Causative glaucoma treatment: promising targets and delivery systems

Raphael Mietzner and Miriam Breunig

Department of Pharmaceutical Technology, University Regensburg, Universitaetsstrasse 31, 93040 Regensburg, Germany

Glaucoma is one of the most common causes of blindness worldwide. Elevated intraocular pressure (IOP) is the major modifiable risk factor of the disease. Conventional therapy suffers from poor compliance, low bioavailability, and the lack of causative treatment options. To improve therapeutic success, it is crucial to identify major mediators of pathological changes associated with elevated IOP and to intervene at the molecular level. Here, we discuss relevant key functions of transforming growth factor-β2 (TGF-β2), connective tissue growth factor (CTGF), integrins, Rho-associated kinase (ROCK), and nitric oxide (NO) with regard to the onset of glaucoma, highlighting new drug delivery approaches for causative treatment.

Introduction

Glaucoma is a chronic, progressive neuropathy of the optic nerve and one of the leading causes of blindness worldwide [1]. In 2013, the number of people affected by glaucoma was estimated to be 64.3 million and this number is predicted to increase to 112 million in 2040 [2]. The disease often proceeds painlessly and is characterized by an increasing loss of peripheral vision until no vision remains. Glaucoma can be divided into open-angle and angle-closure glaucoma. In angle-closure glaucoma, the iridocorneal angle is closed, which prevents aqueous humor from draining out of the anterior chamber of the eye. By contrast, in open-angle glaucoma, the outflow pathway is accessible, but pathological changes in the outflow tissue lead to an increased outflow resistance. This form of glaucoma can occur with an elevated or normal IOP. Secondary forms of glaucoma occur as a consequence of another underlying disease. Primary open-angle glaucoma is the most prevalent form of glaucoma and the focus of this review [3]. Increased IOP is the main modifiable risk factor in the development of the disease [4]. Therefore, standard treatment options are eye drop medications aiming to reduce the IOP and include carbonic anhydrase inhibitors (e.g., dorzolamide), β-blockers (e.g., timolol), sympathomimetic drugs (e.g., brimonidine), and prostaglandin analogs (e.g., latanoprost). They act either by reducing aqueous humor formation and/or by improving its outflow via the unconventional pathway [4,5]. Given their short residence time on the ocular surface, poor corneal permeability, and rapid drainage with the tear fluid, the bioavailability of these treatments is greatly reduced inside the eye to ~1–7% [6,7]. In addition, application frequency up to three times a day reduces their therapeutic success [7,8], and ~50% of patients do not adhere to their medication over 75% of the time [8,9]. Moreover, ~25–50% of patients only respond suboptimally to most commonly used prostaglandin analogs [4]. Another fundamental problem is that most available drugs do not act on the pathological changes in the conventional and/or trabecular outflow pathway that are responsible for the increased IOP in glaucoma. These facts clearly demonstrate the need for new and causative treatment options to improve the therapeutic outcome.

IOP is generated in the anterior chamber of the eye and is well balanced by the production of aqueous humor in the ciliary body and its drainage through the trabecular outflow system (Fig. 1a) [2]. The trabecular outflow pathway anatomically comprises the trabecular meshwork (TM), Schlemm's canal (SC), and the collector channels and/or aqueous veins (Fig. 1b) [10]. The TM is a filter-like tissue and includes, in flow-wise direction of aqueous humor, the uveal meshwork, the corneoscleral meshwork, and, in deeper regions, the juxtacanalicular connective tissue (JCT). The latter, together with the inner wall endothelium of SC and its basement

Corresponding author: Breunig, M. (miriam.breunig@chemie.uni-regensburg.de)

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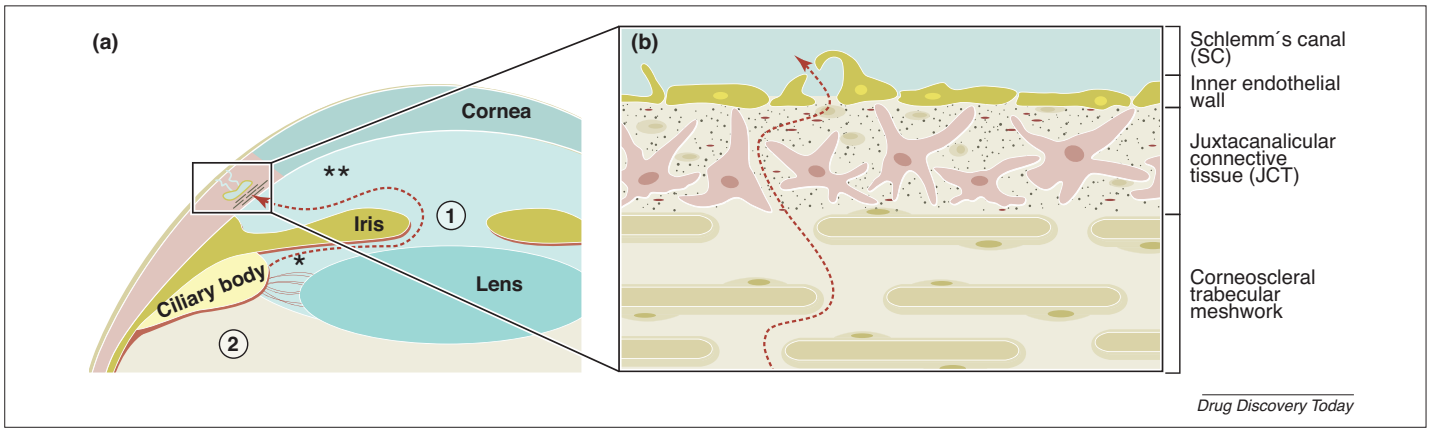

FIGURE 1

Generation of the IOP in the anterior chamber of the eye. (a) Schematic of the aqueous humor dynamics (indicated by the dashed, red arrow). Aqueous humor is secreted by the ciliary body and flows from the posterior chamber (*) to the anterior chamber (**). The major part of aqueous humor is drained via the trabecular outflow pathway. The anterior chamber and posterior chamber are filled with aqueous humor (1) and the vitreous body is filled with vitreous humor (2). (b) Magnified schematic of the trabecular outflow pathway, which comprises the uveal (not shown) and corneoscleral trabecular meshwork, the juxtacanalicular connective tissue (JCT), and the inner wall endothelium of Schlemm's canal (SC), and the lumen of SC.

membrane, are likely to be the key locations for the generation of IOP and responsible for the increase of IOP in glaucoma [10]. Pathological changes accounting for elevated IOP are increased amounts of extracellular matrix (ECM) in the JCT and/or a reduced pore density in the inner wall endothelium of SC [3]. This clinical evidence is explained by significant stiffening of the TM tissue in patients with glaucoma [3]. In turn, a stiffer microenvironment severely affects the mechanobiology of TM and SC cells. Consequently, their cytoskeletons are subjected to massive reorganization, which ultimately leads to significantly enhanced cell contractility [11].

At the molecular level, a complex interplay of various cues and signaling pathways is held responsible for pathological changes during glaucoma development and progression. Here, we identify and highlight the major mediators of pathological changes and describe how these novel targets can be exploited with appropriate drugs and drug delivery systems for causative glaucoma therapy.

Therapeutic targets

Mechanobiological changes in the TM during glaucoma development and progression account for elevated IOP and can be attributed to alterations in the homeostatic balance of certain growth factors, signaling molecules, receptors, and enzymes. Figure 2 provides a simplified overview of the complex interplay at the molecular level and illustrates the corresponding targets that we identified from the literature as potential novel treatment options for causative glaucoma therapy. Here, we discuss the relevant physiological and pathophysiological key functions of major mediators of glaucoma in the anterior chamber of the eye.

TGF-β2 initiates the signaling cascade responsible for the pathological changes associated with glaucoma. TGF-β2 is a multifunctional regulatory protein that is most likely secreted by epithelial cells of the lens, iris, and ciliary body [12,13]. It regulates several processes pivotal for maintaining the physiological balance in the anterior chamber of the eye, including proliferation, apoptosis, phagocytosis, suppression of angiogenesis, and wound healing [14,15]. Most significantly, it modulates the immune privilege of the eye to preserve the transparency of the cornea and guarantee undisturbed vision. [14]. TGF-β2 levels in the aqueous humor of patients with glaucoma were ~1.8-fold higher than those of control cataract groups [16]. The underlying reason is most likely a genetic predisposition [4]. As a consequence, the homeostatic balance in the anterior chamber is disturbed. Fibrotic ECM proteins, such as fibronectin and collagen types III, IV, and VI, are produced at higher amounts, which is a key event in the change of mechanical properties of the tissue [17,18]. TGF-β2 also mediates the irreversible cross-linking of fibronectin by enhancing the expression of tissue transglutaminase, thereby amplifying the stiffness of the TM tissue [13,15,17]. Given that TGF-β2 signaling acts directly on the actin cytoskeleton and also modulates the expression of α-smooth muscle actin (α-SMA) in JCT cells, pathological TGF-β2 levels contribute to the increased cell contractility [13,15,17]. A concomitant effect of elevated TGF-β2 levels is an enhanced expression of connective tissue growth factor (CTGF) and the integrin receptor subunit α_v which also both influence the pathophysiology by themselves [17]. Finally, TGF-β2 is able to increase its own secretion, which decreases the pathological effects [18]. These examples illustrate TGF-β2 as a key player in the structural changes observed in glaucoma.

The profibrotic effects of TGF-β2 are primarily mediated by CTGF, which is a downstream molecule in the TGF-β2 signaling cascade. CTGF is a matricellular protein that is strongly expressed by cells of the TM, ciliary muscle, retina, and others tissues in the eye [17]. Analysis of CTGF in the aqueous humor of patients with glaucoma revealed ~1.2-fold higher levels compared with control individuals; in patients with pseudoexfoliation glaucoma, levels were twofold higher [19]. As discussed for TGF-β2, CTGF also increases the expression of fibrotic ECM and has a similar autoinductive effect to amplify its own expression [18]. In transgenic glaucoma mice with lens-specific overexpression of CTGF, IOP was ~1.3-fold higher compared with control mice [20]. In the same study, significantly increased fibronectin and α-SMA expression were observed; both proteins are involved in the enhanced tissue and cell stiffness. Finally, elevated TGF-β2/CTGF signaling is likely

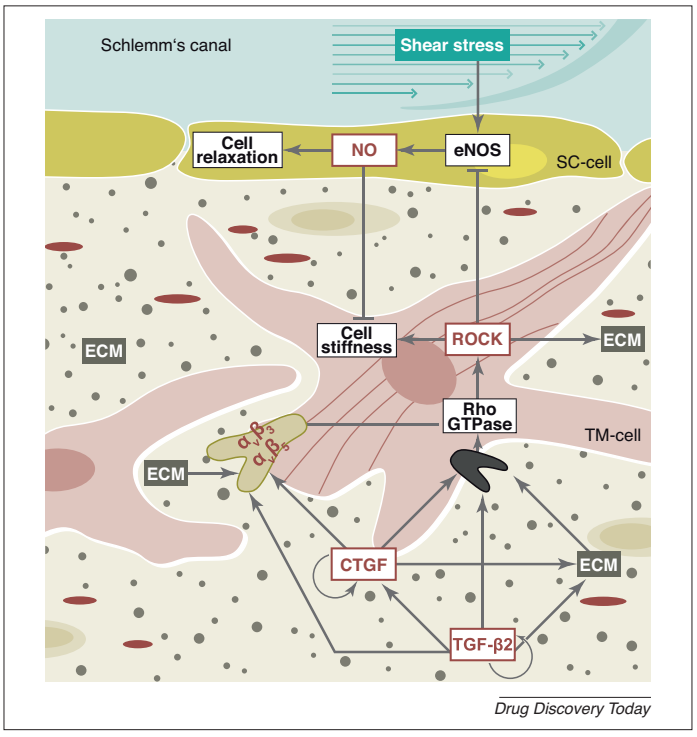

FIGURE 2

Schematic magnification of trabecular meshwork (TM) and Schlemm's canal (SC) cells, which are located in the conventional outflow pathway. Several key factors modulate the homoeostatic balance of the TM to maintain intraocular pressure (IOP). Imbalance of transforming growth factor-β2 (TGF-β2), connective tissue growth factor (CTGF), integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$), Rho-associated kinase (ROCK), and nitric oxide (NO) causes pathological changes in glaucoma, such as increased extracellular matrix (ECM) deposition and cell stiffness. The black receptor represents different types of receptor through which their specific ligands elicit their effects; for example, CTGF or TGF-β2 bind to their corresponding growth factor receptor. Therefore, these factors are recognized as major mediators of increasing aqueous humor outflow resistance and, thus, IOP elevation. Collagen type I, III, IV, and VI and fibronectin are particularly relevant in primary open-angle glaucoma because they are elevated as a consequence of the increased TGF-β2 level. For more details please see the main text.

to induce the trans-differentiation of JCT mesenchymal cells into cells with a myofibroblast-like phenotype [3]. Accordingly, their cytoskeleton and fibrils that are directly associated with the ECM are strengthened, and the cellular tone and ECM accumulation increases. As a consequence of these events that are elicited by CTGF, the TM becomes stiffer and the outflow facility decreases.

Integrins are heterodimeric transmembrane receptors that not only mediate cell adhesion to the ECM, but also act as bidirectional signal transducers between the extra- and intracellular environment. They are critically connected to glaucoma because they are involved in the regulation of many activities, such as matrix assembly, TGF-β2/CTGF signal transduction, apoptosis, and cytoskeletal organization [21]. Different integrins are expressed by cells of the TM, of which we focus on $\alpha_v\beta_5$ and $\alpha_v\beta_3$ because of their prominent roles in glaucoma and because antagonists are available for potential therapy [21,22]. Integrin $\alpha_v\beta_5$ mediates the important phagocytotic activity of TM cells, helping to clear cellular debris from the TM and, thus, facilitating outflow of aqueous humor [22]. Integrin $\alpha_v\beta_3$ regulates ECM assembly and actomyosin cytoskeletal organization in TM cells [21] and, therefore, is as an important interface between the extra- and intracellular environment. If the homeostatic balance is disturbed, overactivation of $\alpha_v\beta_3$ integrin elicited by TGF-β2, CTGF, or fibronectin leads to inhibition of the integrin $\alpha_v\beta_5$-mediated phagocytotic activity of TM cells [22]. Additionally, $\alpha_v\beta_3$ integrin activation was observed to increase the cross-linking of actin networks (CLANs) in TM cells in vitro [21]. CLANs are associated with increased cell contractility and are more frequently present in glaucomatous TM cells compared with controls [21,23]. Finally, integrins can also be regarded as potential mechanosensors because of their sensitivity to shear stress, as occurs during elevated IOP [21].

The Rho family of small GTPases acts as important intracellular downstream mediators of integrin activation and includes RhoA, Rac, and CDC42 [24]. RhoA is found in all cells of the TM outflow pathway and is, besides integrin signaling, activated by external cues, such as TGF-β2 and CTGF [24]. In addition, perfusion of porcine eyes with high pressure (50 mmHg) with the goal of provoking an experimentally elevated IOP revealed that mechanical stretch also activates Rho GTPase signaling (sixfold compared with 15 mmHg perfusion pressure) [25]. Once activated, the downstream effector ROCK phosphorylates several intracellular substrates and, thus, regulates actin cytoskeletal dynamics, actinomyosin contraction, cell adhesion, cell stiffness, cell morphology, and ECM reorganization [24]. When comparing healthy and glaucomatous eyes, no significant difference in RhoA and ROCK expression was measured [26]. However, overactivation of ROCK, as in glaucoma, enhances the contractility of TM cells and alters their ECM production and fibrotic activity. In transgenic glaucoma mice, overexpression of RhoA GTPase in the outflow pathway significantly increased the expression of α-SMA (by ~42%) and collagen type IA (by ~50%) and was accompanied

by an elevated IOP (~22% higher) [27]. By contrast, inhibition of ROCK with daily topical instillation of the ROCK-inhibitor Y-27632 (Mitsubishi Pharma Corporation) significantly lowered the IOP (by ~20%) because of the significant reduced expression of profibrotic proteins in the TM (α-SMA approximately –38%; collagen type IA approximately –28%) [27]. Licensing of GLANATEC® (ripasudil) in 2014 and Rhopressa® (netarsudil) in 2017, emphasizes the importance of ROCK in regulating aqueous humor outflow and, thus, its crucial role in the homeostatic balance of IOP.

NO, which is well known for its role as mediator of smooth muscle relaxation [28], has been recognized as, for example, a damper of daily IOP fluctuations, another important regulator of the IOP [29–31]. NO is produced in JCT and SC cells by inducible NO synthase (iNOS) or endothelial NOS (eNOS) [30]. As an endogenous feedback loop, shear stress triggers the release of NO in shear-sensitive SC cells to counterbalance diurnal pressure peaks [32]. NO rapidly diffuses to JCT cells to produce the second messenger cGMP. Ultimately, cellular relaxation is provoked by diverse biological effects, such as the prevention of actinomyosin interaction [30–33]. Analysis of aqueous humor of patients with glaucoma revealed nitrite ($NO2^-$) levels ~0.73-fold lower than the cataract control group. Nitrite is the stable end-product of NO metabolism. In the same experiments, the level of cGMP decreased by ~0.53-fold [34]. eNOS, which is influenced and inhibited by increased activity of Rho/ROCK pathway in endothelial cells [35], also appears to have an important role during the onset of glaucoma. Its relative abundance was significantly decreased in the TM of patients with glaucoma [29]. In addition, transgenic mice that overexpressed eNOS had a lower IOP and an ~2.3-fold increased outflow facility compared with wild-type mice [31].

Therapeutic approaches

Here, we describe drugs that intervene at the described targets during glaucoma development and progression. In addition, we present and critically examine the drug delivery systems that are needed to protect drugs that are prone to degradation or to enhance their specificity for target cells. For many novel approaches, intracameral (injection into the anterior chamber of the eye) or intravitreal delivery is the preferred route of application because they maximize the bioavailability if corneal penetration is the limiting factor [6,36].

TGF-β2

Given that TGF-β2 is a global player in many physiological processes of the eye and exerts neuroprotective effects in retinal ganglion cells [37], intervention at this level would not be straightforward for permanent glaucoma therapy. However, molecules specifically directed against TGF-β2 have their place in some special indications for short-term use.

A good example is TGF-β2 reduction to prevent scar formation after trabeculectomy, which involves the creation of a guarded fistula connecting the anterior chamber and subconjunctival space through which aqueous humor can drain in a controlled fashion. This surgical process is an option if drug therapy fails [38]. However, scarring is an adverse effect and comparable to profibrotic processes associated with glaucoma. As an alternative to conventional antiscarring agents, the RNA therapeutic ISTH0036 (Isarna Therapeutics) successfully completed a Phase I trial (NCT02406833) [39]. It is a locked nucleic acid-modified antisense oligonucleotide with increased stability and long pharmacodynamic activity (up to 8 weeks) and is applied without any delivery system intravitreally at a final concentration in the vitreous humor ranging from 0.3 μM to 10 μM [39]. Preclinical studies with New Zealand White rabbits demonstrated the rapid distribution of ISTH0036 in tissues of the posterior eye after intravitreal application. Only low amounts were detected in the anterior chamber [40]. Because TGF-β2 is mainly secreted by epithelial cells of the ciliary body or the iris [12], intravitreal injection is the straightforward choice of application route. However, because TGF-β2 mediates important physiological functions, a downregulation for long-term glaucoma therapy must be considered and is, in our opinion, not an option.

CTGF

CTGF reduction does not interfere with the pleiotropic effects of TGF-β2; therefore, intervention at this level of the glaucoma-associated signaling cascade is more specific and the risk of possible adverse effects is strongly reduced. The principle feasibility of interfering with the CTGF-induced ECM production was demonstrated by Wallace *et al*. TM cells were incubated with aqueous humor from patients with glaucoma to elicit glaucoma-specific pathological effects. Pretreatment with the human monoclonal antibody pamrevlumab (FG-3019; FibroGen, Inc.) against CTGF significantly suppressed the expression of profibrotic genes [41]. Potential IOP reduction in vivo remains to be demonstrated. Due to their high molecular weight and polarity, monoclonal antibodies are not able to cross the intact cornea as well as cellular membranes; therefore, intravitreal or intracameral administration would be the application route of choice. Monoclonal antibodies are subjected to physical-chemical degradation and denaturation procedures; thus, a possible bottleneck of an antibody therapy could be their stability in the aqueous and vitreous humor because proteolytic enzymes (e.g., serine proteases) are elevated in the aging human vitreous humor (~2.6-fold higher than in younger control groups) [42].

Another promising drug class allowing for the specific reduction of CTGF production is small interfering RNA (siRNA) [43]. Given that siRNA is subjected to degradation and not able to cross cellular membranes, delivery vehicles, such as nanocarriers, are necessary to therapeutically exploit this principle [44]. Recently, the intracameral delivery of anti-CTGF siRNA by using layer-by-layer-coated nanoparticles was proposed [45]. The nanoparticles were decorated with a final layer of hyaluronan. Because hyaluronan is abundant in the ECM of the TM [46], nonspecific adsorption of particles to ECM components should be prevented. Consequently, nanoparticles penetrated deeply into the outflow region, as confirmed in ex vivo organ cultures of porcine, murine, and human eyes (Fig. 3) [45]. Moreover, by binding of hyaluronan to the CD44 receptor, which is significantly overexpressed by glaucomatous TM and SC cells, it was feasible to deliver the nanoparticles into human TM cells. Therefore, hyaluronan serves as material with dual functionality, first by enhancement of nanoparticles mobility in the ECM of the TM and second by allowing for the targeted delivery of nanomaterials to glaucomatous TM and SC cells. However, the feasibility of this approach remains to be proven in vivo.

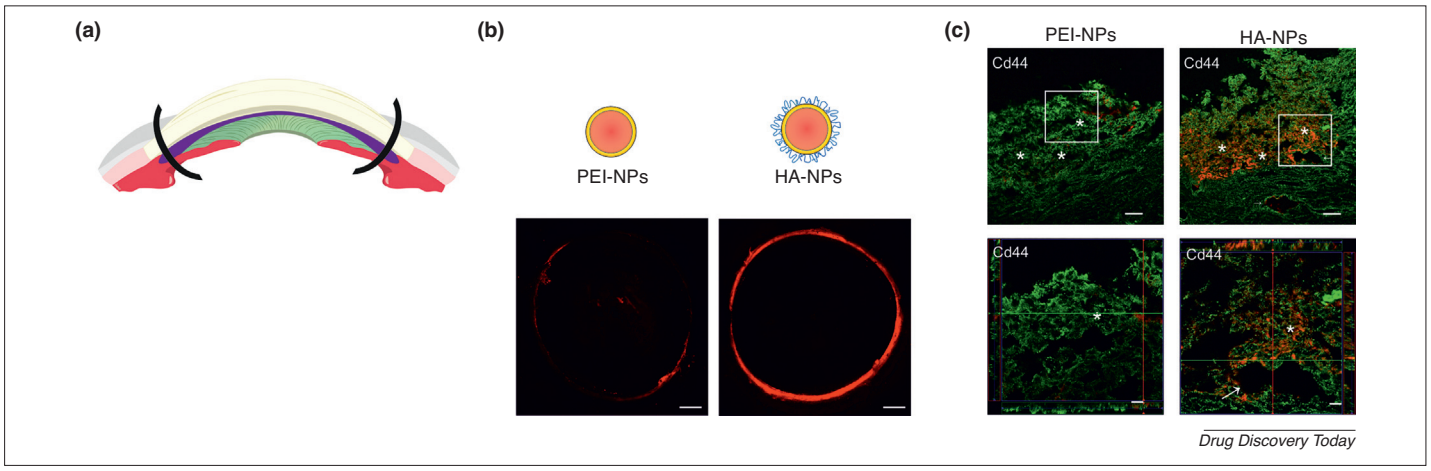

FIGURE 3

Delivery of hyaluronan-decorated nanoparticles to the conventional outflow pathway. Porcine eyes were perfused ex vivo with rhodamine-labeled nanoparticles (NPs). Their surface was modified with hyaluronan (HA-NPs). Poly(ethylene imine)-decorated NPs (PEI-NPs) served as control. (a) The anterior chambers of porcine eyes were dissected after perfusion. (b) Representative fluorescence images of the whole outflow ring showed that the fluorescence intensity of HA-NPs was higher and was detected over the whole outflow ring. (c) Sagittal sections of the outflow ring were labeled with anti-CD44 staining (green). (ii) Magnification of the white rectangle in (i). HA-NPs (red) penetrated deeply into the outflow region, with some particles coming as close as SC. PEI-NPs were detected in lower amounts and only in the entrance region of the TM. Scale bars: 50 μm (i) and 20 μm (ii). The area of the trabecular meshwork (TM) is depicted by asterisks and the Schlemm's canal (SC) by arrows. Reproduced, with permission, from Ref. [45].

Integrins

Controlling integrin activity has been shown to be successful in treating other diseases. For example, various monoclonal antibodies, peptides, or small molecules are approved for the inhibition of integrin $\alpha_2b\beta_3$ for the prevention of thrombotic vascular diseases, of integrin $\alpha_4\beta_7$ for the treatment of inflammatory bowel diseases, and for integrin α_4 for the treatment of multiple sclerosis [47]. This indicates that integrin targeting could be a new therapeutic option for glaucoma. Hennig *et al.* pursued this concept and decorated nanoparticles with the cyclic Arg-Gly-Asp (RGD) pentapeptide cyclo(RGDfC) [48]. The nanoparticles targeted cultured TM cells by binding to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and were then rapidly and efficiently endocytosed. Furthermore, soluble cyclo (RGDfC) efficiently prevented CTGF-mediated profibrotic effects and maintained CTGF signaling at a basal level. Thus, RGD peptides unify two functions within one entity. First, they are powerful ligands to address nanoparticles with high specificity to the TM to potentially deposit antiglaucoma drugs incorporated into the nanoparticle core. Second, they elicit their own therapeutic effect by interfering with CTGF signaling upon arrival. Proof of this concept in vivo is eagerly awaited to see whether RGD peptide-decorated nanoparticles are a new treatment option for glaucoma.

Other integrin-antagonists, including monoclonal antibodies or small molecules, have not yet been tested as antiglaucoma agents [22]. For any potential therapy, it is important to keep in mind that integrin antagonists can act either as agonists or as antagonists depending on their concentration [22,48].

Rho associated kinase

ROCK inhibitors, such as ripasudil (GLANATEC®; Kowa Company, Ltd) and netarsudil (Rhopressa®; Aerie Pharmaceuticals, Inc.), are small molecules that act by interrupting the Rho-signaling cascade and are the first drugs on the market affecting the origin of glaucoma pathology [49]. Rhopressa®, at a concentration of 0.02%, demonstrated a clinically relevant and statistically significant IOP reduction of 16–21% after once daily and 22–24% after twice daily application in three Phase III clinical trials (NCT02207491, NCT02207621, and NCT02558374). The IOP reduction was similar to treatment with timolol (0.5%, twice daily) [50]. The most prevalent off-target effect was conjunctival hyperemia [50]. Roclatan™ (Aerie Pharmaceuticals, Inc.), which is a fixed combination of netarsudil 0.02% and latanoprost 0.005%, is currently under investigation for application in open-angle glaucoma or ocular hypertension in Phase III clinical trials in the EU (NCT03284853) and USA (NCT02674854). A major shortcoming of some topically applied ROCK inhibitors is a low intraocular bioavailability because of hydrophilic physicochemical properties (logP values: ripasudil 0.72; Y-27632 1.0; fasudil (INN) 0.16 [51]). Hence, mucoadhesive polymer films loaded with Y-27632 were tested for the possibility of increasing corneal residence time to ultimately enhance corneal penetration [52]. Unfortunately, the concentration in ex vivo porcine corneas cultures was significantly higher only if corneas had first been cryoprobe treated (threefold higher versus eye drops) [52]. Cryoprobing is a common tool to affect the integrity of corneal endothelial cells, thereby enhancing their permeability. However, after optimization, such polymer films could offer a real alternative because they may improve the bioavailability and reduce the application frequency of topically administrated drugs.

To maximize bioavailability, Koda *et al.* developed Y-27632-loaded PLGA-microspheres for intracameral application (the formulation was intended for the treatment of corneal diseases) [53]. Unfortunately, drug encapsulation efficiency was only ~18% and drug release only lasted over 14 days, making immense particle doses and high application frequency necessary. Switching to more lipophilic ROCK inhibitors (e.g., netarsudil; logP 3.77 [51]) or altering the lactic:glycolic ratio of the polymer could significantly improve the encapsulation efficiency and release profiles. Recently, a biodegradable, injectable poly(ester amide) implant for the intravitreal delivery of the ROCK inhibitor

AR-13503 (Aerie Pharmaceuticals, Inc.) was developed [54]. Again, the formulation was not intended for glaucoma therapy, but rather for the treatment of diabetic macula edema. AR-13503 was released in vitro over 4–6 months. Since ROCK inhibitors also have protective effects on retinal cells [55], and because they are, as a result of their hydrophilicity, most likely eliminated from the vitreous to the aqueous humor, where they could elicit their therapeutic effect [36], such intravitreal releasing platforms of ROCK-inhibitor could bring a great benefit for glaucoma therapy.

Nitric oxide

The NO-signaling pathway offers opportunities to elicit therapeutic effects by increasing the amount of NO itself or by intervening at the enzymatic level. Latanoprostene bunod (Vyzulta™; Bausch & Lomb, Inc.) was recently US Food and Drug Administration (FDA) approved as an eye drop solution. Vyzulta™ combines the leading antiglaucoma drug latanoprost with a novel NO-donating moiety. Following topical administration, latanoprostene bunod is hydrolyzed by corneal esterases to latanoprost acid and butanediol mononitrate, which is further converted to 1,4-butanediol and NO [28]. Latanoprost acid increases aqueous humor outflow through the unconventional pathway, whereas NO increases the outflow facility via relaxation of TM and SC cells and thereby offers an additional 1–2 mmHg of IOP reduction [30]. Because topical application is accompanied by off-target effects, such as conjunctival hyperemia, eyelash growth, iris hyperpigmentation, or even an increased IOP induced by relaxation of the ciliary muscle [28,56], local and controlled delivery of NO is desirable. To follow this goal, Chandrawati *et al*. proposed an intracamerally injectable NO-donating two-component formulation: β-galactosidase encapsulated in degradable poly(methacrylic acid) was co-delivered with a NO-releasing enzyme-prodrug (β-gal-NONOate) incorporated into liposomes (Fig. 4) [56]. Both particles species were delivered to the TM, where β-galactosidase enzymatically released NO. The principle feasibility was demonstrated ex vivo in murine eyes, with the outflow facility enhanced by 84% [56]. Unfortunately, β-galactosidase is not a naturally occurring enzyme in the eye. Exploiting enzymes that are basally expressed in the TM, such as matrix metalloproteinases (MMPs), would be an alternative approach for triggering NO release [57].

Given the risk of developing nitrate tolerance from long term NO exposure, an intervention with the second messenger of NO, cGMP, which is activated by soluble guanylate cyclase (sGC), would be an appealing alternative [33]. IWP-953 (Ironwood Pharmaceuticals, Inc.) stimulates sGC and, therefore, was investigated for its use as an IOP-lowering therapeutic [58]. In cultured human

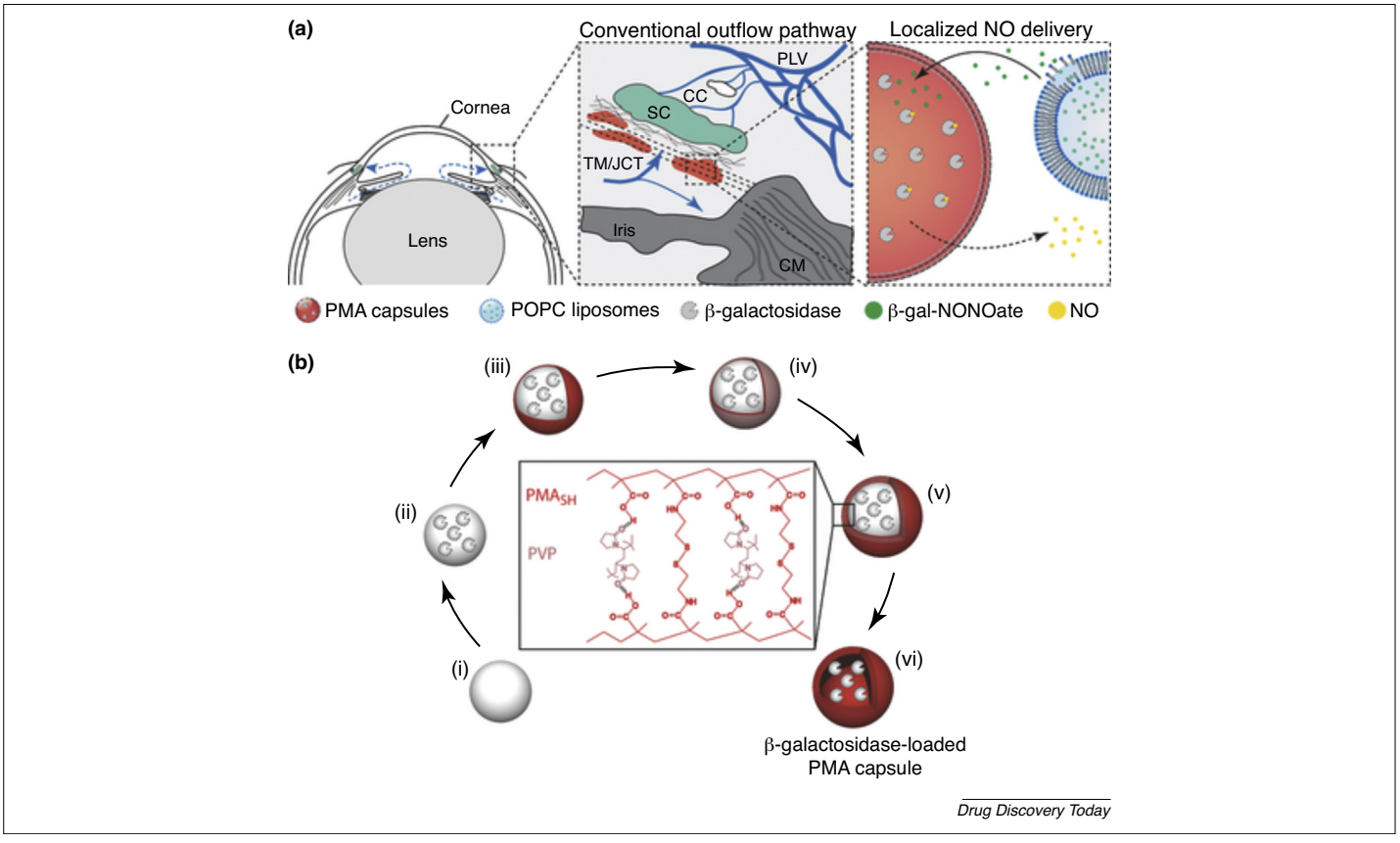

FIGURE 4

Delivery of NO to the conventional outflow pathway via enzymatically triggered release. (a) Schematic overview of localized delivery of nitric oxide (NO) to the trabecular meshwork (TM) outflow pathway via a NO-releasing enzyme prodrug formulation. The delivery system comprises β-galactosidase-loaded poly(methacrylic acid) (PMA) capsules and NO prodrug (β-gal-NONOate)-loaded 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposomes, which are consecutively delivered at a time interval of 48 h by intracameral injection. (b) Schematic drawing of fabrication of β-galactosidase-loaded PMA capsules via the layer-by-layer technique. β-galactosidase is embedded below several thiolated PMA and poly(N-vinylpyrrolidone) (PVP) layers. Reproduced, with permission, from [56]. Abbreviations: CC, collector channels; CM, ciliary muscle; JCT, juxtacanalicular connective tissue; PLV, perilimbal vessels; SC, Schlemm's canal; TM, trabecular meshwork.

TM cells, cGMP production was successfully increased and outflow facility enhanced by ~90% in an ex vivo perfusion model of murine eyes [58]. For in vivo application, a delivery system that allows for a specific transport and/or release of the drug to the TM will be vital. Moreover, in laser trabeculoplasty-induced hypertensive monkey eyes, the sGC activator MGV354 (Alcon) lowered IOP in a dose-dependent manner by ~25% to 35% from baseline after topical instillation [59]. Unfortunately, in a first clinical trial (NCT02743780), MGV354 did not demonstrate statistically significant IOP-lowering effects compared with the control group [60]. Besides a short trial duration (7 days) and possible noncompliant patients, the authors suggested that the preclinical monkey model might not reflect the true nature of the disease and, therefore, led to the early misjudgment of the drug [60].

Concluding remarks and future directions

Over the past few years, molecular mechanisms responsible for pathological changes during glaucoma development and progression have been intensively elucidated, and selected ones are successively being exploited as novel therapeutic approaches. Here, we identified TGF-β2, CTGF, integrins, ROCK, and NO from the literature as interesting mediators of glaucoma and highlighted their role in the pathophysiology. As yet, antagonizing CTGF- or integrin-mediated effects have been tested in a limited number of preclinical studies, whereas a few drugs interfering with the ROCK- and the NO-signaling pathways have already been launched onto the market. Novel drugs can be applied as monotherapy or in combination, depending on their efficacy and the individually desired IOP. By contrast, molecules specifically directed against TGF-β2 are only appropriate for some special indications and short-term use. Few other signaling pathways, which were not emphasized in this review, have also gained increasing attention for the onset of glaucoma and could also be turned into novel therapeutic principles. For example, MMPs have important roles in ECM turnover and, therefore, increased activity of MMP-3 might make the outflow pathway more permeable for draining aqueous humor [61]. Other interesting targets are Smad-7 and-3, which are, similarly to CTGF, downstream effectors of TGF-β2 and, therefore, also involved in ECM remodeling [62]. Strategies that are not specifically directed against glaucoma mediators are also discussed in literature. For example, downregulation of tight junction proteins of endothelial cells lining the SC could improve the outflow facility and significantly contribute to the regulation of IOP [63].

A massive bottleneck in the development of novel therapeutic principles will be the delivery of drugs to their target cells with (i) high specificity; (ii) in a sufficient therapeutic concentration; and (iii) over a prolonged time period. Specific delivery is vital because otherwise, especially after application of highly potent drugs, adverse effects can occur. For example, nonspecific silencing of tight junction proteins, as outlined above, can also disrupt the integrity of the corneal endothelium or blood–aqueous barrier of the ciliary epithelium. In contrast to topically applied conventional medications, drugs such as siRNA or monoclonal antibodies require other routes of administration because of their unfavorable physicochemical properties and instability under physiological conditions. Intracameral administration allows for increasing the bioavailability at the target side to a maximum. However, the relatively rapid turnover of aqueous humor (~2.6 μl/min) and its limited volume (~250 μl) [36,64], would necessitate frequent injection intervals. To address this, an alternative will be to create depot formulations that would be administered in the supraciliary space, the anterior chamber, or even into the vitreous [7]. Given that the vitreous has a high capacity (4 ml) [36] and that hydrophilic or large molecules are preferably cleared from the vitreous into the aqueous humor and then likely transported to the TM [36], the deposition of sustained-release formulations in this part of the eye could be promising for reducing the application frequency of such treatments.

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