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Original article

Potent and selective antisense oligonucleotides targeting the transforming growth factor beta (TGF-β) isoforms in advanced glaucoma: a preclinical evaluation

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Abstract

Purpose: ISARNA Therapeutics is developing highly potent and selective LNA-modified ASO gapmers targeting TGF-β1 and TGF-β2 mRNA. In the field of ophthalmology, glaucoma, the second leading cause for blindness in the US, has been linked to TGF-β activation as a key driver. ISTH0036 is a 14-mer phosphorothioate Locked Nucleic Acid- (LNA) modified antisense oligonucleotide gapmer, targeting the sequence of TGF-β2 mRNA and was developed for therapeutic intervention in ocular diseases. It was shown to effectively and potently downregulate target mRNA in a dose-dependent manner in relevant cell-based assays, as well as leading to target engagement in anterior eye segment tissues upon intravitreal administration (Isarna proprietary information). The aim of this study was to evaluate the therapeutic potential of ISTH0036 in murine models of glaucoma filtration surgery (GFS) following different intraocular administrations.
Methods: A murine model of glaucoma filtration surgery has been used to evaluate the effect of intraocular ISTH0036 administration on post-operative wound healing. Bleb size and bleb survival were determined after different intraocular administra-

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tions of saline, control scrambled oligonucleotide or ISTH0036 (at day of surgery and after two weeks).
Results: Intraocular administrations of ISTH0036 lead to a significant effect on bleb area and survival, as well as decreasing scarring (downregulation of collagen-1 and -3 fibers) in a murine glaucoma filtration surgery model.
Conclusion: Consistent with the expected molecular mechanism of action and demonstrated pharmacokinetic (PK) and pharmacodynamic (PD) properties following intraocular administration, preclinical data with ISTH0036 in murine glaucoma filtration surgery model support the current exploration of the drug candidate in advanced glaucoma patients undergoing trabeculectomy.

Key words: antisense oligonucleotide, glaucoma, intraocular administration, transforming growth factor beta

1. Introduction

In ophthalmology, several diseases have been associated with modulation of transforming growth factor beta (TGF-β) protein expression. In particular, a large body of scientific evidence has been generated for glaucoma, proliferative vitreoretinopathy, posterior capsule opacification, diabetic retinopathy, age-related macular degeneration and corneal diseases such as pterygium and keratoconus.[1-7] Furthermore, single TGF-β isoforms of the TGF-β family (*i.e.*, TGF-β1, -β2 and -β3) appear to be the core pathophysiologic molecular 'driving force' for various key ophthalmic diseases with high unmet medical need. TGF-β2 is the predominant cytokine expressed in the eye and is found in large amounts in the aqueous and vitreous humors, the neuronal retina and the retinal pigmented epithelium in the healthy eye.[8-12] Various studies have shown the potential significance of TGF-β2 signaling by observing that active TGF-β2 protein is significantly increased intraocularly in the ciliary body, the optic nerve and the trabecular meshwork in primary open-angle glaucoma (POAG) patients.[13-21] This effect is also reflected by increase in TGF-β levels 70- to 100-fold above normal in the optic nerve head in POAG patients.[22] TGF-β2-induced changes might contribute to deformation of the optic nerve axons by causing impairment of axonal transport and neurotrophic supply via the remodeling of the extracellular matrix (ECM) in the lamina cribrosa, leading to their permanent degeneration. The increase in intraocular pressure further adds mechanical stress and strain to optic nerve axons and accelerates degenerative changes.[6,23,24] TGF-β also plays a distinct driving role in fibrotic diseases[25] and epithelial-mesenchymal transition and is therefore most probably responsible for the increase of ECM and cellular transformation which is reported for the trabecular meshwork in glaucoma patients. POAG patients show a significant increase in sheath-derived plagues due to fine fibrils and other ECM components which adhere to the sheaths of the elastic fibers in the inner

wall endothelium.[26,27]

Antisense oligonucleotides are small synthetic single strands of DNA or RNA that are complementary to a chosen sequence which can be used to prevent protein translation of certain messenger RNA strands by binding to them and/or to target a specific, complementary (coding or non-coding) RNA. Antisense oligonucleotides have been used for decades to achieve sequence-specific silencing of gene expression, and a wide range of chemical modifications (*e.g.*, locked nucleic acids) have been explored and implemented over the years to improve drug-like properties. ISTH0036 represents a 14-mer fully phosphorothioate Locked Nucleic Acid- (LNA) modified antisense oligodeoxynucleotide gapmer (nucleotide sequence: 5'-**ga**(Me)**c**CAGATGCA**gga**-3'; in which bold italics letters represent LNA-modified nucleotides in a '3+3 gapmer' pattern), for which potent and selective activity on TGF-β2 mRNA has been demonstrated in cell-based assays and *in vivo*, with consequent selective decrease in protein expression (data not shown).

Specifically for TGF-β2, a critical role in the pathophysiology of glaucoma has been demonstrated, making this isoform an obvious therapeutic target of high interest for a disease, which is the second leading cause for blindness in the Western world. As visually summarized in Figure 1, considering the pleiotropic

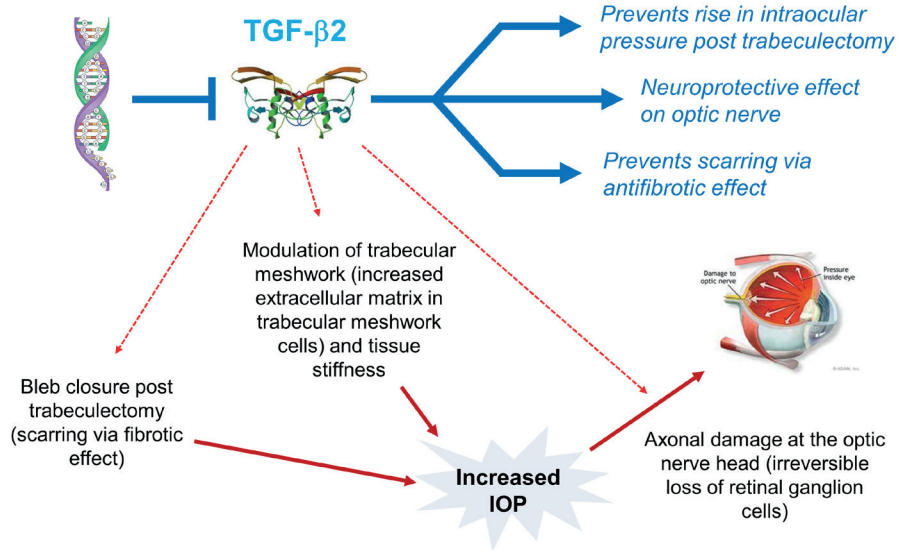

Fig. 1. Pleiotropic physiological mechanisms of action linked to targeting TGF-β2 protein expression claimed to support therapeutic intervention in advanced-staged glaucoma post-trabeculectomy. (Oligonucleotide/mRNA hybrid complex illustration has been adapted from a figure presented by Rigo *et al.* (2012);[28] TGF-β2 protein ribbon structure is from Schlunegger & Grutter (1992);[29] and the illustration of the glaucoma-induced intraocular pressure in human eye is from http://www.ftwortheyedoctor.com/glaucoma.html.)

physiological mechanisms of action, therapeutic intervention targeting TGF-β2 protein expression may have multifold effects on relevant intraocular tissues such as trabecular meshwork (cell invasion/migration), retina (scarring and wound-healing processes) and/or optic nerve head (neuroprotection), and warrant further evaluation in patients suffering advanced glaucoma and undergoing trabeculectomy.

2. Methods

Highly relevant to the initial clinical focus, a murine glaucoma filtration surgery model has been evaluated in which post-operative wound healing after glaucoma surgery is mimicked. Briefly, filtering surgery was performed under anesthesia on both eyes of eight to ten weeks old C57BL/6J mice resulting in a filtration bleb.[30-32] Shortly, the conjunctiva was first surgically dissected to expose the underlying sclera, and a small filtration subconjunctival space was created by running the surgical scissors underneath the dissected conjunctiva. Next, a 30-gauge needle was used to make an incision through the sclera into the anterior chamber of the eye to allow the aqueous humor to escape into the subconjunctival space. Finally, the conjunctiva was closed at the limbus by suturing over the newly created fistula. A topical steroids and antibiotics combination preparation was administered at the end of surgery to avoid opportunistic infections. Intraocular administrations [1-µL intravitreal (IVT) or intracameral (ICM) injections] of saline or about 1 µg of either control scrambled (mismatch) oligonucleotide or ISTH0036 were performed immediately after surgery and repeated after two weeks. Bleb size was measured via digital photographs about three times a week within the one-month experiment duration. Bleb survival was determined at the end of the study, while bleb failure was defined as the appearance of a scarred and flat bleb at two consecutive measurements.

3. Results

As illustrated in Figure 2, although no significant differences were observed between the saline- and the scrambled control oligonucleotide-treated groups on bleb area and survival (with blebs failing at day 17), ISTH0036 was shown to induce a statistically significant increase in bleb area and survival. Interestingly and consistent with the location of the bleb, following ICM administrations, a greater increase in bleb area and survival was observed as compared to IVT administrations, with potentially a significant effect of the second injection performed on day 14. However, it must be noted that considering the very low volume of the aqueous humor chamber (only 1-2 µL), accuracy of doses dispensed by ICM injection as compared to IVT administra-

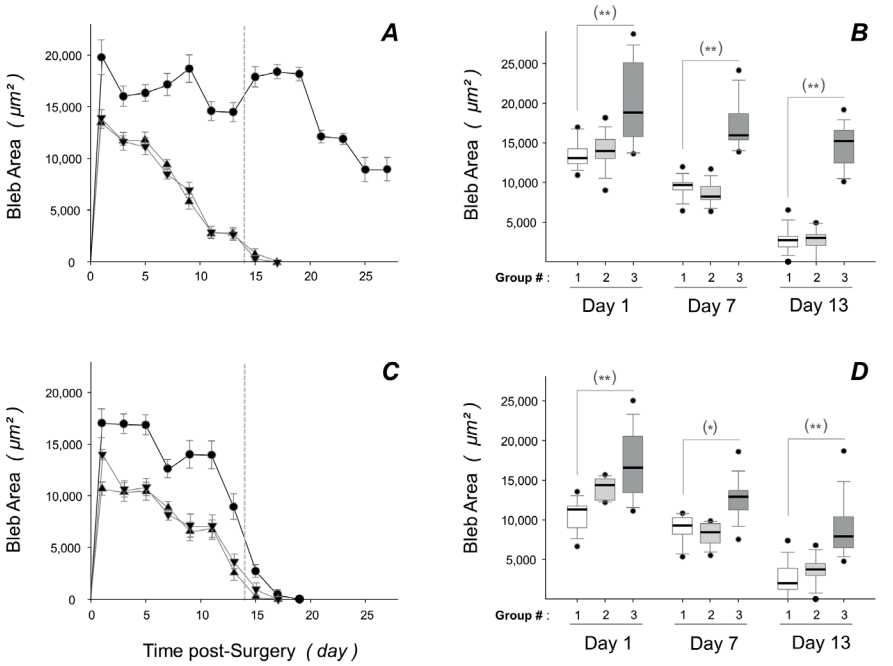

Fig. 2. Effect of ISTH0036 on bleb size and survival in an experimental mouse glaucoma filtration surgery model. C57BL/6J mice were used for a filtration surgery on both eyes, using a technique that has been described previously and that results in a filtering bleb.30 Bleb area (n = 8-10 eyes) was monitored after intracameral (**A, B**) or intravitreal (**C, D**) 1-µL injection(s) of either saline (▲, Group 1), 1-µg irrelevant scrambled mismatch control antisense oligonucleotide (▼, Group 2) or 1-µg ISTH0036 (●, Group 3) to mice after glaucoma filtration surgery (performed on day 0). Injections were repeated on day 14, and bleb size (expressed as μm^2) was measured at the indicated times via digital photographs. Results are presented for each group as bleb area (mean ± SEM) at the indicated times (**A, C**), or as box plot on day 1, 7 and 14 (**B, D**), in which median value (solid line), 25-75% box, 10-90% percentiles and lowest and highest values in each group are presented. All experimental animal procedures were performed in accordance with the standards in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and the EC Directive 86/609/EEC for animal experiments. Non parametric 2-independent samples Wilcoxon-Mann-Whitney test was used to determine the significance of the differences between vehicle control and test item-treated groups (* $p < 0.05$; ** $p < 0.01$).

tion may be challenged. In addition, the very rapid turnover of the aqueous humor fluid (about 100 min[33]) compared to vitreous humor may strongly affect oligonucleotide concentrations over time. Therefore, direct side-by-side dose comparison between the two routes of administration may not lead to conclusive results.

Moreover, to evaluate the deposition of ECM (including collagen) in the bleb area, immunohistochemistry was performed to quantify Sirius Red (SR, collagen-1

and -3 fibers) positive area. Deposition of collagen was determined by measuring the percentage of the collagen positive area in the bleb area. Polarized light was used to distinguish mature from immature collagen fibers. Mature collagen fibers appear bright yellow or orange, whereas immature collagen fibers appear green. Analysis of the Sirius Red positive area in IVT and ICM injected mice showed that no differences in collagen deposition could be seen between saline- and control scrambled mismatch oligonucleotide-treated eyes at day 28. In contrast, treatment with the ISTH0036 was able to significantly reduce the deposition of extracellular matrix, namely collagen, after IVT or ICM injection (Fig. 3).

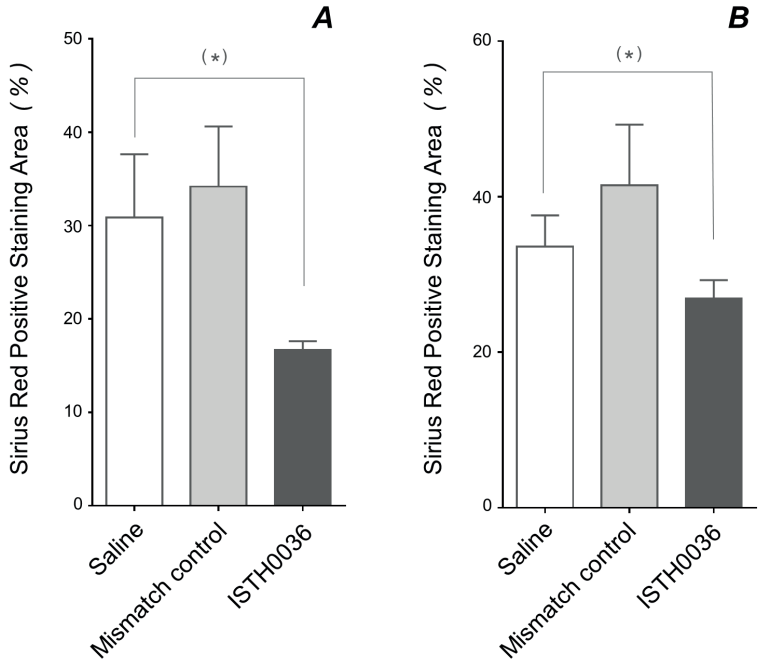

Fig. 3. Effect of ISTH0036 on collagen deposition in the bleb area in a murine experimental glaucoma filtration surgery model. Results represent the Sirius Red positive area (mean ± SD; n = 4) after intracameral (**A**) or intravitreal (**B**) administrations of either saline, mismatch/scrambled control oligonucleotide or ISTH0036 to mice following glaucoma filtration surgery (performed on day 0), as described in the legend of Figure 2. First injections of test items were performed at the time of surgery, and repeated on day 14. Sirius Red positive area (expressed as %) was measured in the bleb area via digital photographs on day 28. Student's t-test was used to determine the significance of the differences between vehicle control and test item-treated groups (* $p < 0.05$).

4. Discussion

We showed that the intraocular administration of ISTH0036, which is leading to sequence-specific TGF β2 downregulation (Isarna proprietary information), showed a significant effect on bleb size and survival, as well as fibrosis, in a murine glaucoma filtration surgery (GFS) model. Treatment with ISTH0036 resulted in prolonged bleb survival and decreased scarring (downregulation of collagen-1 and -3 fibers) in a murine glaucoma filtration surgery model. Considering the pleiotropic physiological mechanisms of action, therapeutic intervention targeting TGF-β2 protein expression in glaucoma patients may have many more effects on relevant intraocular tissues such as trabecular meshwork (cell invasion/migration and scarring), retina (scarring and wound-healing processes) and/or optic nerve head (neuroprotection).

5. Conclusion

These data provide a strong rationale that patients with glaucoma (or other ocular diseases), and potentially specifically those undergoing trabeculectomy, may benefit from treatment with TGF-β2 antisense oligonucleotides. Nevertheless, further comparative studies will be necessary to investigate different doses and treatment schedules. In addition, the reported anti-fibrotic, as well as the potential anti-invasive, anti-angiogenic and neuroprotective effects should be confirmed in other animal models of scarring and ocular diseases. Further studies should include animal models of diabetic retinopathy, wet macular degeneration/human neovascular chorioretinal diseases (choroidal and retinal neovascularization model) and other retinal vascular pathologies (oxygen-induced retinopathy model).

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