitors are new class of important downstream regulators of cellular proteins, which play an important role in several cellular events such as cell proliferation, cell migration, cytoskeletal organization and apoptosis. Kinase inhibitors so far investigated for glaucoma, include kinase signal transduction pathway inhibitors of myosin light chain kinase (ML-9), protein kinase (HA1077), integrin linked kinase, LIM-Kinase 2, cell-cycling-dependent kinase, Src-family tyrosine kinase and Rho-kinase. Of these inhibitors

sub families of tyrosine-kinase and Rho-kinase inhibitors are gaining popularity and later are widely studied.

2.4.1. Myosin light chain kinase (MLCK) inhibitor—Phosphorylation of myosin light chain II, in presence of Ca^{+2} and calmodulin, is known to regulate actomyosin contraction. It is believed that contraction of trabecular meshwork prevents aqueous humor drainage and builds up IOP, while TM cell relaxation may produce the opposite [34–36]. Cultured human trabecular cells contain MLCK [37], which is phosphorylated causing serum stimulation. A MLCK specific inhibitor, 1-(5-chloronaphthalenesulfonyl)-1H-hexahydro-1,4-diazepine (ML-9), demonstrated a significant IOP lowering effect in rabbit model. Inhibition of MLCK phosphorylation with ML-9 improved aqueous out-flow by retraction and dissociation. It also caused disruption of actin bundles, impairing focal adhesion formation in trabecular meshwork. However, this inhibitor did not exert appreciable effect on trabecular meshwork cell morphology. *In vivo* studies demonstrated a dose dependent IOP lowering in rabbits. MLCK inhibition resulted in higher aqueous humor outflow thereby lowering IOP.

2.4.2. Tyrosine kinase inhibitor—Src-family tyrosine kinases (SFTKs) interact with a diverse class of cellular receptors. SFTKs inhibit phosphorylation of MLCK induced by fibronectin, laminin and collagen type IV. SFTK inhibitors include PP1, PP2 and damnacanthal. *In vitro* studies demonstrated similar enzyme inhibitory activity for PP1 and PP2. However, *in vivo* studies in normotensive rabbits with intracameral injection revealed a quite opposite effect with SFTK inhibitors. PP2 demonstrated a high IOP lowering efficacy relative to PP1 [38]. A probable reason may be chemical structure and suboptimal physicochemical properties of PP1 which may have affected tissue permeability leading to lower efficacy relative to PP2 (Fig 3). At cellular level, PP2 appears to induce a diminution in transepithelial electrical resistance (TEER) to reduce cell adhesion of trabecular meshwork cells to culture surface. This result indicates that decrease in TEER may stimulate aqueous humor drainage partly by conventional outflow resulting in lower IOP.

Epiderminal growth factor receptor (EGFR) is a transmembrane protein with intrinsic tyrosine kinase activity. EGFR is absent in mature astrocytes. However, optic nerve insult (acute ischemia, chronic glaucoma and optic nerve transection) may result in rapid upregulation and activation of EGFR, triggering quiescent astrocytes to become reactive astrocytes [39]. Specific inhibitor of EGFR tyrosine kinase includes AG1478 and AG82 [40]. *In vivo* studies in rats demonstrated that optic nerve insult triggered the upregulation of EGFR [41]. Elevation of IOP in rats resulted in significant loss of RGCs (20% in peripheral retina and 10% in central retina). Oral administration of AG1478 (in drinking water) did not appear to provide any IOP lowering effect in normal and IOP elevated rat models relative to control group. However, AG1478 significantly blocked the EGFR activation and demonstrated protection with no RGC loss in a rat model of elevated IOP. Blocking of EGFR activation precluded activation of reactive astrocyte phenotype and consequently resulted in RGC protection [41].

Rapamycin is a mammalian target of rapamycin receptor (mTOR) inhibitor. Both *in vitro* and *in vivo* studies indicate that rapamycin inhibits neurotoxic mediator release from

microglia by modulating nuclear factor-kappa B signaling (chronic ocular hypertensive rat model) [42]. Rapamycin significantly suppressed nitric oxide (NO) and TNF-α production in BV2 microglia. The compound inhibited the microglia activation *in vivo* and suppressed the glutamate induced apoptosis of primary RGCs. Sparing phosphorylation of Akt is critical for cell survival. It can promote neuroprotection of mTOR inhibitor in an experimental glaucoma model [42].

2.4.3. Cell cyclin [correction of cycling]-dependent kinase inhibitor—These inhibitors act by modulating cell contraction-relaxation in trabecular meshwork [43]. Roscovitine (racemic mixture) is an inhibitor of cell cyclin-dependent kinase (CDK)-2, CDK-4 and CDK-5, which are upregulated in stress conditions inducing apoptosis [44]. Also, CDKs regulate collagen production and expression in fibroblasts. Roscovitine inhibits CDKs, induces trabecular meshwork relaxation and improves aqueous outflow. *In vivo* studies in rabbits demonstrated that both isomers (R- and S-) significantly reduce IOP upto 4h relative to vehicle. However, S- isomer was superior to R- isomer in lowering IOP and providing protection to retinal ganglionic cells. The exact reason for such anomalous activity of the R- and S-isomers requires in-depth understanding and exploration.

2.4.4. Rho-kinase inhibitors—Rho family consists of RhoA, B and C guanosine triphosphatases (GTPases) binding proteins which are involved in regulating signal transduction pathways and actin cytoskeleton function [45]. In Rho dependent signal transduction pathway, Rho is activated by GTP which further activates its effector molecules Rho kinase ROCK1 and ROCK2 (isoforms of serine/threonine kinases). ROCK1 and ROCK2 conserve 65% overall sequence homology at amino acid levels and the kinase domains are 92% identical [46]. ROCK 1 and 2 are expressed in human trabecular meshwork, ciliary muscle cells and optic nerve head [47] and have distinct roles [48,49]. Moreover, elevated levels of RhoA are expressed in optic nerve head of glaucomatous eye relative to age match controls [47]. Rho binds to ROCK and enhances catalytic activity by phosphorylating MLCK. This protein induces actin fiber contractility and resistance to aqueous humor outflow. Also, ROCK phosphorylates LIM kinases and reduces cell migration. ROCK inhibitors prevent phosphorylation of MLCK, prevent contractility of trabecular meshwork/Schlemm's canal and aid in drainage of aqueous humor. Therefore, ROCK specific inhibitors which can alter actin cytoskeleton and cell motility of trabecular meshwork, canal of Schelmm and ciliary muscle cell indicate potential new category of ocular anti-hypertensives that can enhance aqueous humor drainage. Y-27632 was the first identified ROCK specific inhibitor [50]. The major difficulty is to create a ROCK specific inhibitor because of structurally similar active binding sites in various protein kinases [46,51]. However, many highly selective ROCK inhibitors with kinase selectivity ~ 1% hit ratio have been developed. Y-27632 and H-1152 are non-specific ROCK inhibitors which demonstrated a rapid and prolonged IOP decrease by competitive inhibition of ROCK with adenosine triphosphates [50,52]. Five different mechanistic pathways for Rho-kinase inhibitors in glaucoma treatment have been identified which include (i) increase aqueous humor outflow by relaxing trabecular meshwork, (ii) improve blood flow to optic nerve, (iii) provide neuroprotection of healthy ganglion cells, (iv) treat glaucoma as an antifibrotic

agent in glaucoma surgery and (v) inhibit corneal endothelial cell dysfunction in humans [34,53–62].

ROCK inhibitors such as Y-39983/SNJ-1656/RKI-983 and INS-117548 were developed for IOP reduction, but, these compounds have limited efficacy and low tolerability. Topical application of Y-39983 (0.05%) in normotensive cynomolgus monkeys and rabbits showed disparity in IOP response. These differences may be due to anatomical/physiological, pharmacokinetic, expression levels of ROCK in various ocular tissues. Other commonly observed adverse effects in both species include punctate sub-conjunctival hemorrhage and conjunctival hyperemia [63]. INS-117548 produced mild reduction in IOP by altering actin cytoskeleton. However, higher doses related side effects include ocular hyperemia, hemorrhage and chemosis [64]. Several other Rho kinase inhibitors currently in clinical trials are listed in Table 1 [65–69] and their chemical structures summarized in Fig 4. Current research is directed towards synthesis and identification of ROCK specific inhibitors. These ROCK inhibitors may be divided into several groups depending on chemical group such as (i) isoquinoline derivatives, (ii) urea derivaties, (iii) indazole derivatives (iv) aminopyrimidine derivatives, (v) chroman-3-amine derivatives (vi) benzimidazole derivatives (vii) quinazolinone derivatives, (viii) indoles and (ix) 7-azaindoles derivatives. Chemical structures and inhibitory activities (IC_{50}) of the representative ROCK inhibitors and their analogs are summarized in Fig 5.

Shröter et al. first described the cell based high throughput screening assay for ROCK inhibitors [70] which led to the discovery of pyridine-thiazole based amide compound. This novel compound is a potent inhibitor of ROCK2 with an IC_{50} of 7.2 nM [71]. The compound display high selectivity against other kinases and therefore was selected for further optimization. In 2008, Chen and co-workers identified benzodioxane scaffold as a lead molecule with a IC_{50} of 2 nM for ROCK2 and favorable selectivity (~100 times) against protein kinase A. However, this compound exhibited low oral bioavailability (F <1 %). To improve the physicochemical properties of the molecule, several derivatives were prepared and screened for microsomal stability and oral bioavailability. Some of these derivatives demonstrated improved human microsomal stability, oral bioavailability and better selectivity against protein kinase A. The compounds retained ROCK2 inhibitory activity similar to the lead compound [72–74]. Moreover, newly developed urea based compounds are potent inhibitors of enzymatic activity. Additionally, biological evaluation of the urea derivatives in rats demonstrated significant IOP lowering effect [75]. Similarly, Pireddu group designed and reported a library of pridylaminothiazole based derivatives by incorporating urea into the parent structure [76]. Benzyl pyridylthiazole urea analogs displayed low nanomolar binding affinity *in vitro*. These derivatives were identified as potent ROCK inhibitors. In 2010, Davis and co-workers using high through-put screening discovered benzothiopene scaffold as a novel ROCK inhibitor with IC_{50} of 1.5 μM [77]. Further derivatization at positions 2- and 5- improved potency and solubility. One of these derivatives was compared for *in vivo* IOP lowering activity relative to Y39983. This novel derivative significantly reduced IOP in ocular hypertensive monkeys after one hour dosing and the effect was sustained for six hours in the hypertensive eye [77]. Similarly, in 2010 Henderson et al., identified 2,3-diaminopyrazines as ROCK specific inhibitors [78]. The

structure activity relationship for the two hit compounds led to the discovery of a series of other compounds. Some of these compounds demonstrated less than 500 nM and 100 nM activity for ROCK1 and ROCK2, respectively. Further, these compounds were studied *in vivo* in rabbits and monkeys, where one of the compounds exhibited higher IOP lowering effect [78]. In 2011, Ray et al., identified thrombin/FactorXa building block as a ROCK1 inhibitor by fragment based NMR screening with the aid of small literature focused library [79]. Fragments from ROCK and other kinases were screened and historical thrombin building block was identified. Further, the identified core was subjected to fragment growth and linker modification. Following this protocol several ROCK1 inhibitors were designed. From the library of inhibitors two compounds appeared to generate favorable binding affinity against ROCK1. One of the compounds (23E) demonstrated better potency *in vitro* but had poor pharmacokinetic profile. Further, this group optimized a compound through removal of aminoisoquinoline basic center. The new compound was equipotent against both ROCK1 and ROCK2 and was found to possess improved selectivity for protein kinase A relative to hydroxyl Fasudil. This compound demonstrated a better *in vivo* efficacy in hypertensive rat model of glaucoma [80].

Molecular modeling technology is emerging as a powerful tool in discovering novel chemical entities against various drug targets. There are several advantages of this computational technology in the early stage of drug discovery of ROCK inhibitors. Among the tools, structure based virtual screening is most popular. A large number of compounds from the database was screened *in silico*, and a few selected candidates emerged for biological activity evaluation. As an example, Gong et al., virtually screened database of 12,280 compounds by using pharmacophore models based on the known representative ROCK inhibitor [81]. A total of 3943 hits were obtained and subsequently molecular docking study was employed which finally resulted in 166 hits. The final compounds were selected and ROCK1 inhibitory activity was measured. Compounds with IC_{50} of less than 1 μM were selected as potential ROCK1 inhibitors. Similarly, Shen et al., employed docking based virtual screening from a total of a total of ~1.1 million structures to identify small molecule inhibitors for ROCK1 from Specs and ChemBridge™ database [82]. All the structures in the database were subjected to docking and scoring repeatedly using Glide SP mode to select the best possible inhibitor structures. Chemical similarity clusters from the 2000 compounds were performed for maximizing chemical diversity for biological assay. From this group a small set of virtual hits (174 compounds) which were subjected to series of assays. Out of all the compounds, 12 compounds demonstrated IC50 values in the range of 7 to 28 μM. In another study, Shen et al. discovered the triazine derivatives as ROCK1 inhibitors and optimized with an integrated computational protocol which includes molecular docking, molecular dynamics, simulation and free energy calculations [83]. The results of these studies revealed crucial and favorable interaction patterns. Several compounds were identified as ROCK1, triazine- or pyrimidine based inhibitors. The interaction study suggested that (i) the cation-Π interactions between scaffold of naphthalene ring and Lys^{105}, (ii) the hydrogen bonding interactions between, pyridine like scaffold and Met156 and piperazin like group and Asp160. Based on predictions from molecular modeling, several derivatives were synthesized and four compounds demonstrated higher potency.

2.4.5. Protein Kinase C inhibitor—Protein kinase inhibitor interferes and inhibits with the downstream effects of actomyosin i.e., contraction. HA1077 (1-(5-Isoquinolinesulfonyl)-homopiperazine inhibits actomyosin and may induce smooth muscle and vascular relaxation [84]. *In vivo* studies in rabbits with topical, intracameral and intravitreal injection of HA1077 demonstrated a significant lowering in IOP with an increase in aqueous humor outflow. Anti-hypertensive effect of HA1077 may be related to alterations in the trabecular facility, changes in the permeability of the chamber angle venous plexus and the iris vasculature. HA1077 acted by similar mechanism to serine/threonine kinase inhibitor -H-7, by disrupting the F-actin bundles. The compound impairs focal adhesion of trabecular meshwork cells causing cell junction disruption and IOP lowering.

2.4.6. Integrin linked kinase inhibitor—Integrin linked kinase (ILK) inhibitor also plays a role in regulating cellular signaling pathways such as integrin activation, fibronectin matrix assembly, viability, differentiation and cell motility [85]. Example of ILK inhibitor includes KP392 and QLT0267 which interrupt ILK signaling. ILK inhibitor regulates actin cytoskeletal organization in cultured human trabecular meshwork cells and diminishes fiber contractility and facilitate aqueous humor outflow through trabecular meshwork, thereby lowering IOP.

2.4.7. LIM-Kinase 2 inhibitor—LIM kinases (LIMK-1 and LIMK-2) are downstream ROCK signaling pathways which regulate polymerization of actin filaments. A series of inhibitors were synthesized to evaluate efficacy in lowering ocular hypertension. LIMK-2 inhibition reduces ocular hypertension by enhancing aqueous humor drainage and associated glaucoma [86]. *Ex vivo* studies with porcine eye demonstrated a 30% increase in aqueous humor outflow. *In vivo* studies in mice indicated a dose dependent IOP lowering. However, the drug 22j (see Fig 6 for structure) at the highest evaluated concentrations did not elicit the same level of response as the β-blocker (timolol). But, the duration was comparable to timolol.

2.4.8. Dual leucine zipper kinase inhibitor—Dual leucine zipper kinase (DLK) *aka* mitogen-activated protein kinase kinase kinase 12 (MAP3K12) belongs to serine/threonine protein kinase family. DLK contains a leucine zipper domain. DLK along with c-*Jun*-N-terminal kinase (JNK) play an important role in RGC apoptosis and degeneration signaling cascade. A high-throughput RNA interference screening with primary RGCs identified DLK as a neuroprotective target [87]. Posttranscriptional upregulation of DLK was observed under the conditions of axonal injury. DLK upregulation activates downstream JNK signaling cascade resulting in RGC apoptosis. Kinase inhibitors that selectively bind to DLK include tozasertib, crizotinib, foretinib, KW-2449, axitinib, and lestaurtinib. *In vitro* studies demonstrated that tozasertib significantly inhibited DLK signaling and demonstrated neuroprotection to RGC. *In vivo* studies in rats with tozasertib microspheres demonstrated a significant activation of RGCs relative to vehicle treated group. Results indicate that DLK is the major pathway for mediating JNK signaling under the conditions of retinal insult. Ihibition of DLK promotes RGC protection. Recently, the parent structure of DLK inhibitor was reported (Fig 7a) [88]. A series of DLK inhibitors were synthesized and their activity was screened for neuroprotection. Out of these analogs, compound 26 (Fig 7b) demonstrated

significant potency, *in vitro* metabolic stability, and better selectivity over other JNK pathway kinases and homologues of DLK. Also, compound 26 demonstrated better potency in promoting RGC cell survival in mouse optic nerve crush model of axonal injury. *In vivo* studies revealed that loss of DLK expression resulted in attenuation of down-stream signaling cascade. Moreover, oral administration of compound 26 significantly protected RGC in a dose dependent manner by reducing p-c-*Jun* expression in retina [89].

2.4.9. JNK inhibitors—c-Jun N-terminal kinase (JNK) belong to mitogen activated protein kinase family which is involved in signal transduction pathways leading to apoptosis, inflammation and carcinogenesis. Phosphorylation of JNK causing activation of signaling cascade may be responsible for RGC death in open angle glaucoma [90]. Examples of JNK inhibitors include D-JNKI-1, L-JNKI-1 and SP600125. IOP elevation (45 mmHg for >6 h) was responsible for activation of p-JNK pathway and retinal insult [91]. However, the JNK pathway activation was blocked by SP600125. *In vivo* studies in rats with elevated IOP produced irreversible damage to optic nerve axon and RGCs. Intraperitonial administration of SP600125 to rats demonstrated significant protection. The compound preserved the RGC density relative to vehicle treated group by inhibiting JNK pathway. These results indicate that JNK activation is another key signaling factor for RGC loss. Inhibition of JNK activation with specific inhibitors may also delay the RGC loss. But, SP600125 is a non-specific inhibitor because of its binding to several protein kinases including JNK [92]. In another study it was observed that JNK pathway was involved in N-methyl-D-aspartate (NMDA) mediated retinal excitotoxicity [93]. D-JNKI-1 minimized the retinal NMDA induced JNK activation. *In vivo* studies demonstrated that a selective dose (5 nmol) of D-JNKI-1 may provide RGC protection. High dose (10 nmol) may induce unwanted and dose-dependent phosphorylation of JNK and c-*Jun*. It is believed that D-JNKI-1strongly inhibits calpain activity and provides RGC protection. D-JNKI-1 is protease-resistant (relative to L-JNKI-1) and highly specific. It binds to JNKs as well as MKK4 and MKK7, because these proteins carry JNK binding domains. D-JNKI-1 may provide a strong and long term RGC survival against excitotoxicity and glaucoma.

2.5. Ion channel blockers/inhibitors

Calcium channel blocking may be considered as an alternative treatment option for glaucoma. Calcium channel blockers can improve ocular blood perfusion, neuroprotection and may cause IOP lowering. Examples of calcium channel blockers (CCB) include diltiazem, nifedipine, verapamil, flunarizine, iganidipine, nimodipine, nilvadipine and lomerizine (Fig 8). Interstingly, betaxolol (β-adrenoceptor antagonist) has been known to attenuate the N-methyl-D-aspartate (NMDA) induced Ca^{+2} influx by calcium channel blocking. It also interacts with NMDA receptors [94]. The result is reduction of Ca^{+2} influx and IOP lowering. Recently, it has been shown that flunarizine reduced IOP in a dose dependent manner in glaucomatous monkey eye by improving conventional outflow facility *via* trabecular meshwork [95]. Human trabecular meshwork expresses voltage-activated L-type calcium channels and flunarizine modulates trabecular meshwork contractility [96]. However, the exact mechanism for IOP lowering by CCB is not known. Administration of oral CCB may not provide sufficient concentrations in the ocular compartments to produce required hypotensive effect. Systemic administration of CCB produced much smaller IOP

reduction in rabbits [97]. Most of the studies have reported the effect of CCB inhibitors with topical administration rather than oral and other systemic administrations. Topical CCB administration resulted in significant IOP lowering and neuroprotective effects in animal models (rabbits, monkeys) and humans [95,98–109]. These results are summarized in Table 2.

3. CONCLUSIONS

Treatments aimed at lowering IOP are important for slowing down the progression of glaucoma and associated vision loss. Recent focus in glaucoma research includes optimization of novel Rho/ROCK kinase inhibitors that increase aqueous humor outflow through trabecular meshwork and provide neuroprotection to optic nerve head with minimal or no adverse effects. Several inhibitors with different molecular targets have been developed to treat glaucoma. These compounds demonstrate improvement in aqueous humor and blood flow to posterior ocular tissues and provide protection to healthy ganglionic retinal cells under ocular hypertensive conditions. Most of the research is currently focused on the development of molecules that interferes with cell signaling pathway resulting in disruption of actin filaments. These compounds appear to dilate the contracted trabecular meshwork, improve drainage and blood circulation to RGC. Till now, the exact etiology of glaucoma has not been completely delineated, which limits the treatment options. However, ROCK specific inhibitors and blocking JNK signaling cascade are promising candidates for lowering IOP and neuroprotection to RGC. Other down-stream inhibitors are also being explored for ocular anti-hypertensive efficacy which includes Src-family tyrosine kinase inhibitors and cell cyclin-dependent kinase inhibitor. These compounds may be further explored for IOP lowering activity with minimal or no local ocular toxicity. Results from these studies suggest that these compounds may require further improvements. Promising drug candidates discussed in this review are efficacious, provide benefit to patients, and have specific mechanism of action. Although, this review is focused on the novel inhibitors for the treatment of glaucoma, combination of drugs that block ocular hypertensive effects with different mechanisms of action on trabecular meshwork/Schlemm’s canal or juxtacanalicular region may be potent agents for reducing IOP thereby preventing the onset of glaucoma.

4. EXPERT OPINION

Glaucoma is a multifactorial disease and its treatment is challenging. Significant research in this area lead to identification of crystal structures for ROCK1 and ROCK2 with key differences in their kinase domains. The knowledge gained about ROCK kinases may assist in optimizing a key molecule with molecular modeling techniques. Such a compound may preferentially bind to ROCK. Current research is focused on inhibiting Rho/ROCK kinase. However, the key cell surface triggering receptors such as EGFR, heterotrimeric G protein coupled receptor, tyrosine kinase receptors, cytokine receptors, frizzled and adhesion receptors which normally trigger the cascade of signaling events in glaucoma must be pursued. The newly identified molecule with the help of molecular modeling techniques may generate superior inhibitory activity towards ROCK but may suffer from suboptimal physicochemical properties such as solubility and stability. Also, the molecule may be able

to possess high cell penetrating ability to translocate cell membrane. Therefore, research should be focused towards identifying a suitable candidate with high stability, better solubility, demonstrating lower toxicity and inhibit the cell surface key receptor. Such a new molecule may simultaneously inhibit key cell surface glaucoma signal triggering receptors with equipotency as observed with compound 23E for ROCK1 and ROCK2. Such a compound can make a significant impact on glaucoma by lowering IOP and simultaneously providing neuroprotection to retinal cells. The research is multi-facet and may involve diverse group of scientists to develop a final product. After identification of lead molecule, the next challenge will be therapeutic concentrations of this drug in anterior and posterior ocular tissues. Research is on-going at a rapid pace to deliver drugs to back of the eye tissues with topical drop administration (nanomicellar formulations) [110–112]. This technology may be helpful in achieving therapeutic levels in retina. Another strategy to treat glaucoma is with micro RNA technology. The micro RNAs are regulators of gene expression and play a major role in both normal and diseased states. The micro RNAs from the “optic nerve head,” a region often affected at the onset of glaucoma development may be considered for intervention. The changes observed may be correlated with the start and progression of the disease. The defective microRNAs intervention may be employed as new drug targets to prevent and treat glaucoma.

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ARTICLE HIGHLIGHTS

- Glaucoma is a multifactorial ocular disease characterized by progressive degeneration of retinal ganglion cells (neuropathy) and irreversible loss of visual field leading to blindness
- Glaucoma is broadly classified into two main categories, “open-angle” and “closed-angled” depending on the iridocorneal angle.
- Treatments aimed at lowering IOP are important for slowing down the progression of glaucoma and associated vision loss
- There a number of drugs under development for treating glaucoma including: carbonic anhydrase inhibitors, acetylcholinesterase inhibitors, angiotensin Converting Enzyme inhibitors, cellular kinase inhibitors and ion channel blockers/inhibitors
- Recent focus in glaucoma research includes the optimization of novel Rho/ROCK kinase inhibitors that increase aqueous humor outflow.
- Defective microRNAs intervention may be employed as new drug targets to prevent and treat glaucoma.

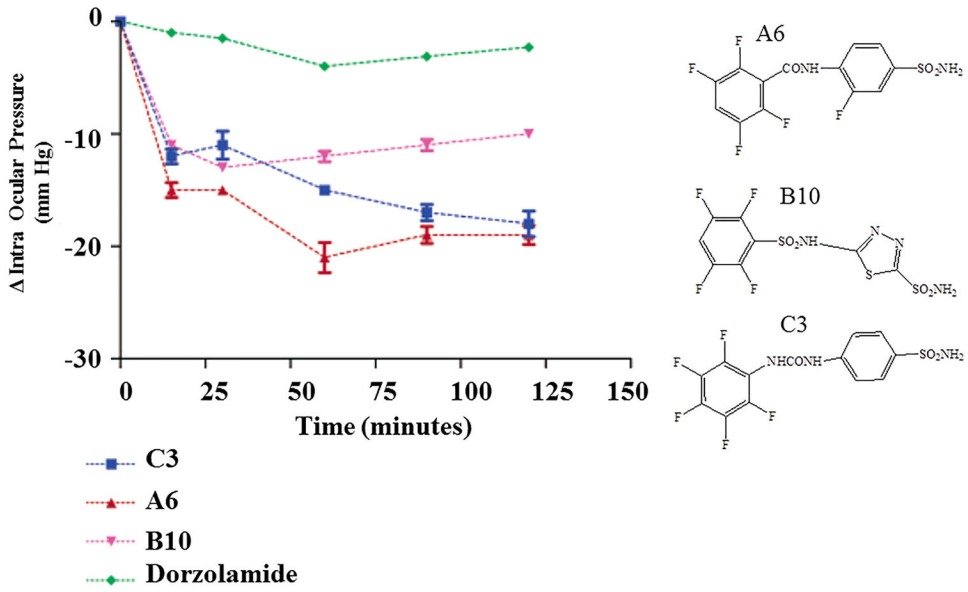

Fig 1.

IOP lowering in hypertensive rabbits (initial pressure in the range of 34 ± 1mm Hg) after topical treatment with one drop (50 μL) of 2% solution of the CAIs dorzolamide, A6, B10 and C3 (mean ± standard error, from three different determinations). Structures for compounds A6, B10 and C3 are provided on the right side. Reproduced from [20] with permission of the American Chemical Society.

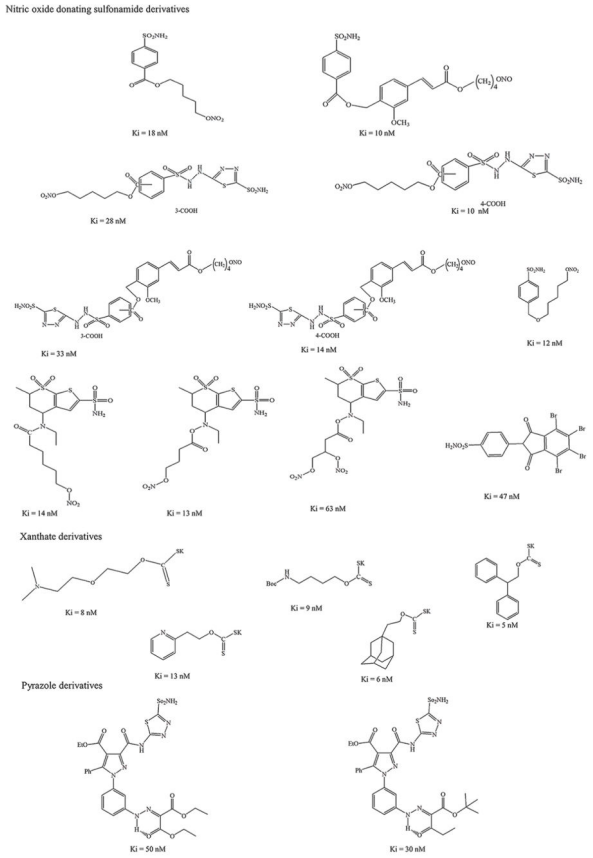

Fig 2.

Chemical structures for nitric oxide donating sulfonamide, sulfonamide, xanthates and pyrazole derivatives with their carbonic anhydrase II inhibitory efficacy (Ki).

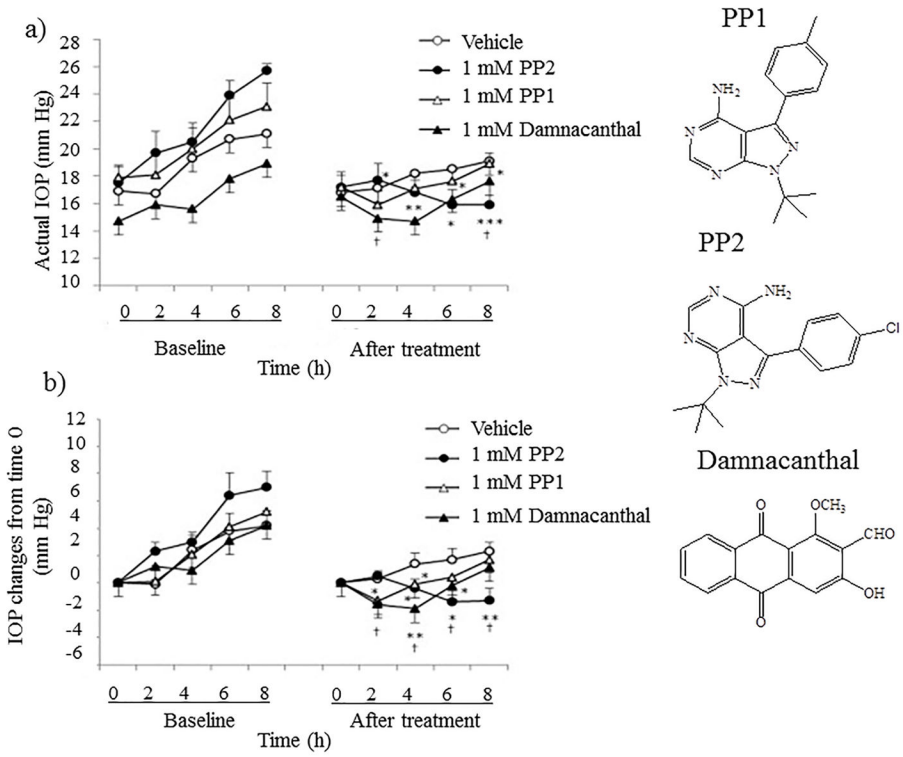

Fig 3.

Changes in intraocular pressure (IOP) after administraton of SFK inhibitors. PP2, PP1 or damnacanthal at 1 mM, or vehicle, was intracamerally injected into one eye in ocular normotensive rabbits on the treatment day. IOP change after drug administration was compared to each scheduled time point of baseline. Baseline was measured 2 days before the treatment day without drug administration. Data represent mean ± SE for 5 animals. *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$ relative to baseline (paired t-test). '$p < 0.05$ relative to the vehicle-treated group (Student's t-test). Reproduced from [38] with permission of Elsevier Limited.

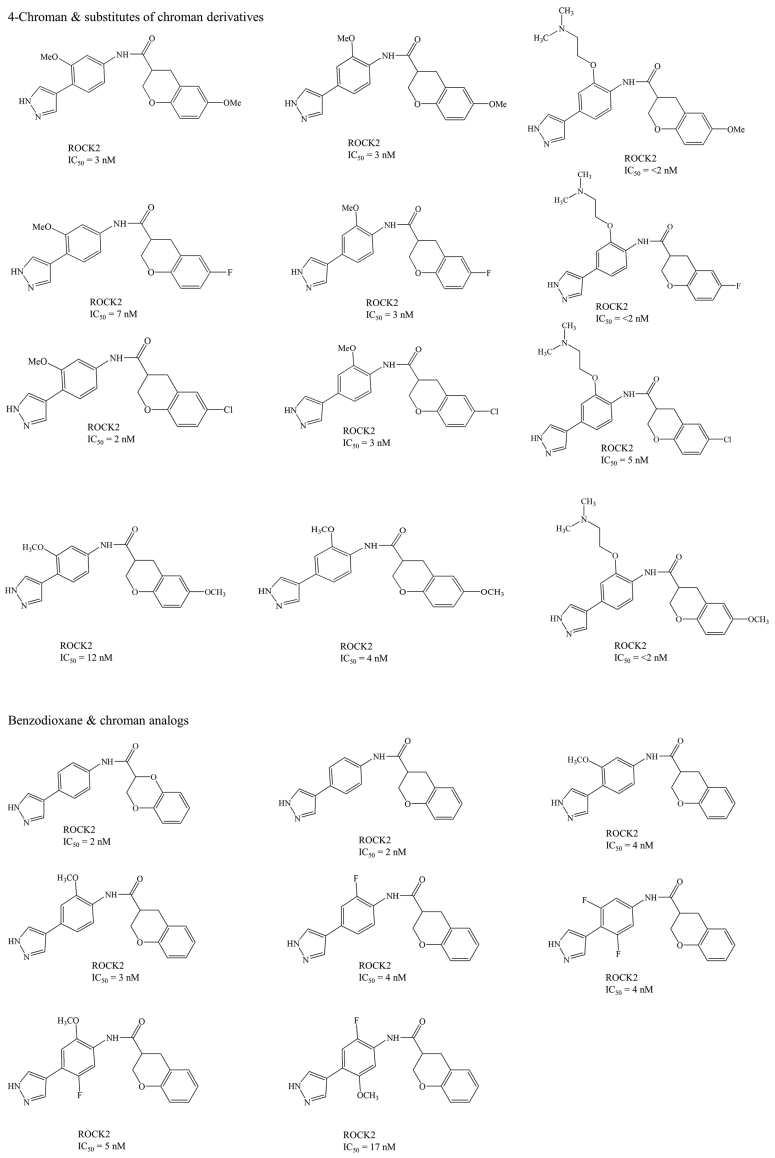

Fig 4.

Chemical structures for Rho-kinase inhibitors in clinical trials and under investigation

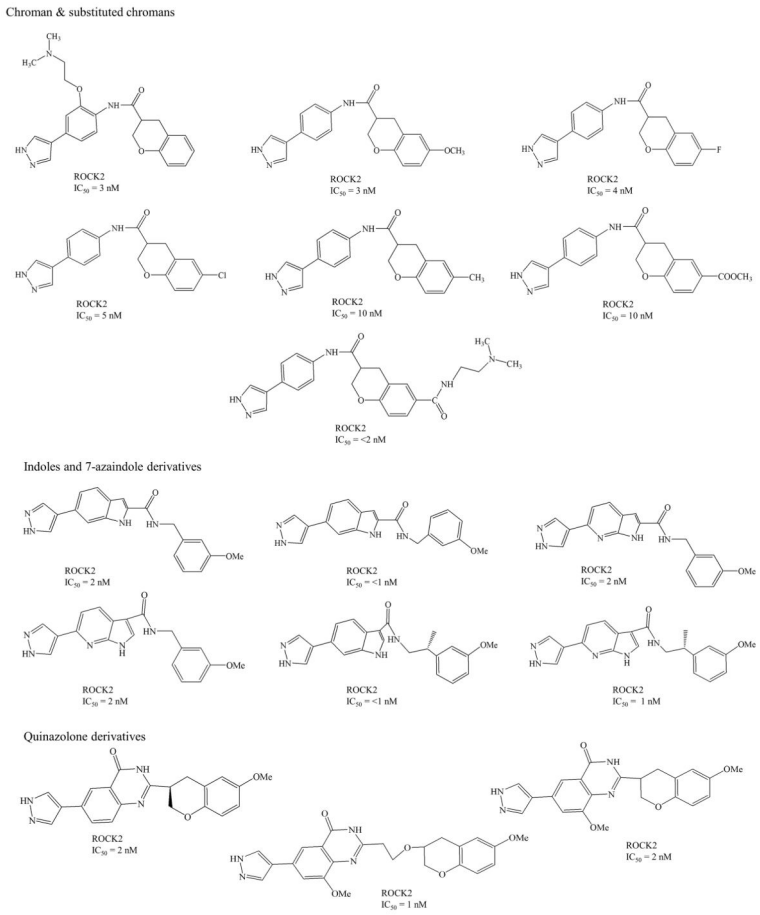

Fig 5.

Chemical Structures for ROCK inhibitor derivatives and their IC_{50}

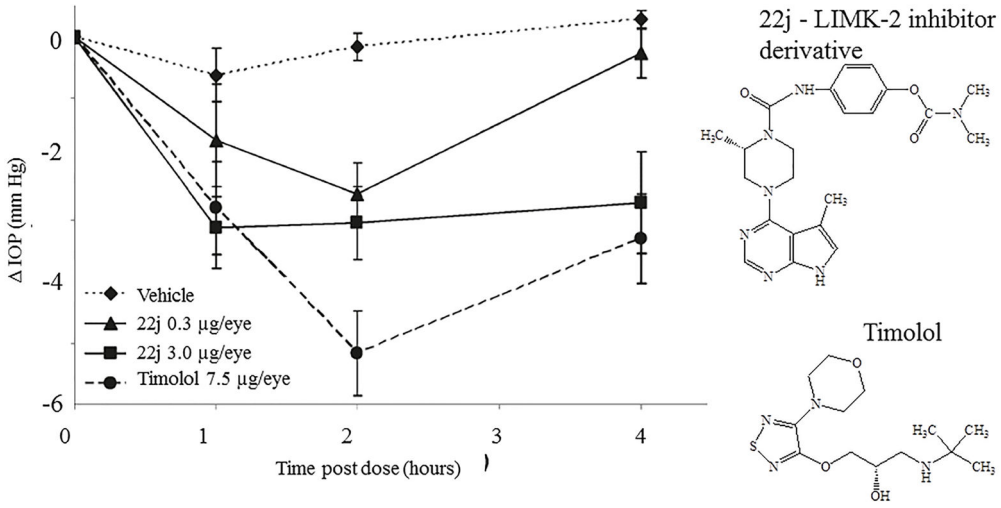

Fig 6.

Change in intraocular pressure (IOP) in a dexamethasone induced ocular hypertensive mouse following topical instillation of 3 µL of a 1 mg/mL or 0.1 mg/mL HPMC based solutions of 22j, or of a 2.5 mg/mL solution of timolol (vehicle, n = 10; 0.3 µg of 22j, n = 10; 3 µg of 22j, n = 9; timolol n= 10). Xanthum gum was used as vehicle. Structures of compounds are presented on the right side. Reproduced from [86] with permission of the American Chemical Society.

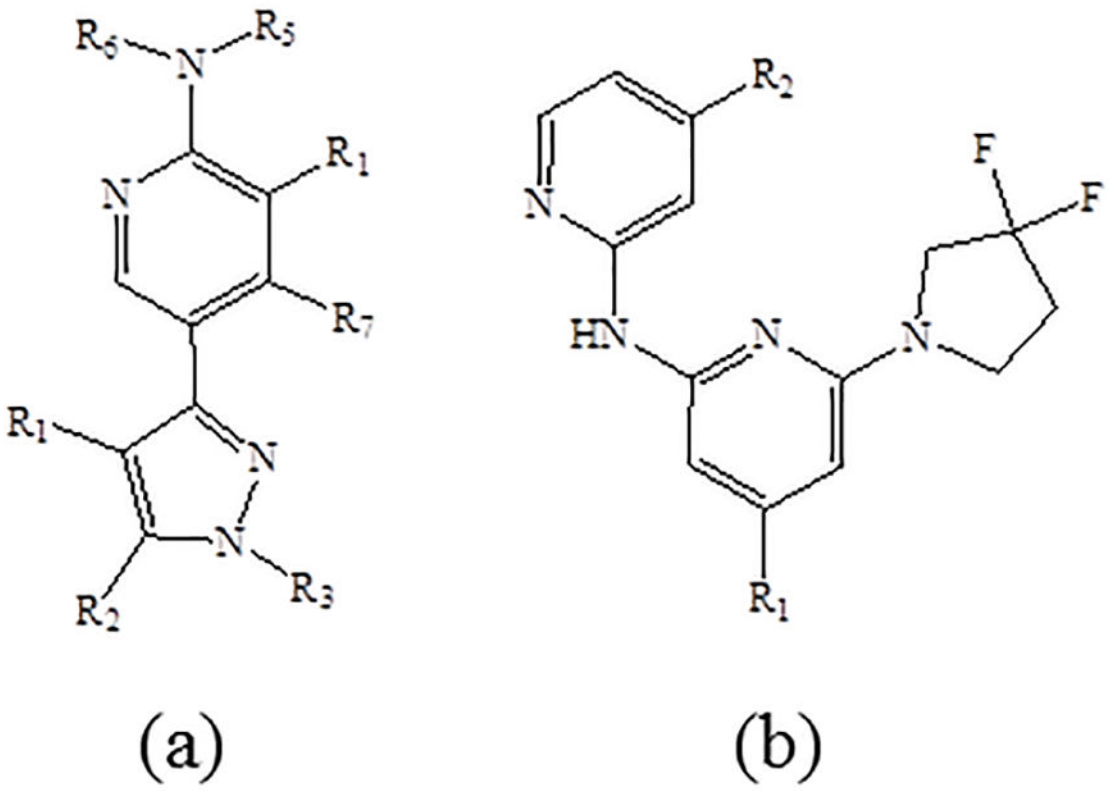

Fig 7.

Chemical structures (a) master scaffold for Dual Leucine Zipper Kinase Inhibitor and (b) compound 26

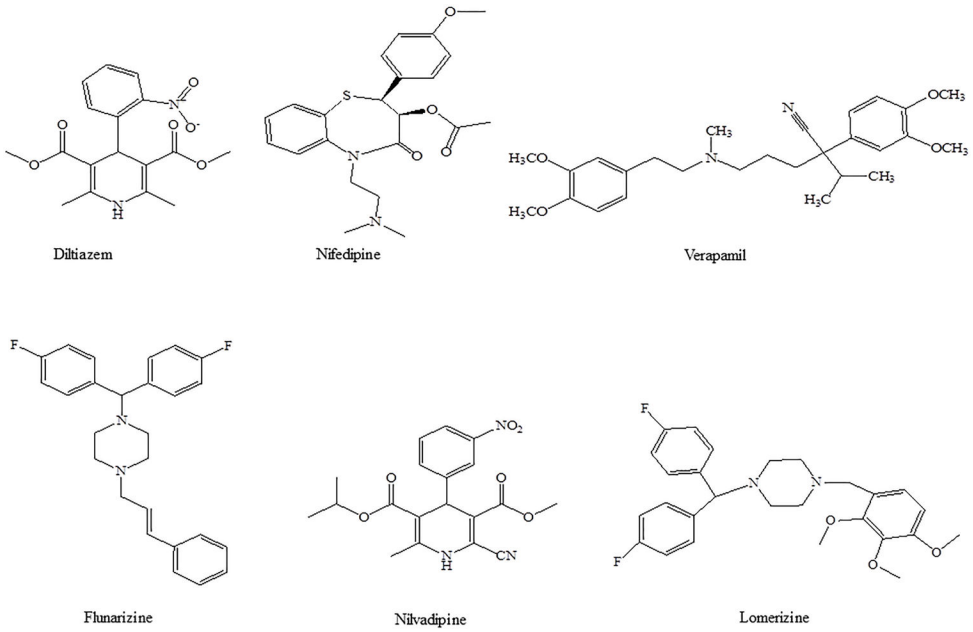

Fig 8.

Chemical structures for calcium ion channel blockers/inhibitors used in the treatment of glaucoma

Table 1

Rho kinase inhibitors in clinical trials and under investigation

Rho-kinase inhibitors	Company	Remarks	Clinical trials stage	References
AMA0076	Amakem, Belgium	Locally acting soft ROCK inhibitor that decreases actin stress fibers, focal adhesion and induce inter trabecular pores size, leading to lower resistance to aqueous outflow. Reduced IOP in normotensive and acute hypertensive rabbit model with significantly low hyperemia relative to Y-39983.	II	[66, 67] http://clinicaltrials.gov/show/NCT02136940
AR-13324	Aerie Pharmaceuticals Inc.	A dual acting ROCK/NET inhibitor that increases the aqueous outflow and lowers inflow.	III	[67] http://clinicaltrials.gov/show/NCT02207621
K-115	Kowa, Japan	An isoquinolinesulfonamide, enhance the fluid outflow *via* trabecular meshwork. Alters actin cytoskeleton and cellular motility of outflow pathways.	III	[67, 69]
PG324	Aerie Pharmaceuticals Inc.	A combination of AR-13324 ande latanoprost demonstrating three mechanisms actions; increase outflow fluid through trabecular meshwork, uveoscleral pathway and reduce fluid production	II	[67] http://clinicaltrials.gov/show/NCT02057575
Y-39983 RKI-983	Senju, Japan; and Novartis pharmaceuticals	Outflow measurements suggest that Y-39983 may affect contractility of TM and SC cells. This results in increased conventional outflow. TM exhibits higher levels of mRNA for ROCK and ROCK substrates in human and monkey eyes, suggesting TM as one of the major sites to regulate of IOP.	II	[67, 68] http://clinicaltrials.gov/ct2/show/NCT00515424?term=RKI-983&rank=2
H-1152		Increase the conventional outflow by increasing the intercellular spaces and alters actin cytoskeleton and cellular adhesions.	Not available	[65]

Table 2

Calcium channel blockers in glaucoma treatment

Drug	Model	Effectiveness (IOP lowering)	Neuroprotection	Remark	References
Diltiazem	Rabbit, mice and Cynomolgus monkey	significant	significant	Significantly lowered IOP in a dose dependent manner by reducing aqueous humor inflow and improving outflow after topical drop administration. Intraperitoneal injection of diltiazem demonstrated neuroprotective effect and preserved visual function in retinal degeneration mice model.	[98 – 101]
Nifedipine	Rat and monkey	significant	significant	Demonstrated protection to retina from ischemic damage in rats. Topical administration was shown to significantly lower IOP.	[98, 102]
Verapamil	Human	significant	N.A	Topical drop administration provided strong ocular hypotensive effect (~12% from baseline) after two weeks of treatment.	[103 – 104]
Flunarizine	Cynomolgus and normal monkey	significant	N.A	Ocular hypotensive effect was pronounced with topical drops. In normal monkeys, the magnitude and duration of ocular hypotensive effect appeared to be more significant with twice daily administration. A dose dependent effect in IOP reduction was observed.	[95, 100]
Nilvadipine	Rat and mice	N.A	significant	Higher permeability into ocular tissues and drug levels produced elevated levels in retina relative to serum after intramuscular injection. Photoreceptor protection was observed in retinal degeneration mouse and Royal College of Surgeons (RCS) models with intraperitoneal injection.	[105 – 107]
Lomerizine	Rat	N.A	significant	Abolish retinal neurotoxicity by removing Ca^{+2} in a dose dependent manner. Oral lomerizine administration prevented central and dorsal retina death at second week in adult Piebald-Virol-Glaxo (PVG) rats.	[108 – 109]

N.A – Not available