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Abstract

RNA interference (RNAi) is a posttranscriptional mechanism of gene regulation present in eukaryotic cells. Inducers of RNAi are small molecules of RNA that act in the cytoplasm where they are able to impair translation of a specific mRNA to protein, hence modifying gene expression. The discovery of this mechanism in mammals led to the development of a new class of therapeutics with the aim of exploiting this endogenous mechanism of action. In the last decade, great efforts have been put into understanding RNAi and translating this accumulated knowledge into the design of modern therapeutics. With several compounds in phase III clinical development, the field is getting closer to its first market authorization. Here we make a thorough overview of the field of RNAi therapeutics in ophthalmology, one of the fields in which RNAi has been most successful.

Keywords: RNAi, eye diseases, ophthalmology, drug development

1. Introduction

Short interference RNAs (siRNA) are small molecules of double-stranded RNA of around 21 base pair long that specifically downregulate the expression of a target gene [1]. This mechanism of endogenous gene expression regulation, present in most eukaryotic cells, has been thoroughly used to study gene function [2]. SiRNAs exert their function in the cytoplasm of the cell, where they assemble with a several proteins to yield the RNA-induced silencing complex (RISC), a multimeric RNA–protein complex that recognizes complementary messenger RNAs (mRNA) and promotes their degradation, thus blocking the synthesis of specific proteins. RNAi may be activated by endogenous siRNAs synthesized in the nucleus of the cell and generated by subsequent processing within the cell cytoplasm to yield siRNAs. Alterna-
tively, siRNAs can be exogenously introduced to mimic the action of endogenous RNAi triggers [3]. Among the benefits of RNAi are the potential of transiently silencing any given gene at any stage of development and to affect gene expression in specific anatomical regions without affecting nontargeted regions. These benefits are being used as a basis to develop a new class of innovative drugs that may reach the market in the next five years; the present report highlights the advances made in RNAi therapeutics on the field of ophthalmology.

2. The special environment of the eye: advantages and disadvantages

The eye has traditionally been considered a good organ for proof-of-concept studies to assess the efficacy of innovative therapies. It has a very particular anatomy that allows the transformation of sensory information into electrical signals that can be thereafter interpreted by the brain. Transformation and partial processing of sensory information takes place in the retina, located at the back of the eye. The correct function of the eye requires light to travel through several anatomical structures in order to reach the photosensitive retina; hence, these structures have to be transparent or semi-transparent to allow passage of light. The environment of the eye is extremely specialized to ensure that transparency is maintained, and there are several mechanisms in place to ensure that this specialized environment is preserved. One of the anatomical characteristics of the eye to allow light to travel through its structures is restricting the blood flow to regions where transparency is not strictly required.

In addition, access to the innermost regions of the eye is controlled by several barriers; these barriers isolate the eye from external aggressions and pathogens but also limit the access of therapeutics. The influence of the particular anatomical features of the eye on drug delivery is further explained in Section 4 of this review. The barriers of the eye do not only isolate this organ from external aggressions or substances but also limit the access of internal elements; as such, the immune system has only limited access to the eye making the eye a partially immune-privileged region. Finally, the aqueous humor, the clear liquid that fills the eye and maintains its shape and pressure, has a very low content in proteins compared to serum. Among the proteins that are significantly reduced compared to other tissues are RNases and elements of the complement cascade. The reduced concentration of RNases increases the half-life of RNAs used as therapeutics, and reduction in the elements of the complement cascade further decreases the likelihood of an unwanted immune reactions to drugs.

In order to preserve its integrity, the eye has efficacious barriers to block the entrance of pathogens and substances that could potentially affect its sensory function. The eye has developed specific features that ensure that light travels through its tissues; this specialization of tissues to preserve visual function is also observed by the immune system [4]. Immune responses change the local environment of tissues and are frequently associated to tissue inflammation. In order to avoid these potentially harmful changes, the eye has a relatively immune privileged status. This immune privilege status is maintained by several mechanisms. Absence of lymphatic and blood vessels in certain areas and abundance of immunosuppressive factors in the aqueous humor are among these mechanisms [5]. On the other hand, the eye
needs to be able to respond to situations in which its integrity can be compromised such as viral or bacterial infections. The innate immune response is the first system activated in response to aggressions; it acts like a watchdog mechanism recognizing pathogen-associated molecular patterns (PAMP). Depending on the molecular characteristics and location of the PAMP, different effectors of the innate response are activated; these responses can be mediated by toll-like receptors (TLR) or independent of these receptors. Toll-like receptors discriminate self-motifs from non-self-motifs [6]. There are ten known TLRs, and they differ in their subcellular localization and in the type of non-self-pattern they recognize. TLR1, TLR2, TLR4, TLR5, and TLR6 recognize components of bacterial walls and are located in the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 recognize oligonucleotides and are sequestered in intracellular compartment. TLR3 binds to single- and double-stranded RNA, TLR7 binds to single-stranded RNA, and TLR9 binds to unmethylated CpG motifs, usually found in bacterial DNA. In the eye, the expression pattern of each TLRs varies depending on the anatomical structure; all TLRs are present in the corneal and retinal pigment epithelia; TLR4 is the predominant TLR in the rest of the eye structures where it localizes in resident antigen presenting cells [7, 8]. TLR-independent response mechanisms to cytoplasmic oligonucleotides include dsRNA-binding protein kinase, the RNA helicase (RIG-I), and oligoadenylate synthase enzyme. These proteins are cytoplasmic dsRNA sensors belonging to the antiviral innate immune system, which plays an important role in antiviral defense in response to viral infection and replication [9].

The first proof-of-concept studies to demonstrate the viability of silencing genes in the eye showed that the injection of siRNAs into the subretinal space or vitreous cavity could indeed downregulate specific genes. In these experiments, the downregulation of genes of the vascular endothelial growth factor (VEGF) family with siRNAs correlated with the inhibition of ocular neovascularization [10, 11]. The first set of these experiments used an adenoviral vector that codified for an siRNA designed against VEGF1. The subretinal injection of this vector 36 h after the induction of coroidal neovascularization (CNV) by laser reduced the areas of neovascularization compared to areas of mice injected with vector codifying for a scrambled siRNA [10]. In a subsequent study, Campochiaro and coworkers [10] demonstrated that the inhibition of ocular neovascularization could also be achieved by injecting a VEGFR1 siRNA directly, without an expression vector, into the vitreous cavity. The siRNA used in this study had the so-called canonical design, which comprises a 21-nucleotide guide strand and a complementary passenger strand annealed to form an siRNA duplex with a 19-bp dsRNA stem and 2 nucleotide 3’ overhangs at both ends [11].

In 2008, Kleinman and coworkers published a study in *Nature* demonstrating that the effect of siRNAs on CNV was mediated by activation of TLR3 rather than an effect on target [12]. The results of these studies showed that the effect of the siRNAs on CNV was dose-dependent but not dependent on sequence. In addition, the authors demonstrated that a minimum length of the siRNA was required in order for the molecules to have an effect on CNV; this length was shown to be at least 21 nucleotides. The study also showed that the internalization of the siRNAs was not required for the inhibition of CNV as cells of the retinal pigment epithelium (RPE) abundantly express TLR3 on the cytoplasmic surface. The authors used several sequences to point out that the inhibition of CNV was mediated...
through an off-target effect. Using docking models, the authors showed that TLR3 and siRNAs were indeed able to interact with each other but the interaction was unstable when siRNAs were shorter than 21 nucleotides. A subsequent study by the same group showed that activation of TLR3 by IVT siRNAs led to caspase-3-mediated degeneration of the RPE questioning the safety these compounds as therapeutics for back of the eye diseases [13]. The findings of Kleinman and coworkers boosted research on alternative designs that could successfully block immune recognition; among the most commonly used strategies are incorporation of delivery systems and 2'-ribose modifications.

Finally, exogenous RNAs are quickly degraded by RNases present in tissues and body fluids [14]. These enzymes cut RNA into smaller components that are subsequently incorporated into the route of degradation of nucleotides. RNAses are present at high concentrations both in tissues, such as in spleen, liver, and pancreas, and in biological fluids, such as in serum [15]. In the eye, the tear fluid is rich in nucleases but the presence of these enzymes is considerably lower in most eye tissues, thus allowing for increased half-life of the siRNAs used for therapeutic purposes.

3. Efficacy studies: Animal models to study the eye

Proof-of-concept studies are required in order to demonstrate that a particular drug has the proposed activity. siRNAs are designed using bioinformatics tools to target specific regions of the human genome. Therefore, assessing the activity of these molecules in animal models requires that the siRNA has biological activity in the species chosen to perform the proof-of-concept study. With the sequencing of the genome of most animal models used in biomedical research, evaluating the homology of a given sequence between two species is nowadays common practice, but this does not warrant finding a fully homologous siRNA for all targeted genes. In cases where homologous sequences cannot be found, a surrogate compound can be used to perform animal efficacy studies; this entails designing a compound that targets the exact same region as the human version but with the sequence of the gene of the animal species to be used. Several animal models can be used to assess the activity of a compound. Here we highlighted the animal models used in the developmental programs of products included in the ophthalmic siRNA pipeline.

3.1. Ischemic optic neuropathy

Ischemic optic neuropathy is a sudden loss of vision caused by interruption or decreased blood flow in the optic nerve. There is a disagreement as to its pathogenesis, clinical features, and management because ischemic optic neuropathy is not a unique disease, but a spectrum of different types [16]. Ischemic optic neuropathy can be primarily of two types: anterior (AION) caused by the interruption of blood flow in the optic nerve head and posterior (PION) involving the posterior part the optic nerve. Both types can be further classified into different subtypes. AION comprises arteritic (A-AION) caused by giant cell arteritis and nonarteritic (NA-AION) caused by other than giant cell arteritis. NA-AION is by far the most common
type and typically affects individuals between 55 and 67 years of age. The incidence of AION has only been thoroughly studied in the USA where there are 2.3–10.3 cases per 100,000 inhabitants; for the nonarteritic type, the numbers are lower: 0.36 per 100,000 inhabitants. NA-AION is characterized by visual loss, optic disc swelling, sometimes with flame hemorrhages on the swollen disc or nearby neuroretinal layer, and sometimes with nearby cotton wool exudates. Visual loss is usually sudden and may progress over several hours to days and even weeks [17].

Animal modes of this disease are used to assess efficacy of pharmaceuticals in development for these conditions and include the optic nerve crush model and the photoembolic stroke model. The optic nerve crush model is a general model in which surviving of the ganglionar cells can be studied in response to a physical damage to the optic nerve [18]. In the photoembolic stroke model, a photosensitive dye, such as rose bengal, is injected intravenously, and laser is specifically applied to the optic nerve head to activate the dye. The activation of the dye results in damage to the endothelial cells of the optic nerve vascular system that ultimately leads to thrombosis of vessels and edema of the optic nerve head [19].

3.2. Glaucoma

Glaucoma is a group of progressive optic neuropathies characterized by vision impairment and degeneration of retinal ganglion cells that if left untreated can lead to blindness. Glaucoma is the second leading cause of global irreversible blindness, and it has been estimated that 60.5 million people were affected by primary open-angle glaucoma (POAG) and primary angle–closure glaucoma (PACG) globally in 2010, a number expected to increase to nearly 80 million by 2020 [20]. The degeneration of the optic nerve is thought to be produced as a result of changes in intraocular pressure (IOP), but specific molecular mediators of these changes have yet to be identified. Because glaucoma may be asymptomatic until a relatively late stage where the nerve damage has already occurred, early diagnosis and treatment are crucial for halting the progression of the condition [21]. Reduction of IOP is the only proven strategy to treat the disease; thus, first-line treatments are aimed toward achieving this goal. There are several compounds currently used to lower IOP, and although most of them are efficacious in lowering IOP, they all come with their own set of side effects and tolerance to the drug is a frequent phenomenon. Tolerance or reduced response of the drug requires change of drug regimen, frequently increasing the dose or combining the prescribed pharmaceutical with another drug [22, 23].

Depending on the specific phase of the disease one wants to model, several animal models can be used [24]. If studying the degeneration of the retina is the main objective, the models mentioned in the previous section can be used. For assessing the IOP lowering efficacy of pharmaceuticals, models with increased IOP are generally used. The increased IOP model induced by oral water overloading in rabbits is a very easy and physiologic model to screen compounds. The basis behind this model is to give the animal an oral overload of water that will result in a transient increase in IOP [25]. Although the specific mechanism behind the increase in IOP following water overloading is uncertain, the model has been extensively used to perform rapid screens of IOP-lowering compounds. The main advantage of this model over other existing models of increased IOP is that the anatomical structure and physiology of eye
structures are preserved allowing a normal response to hypotensive drugs. Other models of increased IOP include laser photocoagulation, intracameral injection of latex microspheres, topical application of prednisolone, light-induced reduced outflow facility, subconjunctival injection of betamethasone, or episcleral vein occlusion [24].

3.3. Dry eye disease

Dry eye disease (DED) is a multifactorial disease of the tear-fluid and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface [26]. Common symptoms of this condition include blurry vision, tearing, and ocular pain. There are several factors that contribute to the etiology of the disease, among them are insufficient tear secretion, excessive evaporation, and alteration in the composition of the tear film [27]. Temporary changes of the composition of the tear film can cause an acute form of DED; if changes persist, the condition can turn into chronic DED. Damage to the ocular surface is usually more severe in the chronic forms than in the acute types. DED is frequently associated to other conditions such as Sjögren’s disease or lachrymal gland dysfunction, but it can also be caused by vitamin deficiency, contact lens wear, and use of several prescription drugs. Acute DED is handled with lubricants and avoiding preservatives in concomitant eye drops. If DED persists, treatment options include procedures that favor tear retention such as punctal occlusion, moisture chamber spectacles, contact lenses, or pharmacologic agents that stimulate tear secretion. More severe forms may require the use of anti-inflammatory therapy [28]. Although some advances have been made toward alleviating some of the symptoms of DED, pain associated to this condition is not usually addressed. Pain in the eye results from stimulation of sensory axons of the trigeminal ganglion neurons innervating the cornea [29]. Animal models to assess the efficacy of ocular analgesics are extremely complex in terms of interpreting efficacy outcomes [30]. One of the commonly used models to study pain is the capsaicin-induced ocular pain model developed by Gertrudis and colleagues [31]. This model is based on the evaluation of animal behavior after topical ocular administration of capsaicin, a selective agonist of transient receptor protein vaniloid type 1 (TRPV1). Capsaicin applied locally to the eye activates TRPV1 inducing palpebral closure. Latency to open the eye and time required for complete palpebral opening can be used as measurements of the discomfort caused by capsaicin. Reference products used in this model include analgesics, in particular, capsazepine, the antagonist of TRPV1 channels.

3.4. Ocular allergy

Ocular allergies constitute a heterogenic group of diseases with a broad spectrum of clinical manifestations and include mild forms such as seasonal allergic conjunctivitis (SAC) and perennial allergic conjunctivitis (PAC), and more severe manifestations such as vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), and giant papillary conjunctivitis (GPC). The severe forms can be associated to complications such as corneal damage and may cause vision loss. SAC and PAC are commonly IgE-mast cell-mediated hypersensitivity reaction to external allergens, whereas AKC and VKC are characterized by chronic inflamma-
tion involving several immune cell types. In SAC and PAC, allergens, with the help of antigen presenting cells, trigger a Th2-predominant immune response that induces B cells to release IgE. In SAC and PAC, allergen-induced local release of IgE prompts infiltration and degranulation of mast cells in Ca\(^{2+}\)-dependent mechanism. Mast cells liberate preformed inflammatory mediators such as histamine and leukotriene 4 that subsequently attract eosinophils amplify the allergic response [32]. The prevalence of ocular allergies in the general population is estimated to be around 40% in the United States [33] and up to 35% in Europe and the Middle East [34], but it is probably underestimated in most epidemiologic studies [35]. The primary treatment for ocular allergies includes avoidance of allergens, cold compresses, and lubrication. In persisting cases, symptoms can be treated using topical and oral decongestants, antihistamines, mast-cell stabilizers, or anti-inflammatory agents [36]. Allergic conjunctivitis can be modeled in animals by exposing them to allergens in the presence of an adjuvant [37]. The model developed by Magone and coworkers uses Female Balb/C mice that are sensitized with short ragweed and alum and several days later animals receive a topical dose of short ragweed pollen in the eye. A prescreening of mice can be performed in order to select only those animals that respond to allergens.

3.5. Age-related macular degeneration with choroidal neovascularization

Age-related macular degeneration (AMD) is the leading cause of severe vision loss in individuals over 50 years of age [38, 39]. AMD is caused by a combination of genetic and environmental factors. Risk factors include hypertension, cardiovascular disease, smoking, and high BMI. Among the genetic factors that confer susceptibility to developing AMD are variants in genes encoding complement pathway proteins [40, 41].

The underlying cause for AMD is accumulation of drusen or residual material produced by the renewal process of the external part of the photoreceptors of the retina in the retinal pigment epithelium (RPE). The accumulation of this material in the RPE leads to the production of inflammatory mediators that cause photoreceptor degeneration in the macula and severe vision loss [42]. In the early stages of the disease, accumulated drusen are small; the size and amount of this material increase as the disease progresses and central vision deteriorates.

There are two types of AMD: dry or wet. Dry AMD is characterized by the degeneration of the RPE and photoreceptors along with changes in pigmentation of the RPE. In the wet form, or choroidal neovascularization (CNV), fragile blood vessels of the choriocapillaris grow into the RPE and frequently leak blood and fluid that accumulate between RPE and choriocapillaris. As a result of these abnormal growths, dense scars are formed in the macula, and the RPE can detach. The wet form is more severe than the dry form and sometimes dry AMD can develop into wet AMD [43].

The characteristic invasion of leaky blood to the RPE in wet AMD is mediated by VEGF. The discovery of the relationship between VEGF and changes in vasculature in AMD led to the development of different approaches aimed to decrease the levels of this growth factor. Antibodies targeting VEGF are currently the first-line treatment for wet AMD [44]. The hallmark of wet AMD is CNV; thus, this is the lesion most extensively modeled in animals to assess efficacy of compounds targeting this disease. The laser-induced CNV model is by far
the most used animal model. This model, initially developed for nonhuman primates (NHP), was later adapted into rodents. The basis for this model is to induce a break in Bruch’s membrane using a high-energy laser. The experimental CNV can be analyzed in vivo using fluorescein angiography or optical coherence tomography or postmortem studying the retina explants. The model has been successfully transferred and validated to rat and mouse, and in both species, the chain of events taking place after lesion induction resembles the events that take place in humans with the disease. Other models include the injection of subretinal materials such as Matrigel, angiogenic substances, macrophages, lipid peroxides, or polyethylene glycol. Although these models are promising, they have yet to be appropriately validated in order to be used as a proof of concept tools [45].

3.6. Diabetic retinopathy

Diabetic retinopathy (DR) is an ocular complication of diabetes mellitus characterized by microaneurysms in the retinal vasculature that eventually lead to ischemia and macular edema. Changes in the retina can cause rapid vision loss, and this complication is the main cause of visual loss in working-age individuals [46, 47].

The initial phase of DR, known as nonproliferative DR, is characterized by the thickening of the capillary basement membrane and apoptosis and migration of pericytes. These microchanges cause microaneurysms and small leakages in the vessels that irritate the retina. As the disease progresses, interaction between endothelial cells and pericytes weakens and the capillaries become permeable; subsequent accumulation of fluids in the macula leads to edema. The microaneurysms in the retinal capillaries cause occlusions that compromise blood flow through the retina and cause ischemia. Local hypoxia upregulates angiogenic factors that cause capillaries to grow into the retina, preretinal space, and vitreous cavity; stage known as proliferative DR [48]. Among the upregulated angiogenic factors, one of the most critical is VEGF; the newly formed vessels are structurally deficient and very responsive to this growth factor. As such, antibodies used to treat AMD are also used for the treatment of diabetic retinopathy. DR is usually treated with laser photocoagulation, a procedure that does not cure the disease but mitigates the damage. IVT steroids can also be used to reduce accumulation of fluids within the retina. If accumulation of blood in the vitreous humor physically impedes laser photocoagulation, a vitrectomy has to be performed in order to remove the blood accumulated in the vitreous prior to laser photocoagulation.

There are several animal models of diabetic retinopathy, each of them comes with its own set of advantages and disadvantages. One of the most extensively used is the streptozotocin (STZ)-induced diabetes model. Intravenous or intraperitoneal injection of STZ causes a rapid and selective destruction of β-pancreatic cells leading to hyperglycemia and development of type I diabetes. The model has been used successfully in several animal species including rat, mouse, rabbit, dog, and monkey. Nonproliferative DR develops in this model, but microaneurysms and neovascularization are seldom observed; hence, this model can be complemented with the laser-induced CNV model explained in the AMD section. Larger animal models can be generated by surgically removing the pancreas, but this model is significantly more complicated to generate than the STZ model and has the same drawbacks. Alternatively, animals can be fed a high-galactose diet, but the induction of diabetes is considerably slower [50].
4. Biodistribution studies

Despite the extraordinary potential that RNAi technology displays in the treatment of ocular conditions, the transition of siRNAs programs to the clinical setting still presents challenges. The *in vivo* efficacy of therapies based on siRNAs depends on the ability of a given siRNA to reach the cytoplasm of its target cell in sufficient quantities to achieve its desired biological effect. The intrinsic characteristics of siRNAs such as their sensitivity to degradation by endogenous enzymes, their relative large size, and its negative charge limit their ability to cross biological barriers and reach the cytoplasm. Approaches used to overcome the hurdles associated to the use of siRNAs range from delivery strategies to chemical modifications aimed towards improving the pharmacological properties of the therapeutic siRNAs.

Drug delivery into the eye is challenging due to the presence of static and dynamics barriers that protect the internal tissues. The eye consists of two anatomically differentiated regions: the anterior and posterior segments. The anterior segment includes the cornea, conjunctiva, iris, ciliary body, lens, and anterior and posterior chambers; this segment occupies approximately the anterior third of the eyeball. The posterior segment is of greater size and comprises the sclera, choroid, retina, and vitreous cavity. There are significant anatomical, molecular, and immune differences between the two segments; thus, strategies to deliver molecules to the eye will be very different depending on the targeted segment [51]. The anterior region of the eye is protected from exterior aggressions by the cornea and tear film. The former is a specialized tissue composed of five layers that constitutes the main physical barrier to external molecules; the latter is an enzyme-rich fluid that degrades many biological molecules, lubricates the eye surface, and washes away materials from the cornea. In addition, many components of the tear film impede adhesion of molecules to the eye surface further restricting the access of external molecules to the inside of the eye.

Topical ocular administration of drugs is a patient-friendly administration route typically used for the treatment of pathologies affecting the anterior segment of the eye. However, molecules applied as eye drops are quickly cleared from the ocular surface being the bioavailability of a compound administered via this route less than 5% of the initially applied dose. The standard volume of a commercial eye drop is approximately 40 µL whereas the normal volume of the tear fluid in the ocular surface is 7-9 µL. Once an eye drop is instilled in the inferior conjunctival sac, there is a transient increase of volume that activates the blinking reflex and increases the turnover of the tear film. Most of the content of the eye drop is spilled out by the blinking process or drained via the nasolacrimal duct, drastically reducing the amount of compound available to the eye.

The cornea is a specialized tissue covering the anterior part of the eye whose main functions are protecting against harmful agents and provide the eye with a refractive surface that allows the entrance of light. The human cornea is approximately 0.5–0.8 mm thick, and it is comprised of three layers: the outer five cell layer-epithelium, a thick stroma rich in type I collagen fibrils and glycosaminoglycans, and the innermost endothelium consisting in a single layer of cuboidal cells. The corneal epithelium is separated from the stroma by the Bowman’s membrane, while the stroma and the corneal endothelium are separated by the Descemet’s
There are no blood vessels irrigating the cornea; this provides the required transparency for the transmission and refraction of light. Drugs can take two paths to penetrate the corneal epithelium: the intracellular path crossing through the cells or the paracellular path bypassing between cells. The cells of the corneal epithelium are tightly attached to each other with gap and tight junctions that restrict the diffusion of large molecules between them [52–54]. The cross-cellular pathway requires molecules to be able to cross cell membranes; thus, lipophilic molecules have an easier access through this route. The stroma is an aqueous matrix composed mainly of hydrated collagen and proteoglycans with few keratinocytes interspersed [55]. The hydophilicity/lipophilicity index determines the diffusion of molecules through this layer [56]. The remaining layers of the cornea do not significantly hamper the diffusion of molecules.

Contrary to the cornea, the conjunctiva is a highly vasculated tissue that covers the sclera and lines the inner surface of the eyelids. Its main functions are producing mucus and tears to lubricate the eye surface and preventing the entrance of pathogens. The human conjunctiva is composed of three layers: the outer epithelium, the substantia propia, containing nerves and blood vessels, and the submucosa layer, which provides a lightweight attachment to the underlying sclera [57]. The histology of the stratified outer epithelium varies among the different regions of the conjunctiva, but it is always its apical portion that controls the permeability of the conjunctiva. The conjunctiva offers an attractive route for drug delivery when compared to the cornea as it presents an extended exchange surface as well as a superior rate of permeation to large hydrophilic molecules. The sclera is structurally continuous with the cornea and extends posteriorly from the limbus. The composition of the sclera is similar to that of the corneal stroma, mainly collagen and mucopolysaccharides leaving numerous channels through which drugs can freely diffuse [58]. The sclera is poorly vascularized and significantly more permeable than the cornea but less permeable than conjunctiva. There are contradictory reports on the ability of charged molecules to cross the sclera. Some authors suggest that this layer is more permeable to negatively charged molecules [59, 60], whereas other studies suggest that positively charged molecules cross the sclera more easily [61, 62]. Ranta and colleagues suggested that the negative charge of mucopolysaccharides in the sclera prevented the diffusion of negatively charged molecules as a consequence of charge repulsion. Other studies have shown that negatively charged molecules are indeed able to cross the sclera, pointing out that size is the limiting factor in drug diffusion through this layer [52]. It should be noted, however, that scleral drug binding does not necessarily impair drug delivery to inner structures of the eye; it can also act as a drug-depot if the molecules are subsequently released [63].

Ophthalmic drugs topically administered to the eye can thus be absorbed through two pathways: crossing the cornea to reach the aqueous humor or through the conjunctival-scleral pathway reaching the uvea. The relative quantity that enters through each of the above-mentioned routes varies significantly depending on the size and hydrophilic/lipophilic ratio of the molecule. Generally, the conjunctival route is favored for large hydrophilic molecules, whereas small lipophilic drugs are mainly absorbed through the cornea. The ability of siRNAs to penetrate the cornea has been thoroughly demonstrated as well as the ability of these compounds to enter the cytoplasm of cells within the cornea. However, the capacity of siRNAs to cross the cornea is limited, as shown by the limited amount of siRNAs detected in the aqueous humor following eye drop instillation [64].
Increasing the amount of compound in the anterior part may be of interest for treating specific conditions. For this purpose, several strategies can be used in order to improve delivery: (a) increasing the residence time of the compound within the eye surface, (b) directing the molecule to a specific region to increase the concentration locally, and (c) increasing absorption by using physical methods. Increasing the contact time of the molecule with the eye surface can be achieved by the use of formulations or depots. Formulations that increase viscosity and/or mucoadhesion of ophthalmic solutions are generally believed to increase absorption into the eye. Polymers such as methylcellulose or polyvinyl alcohol can be added to solutions to increase viscosity and consequently increase residence and reduce clearance time. Mucoadhesion may be increased by formulating the oligonucleotides in polymers such as chitosans. These polymers have been used to deliver DNA vectors into the eye [65]. Encapsulation in liposomes and in thermostensitive gels has also been attempted as a means to increase the absorption and residence time of oligonucleotides in the eye [53]. In these studies, a 16-mer was formulated in liposomes, a thermostensitive 27% poloxamer gel, and HEPES; the results of these studies showed that the amount of compound reaching external tissues such as the conjunctiva or the cornea was higher when the compound was prepared in HEPES. By contrast, access to deeper regions of the anterior chamber such as the sclera or the iris benefited from the increased viscosity of the gel formulation [53]. One of the main drawbacks of biodistribution studies to assess the fate of a given siRNA in a formulation is that most of these studies focus on the fate of nanocarrier rather than on that of the oligonucleotide and the relative distribution of the molecule among the tissues of the eye. Therefore, thorough biodistribution, studies are required to address the specific characteristics required for improving delivery for specific conditions. Targeting has scarcely been used to deliver oligonucleotides into the eye; there are a few reports using dendrimers with the goal of increasing the intracellular concentration of therapeutic oligonucleotides in specific regions of the eye, but advances toward this goal are as of today very limited [66]. Physical methods such as iontophoresis have also been studied aiming to increasing the amount of molecule that crosses the cornea and/or the sclera. Although iontophoresis certainly increases the amount of transcorneal and transcleral delivery of oligonucleotides mainly to the anterior chamber but also to some degree to the posterior chamber, the use of this method has not been extensively used most likely because the equipment required to apply the required current would entail in-office administration, which would significantly complicate repeated administrations [67].

Drugs administered systemically enter the eye from the bloodstream crossing the capillaries of the choroid. The choroid is a vascular layer composed of capillaries and supported by Bruch’s membrane, a connective membrane of 2–4 µm thickness. Bruch’s membrane separates the choroid from the retina, forming the main barrier to permeation across the choroid-Bruch’s bilayer [68]. The permeability of Bruch’s membrane is relatively high; charge and size do not generally affect drug diffusion through this membrane unless molecules are very big; in this particular case, size can reduce the rate of permeation [52]. The choroid is a thin and permeable membrane that is rich in melanin. Melanin has the ability to bind and retain many drugs hampering their entrance to the retina and inner tissues. Other drug-binding proteins, depending on the kinetic of binding/unbinding retention of drugs by melanin, can completely block the entrance or act as a reservoir for slow release [69]. Studies to assess the binding of oligonucleotides to melanin have yielded different results suggesting that at least some
oligonucleotides bind to melanin reducing the rate of entrance to the retina; this is however not the case for all oligonucleotides [52, 70].

The main restriction to free permeation of molecules from systemic circulation to the eye is the blood–retinal barrier (BRB). The BRB is composed of the inner BRB and the outer BRB. The former includes the vessels of the retina, whereas the latter is constituted by the retinal pigment epithelium (RPE). Both barriers possess cells with well-developed intercellular junctions that control the permeation of substances through them. Larger molecules, such as proteins and nucleic acids, are mostly able to permeate through the choroid but have limited ability to cross the inner BRB; thus, drugs need to exit the choroid and penetrate the eye crossing the outer BRB. Crossing through the outer BRB usually requires high systemic doses increasing the likelihood of systemic side effects [71]. Delivery to the posterior segment of systemic or topically applied drugs requires thus crossing several biological barriers. Therefore, invasive administration procedures are frequently used to deliver drugs to the posterior segment. In addition, the outflow mechanisms of the eye rapidly remove drugs from the posterior chamber; thus, reaching clinically meaningful concentrations is challenging. Most of the programs developing siRNAs for eye conditions target the back of the eye; consequently, the route of administration is IVT injection. The concentration of siRNAs administered IVT is highest in the vitreous body, but they are also found in the RPE, choroid, and retina. Depending on the stability of the siRNAs, the compound can also be found in systemic circulation.

There are numerous reports describing strategies that can be of benefit for increasing the concentration of drugs in the back of the eye. Several nonbiodegradable (Retisert™, Illuvien™, and Vitrasert®) and one biodegradable (Ozurdex™) intravitreal ocular inserts are currently used in the clinical practice for delivering small molecules to the back of the eye. It is expected that these advances be soon incorporated into the pipelines of larger molecules such as proteins and oligonucleotides.

5. Toxicology

siRNAs are chemically synthesized oligonucleotides and are considered New Chemical Entities (NCEs) by the US and European Regulatory Authorities since 2009 when the European Commission excluded siRNAs from the definition of advanced medicinal products [72]. Toxicology assessment of RNAi-based drugs should be carried out following guidelines for NCEs, and the complete toxicology battery is usually performed following the recommendations of the ICH M3 (R2) guideline [73]. The guideline recommends the assessment of toxicology in two species, a rodent and a nonrodent, at three dose levels and for a duration that should be similar or superior to the clinical trial to be carried out. This assessment should include acute or maximum tolerated toxicology studies and repeated-dose toxicity studies. Additionally, pharmacokinetics, safety pharmacology, genotoxicity, carcinogenicity, and specific toxicology studies should be carried out depending of the nature, indication, and route of administration of the product. On the other hand, some aspects of RNAi-based products are closer to new biological entities (NBEs) rather than NCEs; therefore, some of the requirements
of the ICH S6 guideline also apply to the design of their developmental programs [74]. As such, a tailored toxicology assessment program should be designed combining the recommendations outlined in the above-mentioned guidelines and the accumulated experience of numerous compounds tested in preclinical and clinical development.

Toxicology of ocular products depends on their biodistribution and on their biological activity. Moreover, the disease process, age, sex, or eye pigmentation are other potential factors affecting the toxicity profile of the ocular drug assessed. Additionally, the bioavailability of the RNAi compound will depend mainly on the route of ocular administration (topical versus injected) and on the physicochemical characteristics of the drug.

Toxicities arising from oligonucleotides, including siRNAs, can be classified into hybridization-dependent toxicities and hybridization-independent toxicities. Hybridization-dependent toxicities can be caused by (a) exaggerated pharmacology: excessive activity on the intended target or by (b) off-target effect: modulating gene expression of an unintended target by an RNAi-mediated mechanism. Hybridization-independent toxicities are often associated to the chemistry of the siRNA. Identified hybridization-independent toxicities include prolongation of activating partial thromboplastin time (aPTT), complement activation, and immunostimulation [75, 76, 77].

5.1. General toxicology

Up to date, numerous siRNAs indicated for different eye conditions have entered clinical trials (Table 1); the administration route of four of these compounds is by IVT injection, whereas the remaining two compounds are topically administered in eye drops. The toxicology assessment of these products follows the traditional schedule for NCEs; this schedule entails general toxicology studies in two species, a rodent and a nonrodent species of variable length. Most programs up to date have used NHP as the nonrodent species; this is because siRNAs are species specific, and it is likely that the assessment of toxicology was performed in the only species in which the compound was pharmacologically active. The rabbit is very frequently used to assess toxicology of compounds under development for eye conditions. Many sponsors of programs using NHP or dog as nonrodent species chose to use the rabbit as second species, although this animal is not a rodent per se. Reasons behind this choice include the similarity of the volume of the eye to that of humans and the difficulty of administering controlled doses to smaller animals. This is particularly relevant when the compound is administered by IVT injection. This rationale has also been followed for developing siRNAs for eye conditions; only in one case, PF-04523655, rats were used as the rodent species, and the rest of the programs developing siRNAs for eye indications used the rabbit (New Zealand White rabbits or Dutch Belted rabbit) as second species for toxicology assessment.

Most programs developing siRNAs for eye conditions include acute/maximum tolerated dose and repeat-dose toxicology studies. The length of these studies is determined by the indication, stage of development, and envisioned duration of treatment. In addition, most programs do not only perform toxicology studies using the envisaged route of administration but also include studies using intravenous route to challenge the systemic exposure to the drug and assess potential dose limitations and target organs.
5.2. Genotoxicity

As mentioned in the previous section, both the ICH M3 (R2) and the ICH S6 guidelines apply to programs developing siRNAs [73]. The ICH S6 states that the range and type of genotoxicity studies routinely conducted for NCEs are usually not applicable to NBEs; pointing out that performance of these studies is only required when there is a cause of concern. The European Medicines Agency (EMA) issued a reflection paper on the assessment of the genotoxic potential of antisense oligodeoxynucleotides in January 2005. This paper recommends addressing at least two issues in regards to oligonucleotides which may indicate a cause of genotoxic concern: (a) analyzing the potential of incorporation of phosphorothioated (PS) oligonucleotides into the DNA and (b) addressing the potential of triplex formation of oligonucleotides with the DNA fiber [78]. Several years of experience with siRNAs indicate that full-length molecules are very unlikely to interact with the DNA. Thus, the potential cause of concern may arise from the genotoxic potential of metabolites or chemical contaminants. The metabolism of nonmodified oligonucleotides yields naturally occurring nucleotides that are subsequently incorporated into the natural degradation pathways of endogenous nucleic acids; thus, toxicities derived from these degradation products are not expected. Modified oligonucleotides, on the other hand, incorporate very frequently backbone modifications to reduce nuclease activity and improve other pharmaceutical properties of the molecule. The most commonly used backbone modification is the replacement of a nonbridging oxygen on the backbone between two ribonucleotides with a sulfur to create a PS linkage [79]. Extensive genotoxicity studies performed with Vitravene, a PS antisense oligonucleotide administered by IVT injection, indicate that oligonucleotides with a PS backbone do not pose genotoxic potential [80, 81]. These results are in line with those obtained in the analysis of over 30 compounds studied in the standard battery, all of which have yielded negative results. Other modified nucleotides could potentially be incorporated into nucleotide pools and be thereafter used to synthesize DNA. The standard battery of tests would detect eventual damaging potential of these degradation products.

The EMA reflection paper also recommends assessing the potential of triplex formation with the DNA fiber. For this to happen, siRNA molecules would have to enter the nucleus of the cell and their structure should include an uninterrupted homopurine stretch of at least 10–12 base pairs that should be homologous a given region of the DNA. In silico design of siRNAs usually addresses these issues and candidates with the ability of forming triplex are avoided prior to lead selection.

5.3. Carcinogenicity

Standard carcinogenicity studies are generally not required for NBEs, but these studies may be required for siRNAs depending on their chemical structure, clinical dosing, patient population, or biological activity. If the in vitro test genotoxic studies indicate that there is cause of concern for carcinogenic potential, further studies should be required in relevant models.
For RNAi products under development for eye conditions, the systemic bioavailability of these products is usually very low, and a waiver to perform these studies may be justified. Strategies should be discussed in a case-by-case base with the competent health authorities.

5.4. Reproductive and developmental toxicity

The assessment of reproductive and developmental toxicity is required to support the use of a given pharmaceutical in pregnant women, women of childbearing potential, or children. These studies are regulated by the ICH S5 guideline [82], which recommends assessing the effect of drugs on all phases of the reproductive cycle. These recommendations apply to siRNA-based products. Nevertheless, due to the unique features of these compounds, a case-by-case approach should be followed for each product, and the requirements for these studies should be discussed with the competent authorities. The target, indication, chemical modifications, and systemic bioavailability of the RNAi-based drugs are features that may influence in the nature of the required studies.

Because the toxicity of siRNAs can be caused by exaggerated pharmacology whenever reproductive toxicity studies are required, they should be performed in a pharmacologically active species. Standard reproductive toxicity species in rodents or rabbits can give information on toxicity related to chemical structure. However, if the compound is not active in these species or if the biological activity is not deemed to be equivalent to the foreseen activity in humans, the assessment of reproductive risk may be conducted using an active analog or in a nonrodent species in which the compound has biological activity. If the former strategy is chosen, the toxicity and toxicokinetic profile of the surrogate should be taken into account when interpreting the results. If the compound is only active in NHPs, studies should only be performed in cases where there is cause for concern. In these particular cases, the number of animals should be optimized, and a combined enhanced pre- and postnatal developmental study can be performed as recommended for NBEs. In NHP studies, the assessment of reproductive toxicology is usually studied by histopathologic examination of the reproductive organs as part of the general toxicology studies of at least three months. The timing of reproductive and developmental studies depends on when women of childbearing potential are to be included in clinical trials. If NHPs are required for the assessment, the timing is more flexible due to the length and complexity of the studies [83].

5.5. Local tolerance

Local tolerance studies are required for all topically administered drugs. In most cases, the potential adverse events caused by local tolerance issues are evaluated in the single or repeated-dose toxicology studies, reducing the number of animals required for the program.

5.6. Safety pharmacology

According to ICH S7A, safety pharmacology studies can be reduced or eliminated for locally applied products as well as for NBEs that achieve highly specific receptor targeting [84]. For siRNAs under development for ophthalmology indications, separate safety studies are not
usually required; instead, functional safety end points are incorporated into the repeated-dose toxicity studies. If the results of the toxicology studies indicate that there is cause of concern, separate safety pharmacology studies should be performed.

6. Programs in development and future ahead

Table 1 summarizes the status of siRNA-based therapies under development for ocular conditions. As mentioned in Section 1, the eye offers multiple advantages for developing innovative therapies; therefore, studies in the eye pioneered the field of siRNA therapeutics. The first siRNA to enter clinical development for an ophthalmology indication was bevasiranib in 2004 shortly followed by sirna-027. Bevasiranib targeted VEGFA, whereas sirna-027 targeted VEGFR1. These compounds were being developed for the treatment of AMD as both showed a dose-dependent inhibition of experimental CNV in animal models that correlated with knockdown of their respective target genes [10, 85]. As mentioned in Section 1, in 2008 Kleinman and coworkers published a study demonstrating that the effect of siRNAs targeting VEGF and VEGFR on CNV was not mediated by an on-target effect but by activation of TLR3 [12]. The results of these studies indicated that the effect of the compounds on CNV was sequence-independent and mediated by siRNAs of 21 base pairs or longer. The study also showed that the internalization of the siRNAs was not required for the inhibition of CNV as cells of the RPE abundantly express TLR3 on the cytoplasmic surface. The authors used several sequences, including those of the siRNAs undergoing clinical trials at the time, to point out that the inhibition of CNV by both bevasiranib and sirna-027 was mediated through an off-target effect. A subsequent study by the same group showed that activation of TLR3 by IVT siRNAs led to caspase-3-mediated degeneration of the retinal pigment epithelium (RPE) questioning the safety of these compounds as therapeutics for back of the eye diseases [13].

The clinical development of bevasiranib was halted in 2007 and of sirna-027 in 2009 both as a result of not reaching or being unlikely to reach their respective efficacy end points in phase III trials.

The findings of Kleinman and coworkers boosted research on alternative designs that were not able to activate TLR3, and as result, a new generation of compounds is currently undergoing clinical trials. Currently, the most advanced siRNAs-based programs for ocular indications are Quark’s QPI-007 and Sylentis’ bamosiran (SYL040012). QPI-007 is a 19-nt modified siRNA-targeting caspase 2 currently in phase II/III for the treatment of nonarteritic anterior ischemic optic neuropathy (NAION) [86]. QPI-1007 has shown to be safe when IVT injected to animal models and humans. The ongoing phase II/III trial for this compound analyzes the potential of multiple IVT doses to improve visual acuity in patients suffering NAION [87, 88]. Bamosiran is a canonical-designed naked siRNA-targeting β2-adrenergic receptor (ADRB2) under development for the treatment of increased IOP associated to glaucoma [64, 89–91]. Glaucoma is a degenerative, chronic disease of the optic nerve that can lead to blindness if left untreated [92]. The mechanistic details of optic nerve degeneration observed in glaucoma are yet to be fully detailed, but it is well established that reduction of intraocular pressure avoids...
development of the disease. ADRB2 controls the production and release of aqueous humor. The aqueous humor is responsible for maintaining optimal IOP. Treatment with topical beta-blockers has shown to efficiently reduce intraocular pressure, but currently approved beta-blockers are small molecules and are thus able to reach systemic circulation and systemic organs were they cause unwanted effects. The rationale behind bamosiran is developing a locally active compound that efficiently knocks down ADRB2 in the eye but that is not able to reach systemic tissues reducing the likelihood of side effects. The compound is administered in eye drops and has been shown to be well tolerated in animal models and humans [64, 93]. Three different doses of bamosiran are currently being studied in an active controlled phase IIb trial. Previous clinical trials with this compound have shown promising results in healthy individuals and patients with ocular hypertension [91, 93].

SYL1001 is a naked 19-bp siRNA-targeting transient receptor potential vanilloid-1 (TRPV1) for the treatment of ocular pain. TRPV1 is a cation channel permeable to calcium activated by heat, low pH, and capsaicin among other signals. This receptor is present in several structures of the eye where it has been related, among other roles, to nociception [94]. SYL1001 has shown to be safe when administered in eye drops to animals and humans and to have analgesic effect in the capsaicin-induced eye pain model. The compound is currently undergoing a phase I/II for the treatment of ocular pain associated to dry eye disease, a condition for which no specific treatment currently exists [89].

PF-655 is a chemically stabilized siRNA-targeting RTP801, a stress-induced adaptor protein that inhibits mTOR function upstream to TSC1/TSC2 complex in response to a variety of stresses. Expression of RTP801 is upregulated in response to ischemia, hypoxia, and/or oxidative stress. Intravitreal injection of PF-655 in preclinical animal models of laser-induced CNV leads to silencing of RTP801 via a RNAi mechanism without TLR activation and reduction of CNV volume, vessel leakage, and infiltration of inflammatory cells into the choroid [95–97]. This compound has undergone phase II clinical trials for the treatment of diabetic macular edema and wet AMD. Treatment with PF-655 of patients with diabetic macular edema over a period of 12 months caused a dose-dependent improvement in visual acuity compared to the visual acuity observed in patients treated with laser photocoagulation [98]. A subsequent phase Ib trial was conducted with a new set of doses but terminated because the primary end point was not likely to be achieved. The compound was thereafter tested in combination with ranibizumab, a monoclonal antibody fragment that targets VEGF and is the current gold standards for treatment of the disease. The results of this study have not yet been disclosed. PF-655 has also been studied in patients suffering wet AMD. In this indication, the compound did not show improvement as a single agent or in combination with ranibizumab in mean visual acuity after 3 months of dosing.

Self-delivery rxRNAs (sd-rxRNAs) incorporate 2’-F and 2’-O-Me modifications and a sterol conjugate on the sense strand with the goal of improving stability and cellular uptake. These compounds have a 19-nt antisense strand and a sense strand usually shorter than 15 nt resulting in an asymmetric duplex with a phosphorothioated single-stranded tail on the antisense strand [99]. These compounds have been tested in vitro where they have shown to be able to induce target knockdown in different cell lines. In vivo analysis of their activity
showed these compounds are readily taken-up by retinal cells and that the compound is evenly distributed throughout the mouse retina. Several of these compounds are under development for different eye conditions and are expected to enter clinical development shortly.

SYL116011 is a naked 19-bp siRNA targeting the calcium release-activated calcium modulator 1 (ORAI1). Store-operated Ca\(^{2+}\) entry (SOCE) is activated in response to depletion of endoplasmic reticulum Ca\(^{2+}\) pools. Activation of SOCE induces Ca\(^{2+}\) entry from extracellular compartments, and this is mediated by store-operated CRAC channels. CRAC channels are composed of calcium sensing proteins called STIM (stromal interaction molecule) and pore-forming subunits named ORAI [100]. Mammalian cells have three ORAI isoforms: ORAI1, ORAI 2, and ORAI3; although ORAI2 and 3 fulfill the same role as ORAI1, the Ca\(^{2+}\) currents generated by these proteins are around two- to threefold smaller than the ones generated by ORAI1 [101]. There is growing evidence that indicates that short-term and long-term activation of immune cells in allergic responses is mediated by influx of Ca\(^{2+}\) to immune cells from the extracellular compartment. Short-term responses include the degranulation of mast cells and the activation of effector cytolytic T cells. Indeed, mast cells lacking either STIM1 or ORAI1 show a considerable defect in degranulation [102, 103]. Long-term responses involve the modulation of gene expression that controls B and T cell proliferation and differentiation. SYL116011 is being developed for the treatment of ocular allergies and has shown to reduce immediate clinical signs in a mouse model of ragweed pollen-induced ocular allergy. The decrease in clinical signs was accompanied by a reduction in the number of infiltrating eosinophils in the conjunctiva and reduction of allergy biomarkers.

TT-211 is an AAV-encapsidated construct that expresses a single shRNA modeled into a miRNA backbone that inhibits the expression of VEGF-A for the treatment of wet AMD. VEGF-a protein is responsible initiating a signaling cascade that stimulates the growth of new blood vessels, a hallmark of wet AMD. TT-231 is a second-generation candidate designed to express three shRNAs, which target three different genes, VEGF receptor 2, PDGF-β, and human complement factor B, proteins that play a major role in the progression of wet AMD. Both these compounds are yet in a preclinical phase; IND filing is planned for 2017.

STP601 is a multitargeted siRNA cocktail nanoparticle formulation administered by IVT injection under development for treatment of wet AMD, proliferative diabetic retinopathy, and herpetic stromal keratitis. The cocktail includes three 25-mer siRNA duplexes targeting VEGF, VEGFR1, and VEGFR2. Inhibiting this clinically validated pathway at the endothelial cells lining the interior of the growing blood vessels is thought to halt the progression of AMD. This product is currently in preclinical stage.

AQA001 is a single-stranded long chain nonmodified ribonucleotide connected by a proline-derived linker that self-anneals to form a shot-hairpin structure within the molecule. The compound targeting periostin acts through an RNAi mechanism and is being developed for the treatment of diabetic retinopathy. The compound has shown positive result in a proof-of-concept study of CNV [104].
<table>
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<th>Name</th>
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Table 1. siRNAs in development for ocular indication.

7. Conclusion

RNA interference is on the verge of becoming a new class of therapeutics [105]. The field of ophthalmology has played a major role in advancing siRNAs from laboratory tools to the clinic. In the last few years, significant advances have been made in the understanding of how these molecules enter and exert its action in the eye and in the identification of the main hurdles that still need to be addressed. The introduction of chemical modifications as well as the understanding of the immune activation in the eye has significantly improved the pharmaceutical properties of compounds for eye conditions. However, the following years will tell whether
improvements on these molecules are enough to be of therapeutic value in the field of ophthalmology or not.

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