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# Recent Advances in Ocular Nucleic Acid-Based Therapies: The Silent Era

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Additional information is available at the end of the chapter

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## 1. Introduction

The central dogma of biology describes the transfer of biological information from DNA through to protein (1). In the first phase, known as transcription, DNA is converted into a complementary sequence of messenger RNA (mRNA). This mRNA allows the genetic message to be communicated outside of the cell nucleus, to other areas of the cell, where it is then translated into protein by ribosomes. Post-transcriptional regulatory events take place after an RNA molecule is formed; thereafter the resulting RNA molecule is decoded to produce a specific protein. Protein production depends on the length of survival of RNA in a cell and the efficiency of its utilizations.

Since Paterson et al. first demonstrated the utility of nucleic acids in modulating gene expression over 30 years ago (2), and in 1978 Zamecnik and Stephenson showed the capacity of antisense molecules to inhibit viral replication (3, 4), nucleic acids have emerged as a potent force both as R&D tools and as therapeutic agents.

Indeed, the field of nucleic acid therapeutics has evolved considerably with numerous gene targets and methods having been applied *in vitro* and *in vivo* in a variety of contexts with varying degrees of success. Strategies have included ribozymes, DNA enzymes (DNAzymes), antisense oligonucleotides (ASON), decoys, aptamers and siRNAs, all of which attenuate gene expression by interfering with cytosolic mRNA or translated protein. Currently, a number of these approaches are being evaluated in human and animal trials and are poised to offer considerable inroads and additions to our current therapies.

Ribozymes are catalytically active RNA molecules capable of site-specific cleavage of target mRNA and can occur naturally (5). They must contain antisense sequences that will bind to the target, and also a sequence that will fold into a structure with ribonuclease activity. Such sequences are found in natural hammerhead or hairpin ribozymes. Consequently,

ribozymes don't depend on cellular nucleases for activity (6). The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications.

DNAzymes, like ribozymes, may be perceived as gene-specific molecular scissors. They appeared as a development in the study of ribozymes using analogous deoxyoligonucleotides, given that catalytic DNA has not been observed in nature (7). All existing molecules have been derived by *in vitro* selection processes similar to those used to identify aptamers (see below). The most well-characterized DNAzyme is the "10-23" subtype comprising a cation-dependent catalytic core of 15 desoxyribonucleotides that binds to and cleaves its target RNA. This core is flanked by complementary binding arms of 6 to 12 nucleotides in length that confer target mRNA Specificity (8).

ASONS are single-stranded segments of DNA or RNA generally 15 to 25 nucleotides in length designed to mirror specific mRNA sequences and block protein production. Although their precise mechanism of action is not fully understood, their function is mediated by interaction with target mRNA via hydrogen bonding, blocking translation into protein by steric hindrance of ribosomal movement or by activation of endogenous RNase H for targeted destruction of the DNA/RNA heteroduplex, resulting in mRNA degradation (9). Unmodified ASONs molecules are prone to degradation, and their negative charge makes cellular membrane penetration inefficient. As such, these molecules have evolved with a variety of modifications that enhance stability and efficacy. Around 50 clinical studies have used antisense strategies spanning a variety of disease processes, including cancer, cardiovascular disease, inflammation and infection (10). Fomivirsen or Vitravene®, which targets the immediate-early RNA encoded by human cytomegalovirus (CMV) DNA, has been approved by the FDA for use in humans for treatment of CMV retinitis (11).

In contrast to antisense approaches that target mRNA, oligonucleotide decoys are short, double-stranded DNA molecules that contain binding elements for a variety of protein targets that competitively inhibit promoter binding and gene expression. Although several types of decoys have been developed there are important issues to take into account affecting the potential clinical use of these molecules, including susceptibility to nuclease degradation, propensity to induce a host immunological response, and cell transfection difficulties with higher concentration requirements (12). Currently, decoy oligonucleotides are not pursued as aggressively as other forms of therapeutic oligonucleotides (13).

Aptamers are single-stranded oligonucleotides which may fold into complex secondary and tertiary structures and bind their target protein with high affinity and specificity, inhibiting its function. They are derived by *in vitro* selection from a combinatorial library of nucleic acid sequences (14). Recent developments demonstrate that aptamers are valuable tools for diagnostics, purification processes, target validation, drug discovery and therapeutics. Macugen® is an aptamer approved by the FDA for treatment of wet AMD (15).

RNA interference (RNAi) is one of the gene silencing strategies which has received most attention in recent times. Its initial discovery in 1998 by Nobel laureates Fire and Mello (16)

in *Caenorhabditis elegans*, led to the subsequent finding of the mechanism in mammalian cells and the understanding that it was triggered by small double stranded RNA duplexes of 19-23 nucleotides in length (17). In the following decade siRNA (short interfering RNA) have become widely used tools for silencing gene expression, as they make use of a naturally occurring regulatory mechanism that uses these molecules to direct homology-dependent mRNA degradation via a multienzymatic complex termed RISC (RNA-induced silencing complex). Cells use their own small RNA duplexes as a form of gene regulation. These novel molecules have enormous therapeutic potential and at present there are close to 20 compounds which have reached clinical trials (18).

## 2. Ocular diseases addressable by nucleic acid-based drugs

### 2.1. Glaucoma

Glaucoma is the second leading cause of blindness globally (19). By year 2020 almost 80 million people are estimated to be affected by primary open-angle glaucoma (POAG), the most common type of glaucoma. Glaucoma is defined as the process of ocular tissue destruction caused by a sustained elevation of intra ocular pressure (IOP) above its normal physiological limits (20). In open angle glaucoma (OAG), elevated IOP causes a progressive optic neuropathy due to loss of retinal ganglion cells that ultimately leads to blindness (21). In angle-closure glaucoma the sudden high rise in IOP often renders the eye blind. Blindness in glaucoma is caused by a degenerative process of the retina and optic nerve, but is functionally associated with impairments in the balance between aqueous humor (AH) secretion and outflow. Mechanistically, the changes observed in the trabecular meshwork include loss of cells as well the deposition and accumulation of extracellular debris including protein plaque-like material (22). The loss of vision is not usually evident until significant nerve damage has occurred. For this reason, up to half of glaucoma sufferers are unaware of their condition. Because of this, early diagnosis and treatment is crucial in halting the progression of this pathological condition (23). Risk factors associated with glaucoma include high intraocular pressure, advanced age, family history of glaucoma, African ancestry myopia, hypertension, morphologic features of the optic disc, thinness of the cornea, eye trauma, concomitant use of drugs, diabetes mellitus, hypothyroidism, cardiovascular and haematological abnormalities (24-28). Treatment of glaucoma is mainly focused on lowering IOP. The first line of treatment is medication, followed by surgical and laser treatment. Pharmacological compounds for treating glaucoma fall into four main classes of drugs: parasympathomimetics, antagonists of  $\alpha$  and  $\beta$ -adrenoreceptors, inhibitors of carbonic anhydrases and prostaglandin analogues. Clinicians prescribe medications in a stepwise manner to achieve the target goal of IOP and maintain a balance between medication effectiveness, tolerability and safety. Beta blockers and prostaglandins are the standard first line treatment for POAG (29). Both groups of drugs are very efficient in lowering IOP but beta blockers are not recommended in patients with cardiovascular and respiratory problems and prostaglandin-analogs have local tolerability issues (30, 31). Second-line drugs of choice include alpha-2-adrenergic agonists and carbonic anhydrase inhibitors. When IOP is not adequately regulated with monotherapy it is common to

combine different antiglaucoma drugs, these combinations appear to have the advantage of greater efficacy, better cost and safety, but limit the individualization of dosing. The efficacy of current IOP-lowering therapies is relatively short lived, requiring repetitive dosing throughout the day and in some cases the efficacy decreases with time. Such negative effects may lead to decreased patient compliance or to the end of treatment. If no efficacy in reducing IOP is achieved with any of these drugs, laser therapy may be applied to the trabecular meshwork in order to increase AH outflow. The last therapeutic resource is surgical intervention to create a new route for AH outflow (32).

Novel nucleic-based therapies seek improving the limitations of current antiglaucoma treatment. These are the goals pursued by Sylentis' SYL040012, an siRNA-based drug for the treatment of glaucoma, currently in phase II clinical trial. SYL040012 is a chemically synthesized double-stranded oligonucleotide, specifically designed to target and inhibit synthesis of  $\beta_2$ -adrenergic receptors. The compound has proven efficacious in inhibiting the expression of its target in cell cultures and in lowering IOP in normotensive and hypertensive rabbits (Martínez et al, unpublished results). The efficacy of SYL040012 in reducing IOP is similar to that of commercially available drugs (34). On the other hand, when commercial drugs are used, sustained reduction of IOP relies on the continuous application of the drugs. This is not the case with SYL040012, whose effect in animal models not only lasts 15 times longer than the effect of current treatments, but is also able to maintain the reduction in IOP levels even when the compound is not administered for a period of up to 72 hours. This feature is very attractive since it would protect against the eventual optic damage caused by a reboot effect on IOP in case of poor compliance with the treatment. Moreover, SYL040012 is stable up to 24 h in rabbit aqueous humour, but stability rapidly decreases in rabbit serum. The stability properties of SYL040012 support the use of a siRNA-based therapeutic approach for glaucoma since the compound should be stable enough to exert its effect in the eye but should be rapidly degraded when reaching general circulation, hence anticipating low systemic side effects.

Using a completely different approach, Quark pharmaceuticals is developing QPI-1007, a synthetic chemically modified siRNA designed to temporarily inhibit the expression of the pro-apoptotic protein Caspase 2. QPI-1007 is currently undergoing clinical trials for optic nerve atrophy and non-arteritic ischemic optic neuropathy, but the company has expressed interest in developing adequate delivery systems for the potential use of this drug as a neuroprotectant for glaucoma (35).

## 2.2. Age-Related Macular Degeneration (AMD)

Age-related macular degeneration (AMD) is the leading cause of severe vision loss in the Western world, it occurs primarily among individuals over 50 years of age (36). AMD was a rare disorder in the 19th century but increase in life expectancy together with changes in life-style have enormously affected the prevalence of this disease (37). According to the 2005-2008 National Health and Nutrition Examination Survey 6.5% of the US population over 40 years of age had signs of AMD (38). Extensive epidemiologic and genetic studies indicate that development of AMD is the result of a combination of several aspects such as



genetic predisposition and environmental factors. Tobacco smoking seems to be the most consistent and modifiable risk factor (39). Other risk factors include hypertension, cardiovascular disease and high body mass index (40). Among the genetic factors that confer susceptibility to developing AMD are variants in several genes encoding complement pathway proteins (41).

The underlying cause for AMD seems to be accumulation of 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 undegraded material, known as *drusen* in the RPE leads to production of inflammatory mediators that cause photoreceptor degeneration in the central retina, or macula (42). The center of the macula, named fovea, mediates high acuity vision; hence its degeneration causes severe vision loss. In the early stages of the disease the accumulations of *drusen* are small and often observed along with hypo- or hyperpigmentation of the RPE. As the disease progresses both the size and the amount of *drusen* increase. Advanced AMD is characterized by the presence of large or several medium size *drusen* and loss of central vision and can take two forms: dry or wet. The dry form is characterized by a sharply delineated area of RPE atrophy along with loss of photoreceptors and changes in pigmentation of the RPE. In the wet form, or choroidal neovascularisation (CNV), fragile blood vessels of the choriocapillaris grow into the RPE and frequently leak blood and fluid that accumulate between RPE and the choriocapillaris. As a result of these abnormal growths, dense scars are formed in the macula. Detachment of the RPE is also a frequent feature of this form of AMD. The wet form is more severe than the dry form and sometimes dry AMD can develop into wet AMD.

Great advances have been made in recent years in the treatment of wet AMD; on the other hand treatment options for dry AMD are currently limited to dietary supplements and lifestyle changes. Several pharmaceutical companies are developing compounds for dry AMD by targeting different aspects of the physiopathological progress of the disease. As mentioned above, inflammatory mediators are generated during the process leading to dry AMD, for this reason it has been suggested that antioxidants may play a role in minimizing progression of the disease. The Age-Related Eye Disease Study (AREDS) demonstrated that oral supplementation of antioxidants in patients with unilateral intermediate or advanced AMD reduced vision loss by 19%. In addition these patients showed a decrease of 25% in the chances of developing AMD in the other eye (43, 44).

Other therapeutic approaches for dry AMD currently in clinical development are aimed towards reducing the accumulation of toxic metabolites, diminishing activation of the complement system or avoiding loss of neurons in the retina using neuroprotectants.

As of March 2012 there were approximately 10 clinical studies for pharmaceuticals under development to treat dry AMD registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) with status "recruiting" or "active". Among these clinical trials, only one is using an oligonucleotide based approach to treat dry AMD. ARC1905 is a pegylated aptamer that is currently in phase I study sponsored by Ophtotech Corporation. This compound antagonizes the cleavage of complement component C5 into C5a and C5b, thus inhibiting complement activation.

ARC1905 has shown to be safe when administered by intravitreal injection in combination with ranibizumab for wet AMD (45).

Perhaps one of the facts preventing more oligonucleotide based therapies being developed for dry AMD is that doublestrand RNAs as short as 21 base pair are able to bind and activate Toll Like Receptor 3; and activation of this receptor has been associated with progress of dry AMD (46, 47).

Wet AMD is characterized by the invasion of leaky blood vessels into the RPE. This mechanism is mediated by the action of vascular endothelial growth factor (VEGF). The biological activity of this growth factor has been the target of several therapeutic strategies in the past few years and the pharmaceuticals developed under these programs are the current first line treatment for wet AMD.

Pegaptanib sodium (Macugen<sup>®</sup>) is a 28-base pegylated aptamer designed to target VEGF (48) by Gilead Pharmaceuticals, licensed to Eyetech Pharmaceuticals/OSI. This drug was approved by the FDA in December 2004 for the treatment of all subtypes of wet AMD by intravitreal injection every six weeks. Pegaptanib specifically binds to VEGF<sub>165</sub>, the most pathogenic of the four isoforms of VEGF that are generated by alternative splicing of a common mRNA. The union of pegaptanib to VEGF prevents activation of either of the two receptors for VEGF (VEGFR-1 and VEGFR-2) present on the surface of epithelial cells; hence inhibiting its biological activity. The VEGF Inhibition Study in Ocular Neovascularizations trial demonstrated that pegaptanib sodium injection reduced the risk of moderate vision loss from 70% to 55% and of severe vision loss from 22% to 10% without serious systemic effects and a low rate of serious ocular adverse events (49).

In July 2006 the second drug based on anti-VEGF therapy was approved by the FDA. Ranibizumab (Lucentis<sup>®</sup>) is a recombinant, humanised, monoclonal anti-VEGF antibody fragment with high affinity for all isoforms of VEGF developed by Genentech that is administered by intravitreal injection. Two clinical trials (MARINA and ANCHOR) support the safety and efficacy of ranibizumab for wet AMD (50, 51). The results of these trials showed that almost 95% of patients receiving the drug avoided moderate visual loss versus the 62.2% that managed to do so in the control group (receiving veteporfin). In addition, ranibizumab was not only able to stall progression of wet AMD but even to improve visual acuity in 35.7% of the patients treated with the low dose and in 40.3% of the patients treated with the high dose.

The third anti-VEGF compound currently used in the clinic for treatment of wet AMD is bevacizumab (Avastin<sup>®</sup>). Bevacizumab is a full-length monoclonal antibody that binds to and inhibits all isoforms of VEGF also developed by Genentech. Bevacizumab is in fact the full length antibody that was modified to develop ranibizumab. Ranibizumab was developed because preliminary results indicated that the size of the full length antibody would not allow for appropriate distribution within the retina. Additionally, the longer half life of bevacizumab raised concerns in terms of systemic toxicity. Bevacizumab is currently approved for the treatment of metastatic colorectal cancer and several other malignancies,

and it is used off-label for wet AMD (52). The reduced cost of bevacizumab compared to ranibizumab has extended the off-label use of this drug, for this reason several clinical trials have started in the last four years in order to gather evidence on the efficacy of bevacizumab in wet AMD (53, 54).

In addition to pegaptanib, several other attempts have been made to treat wet AMD with oligonucleotides. Bevasiranib was the first small interfering RNA agent developed for this condition. Developed by OPKO Health, this agent is a naked 21-nt siRNA designed to target VEGF administered by intravitreal injection (55). The results of clinical trials conducted for phases I and II were positive and OPKO Health initiated a phase III clinical trial to examine the safety and efficacy of the combination of bevasiranib with ranibizumab. In March 2009 OPKO terminated the phase III trial because the primary endpoint was not likely to be met: improved efficacy over ranibizumab.

Following the same VEGF-inhibition strategy Sirna Therapeutics (now Merck) developed Sirna-027; a modified siRNA that targets one of the receptors for VEGF, VEGFR-1 (56). In vivo studies demonstrated that Sirna-027 was able to reduce the mRNA and protein levels of VEGFR-1 and to reduce the areas of neovascularization in a mouse model of ischemic retinopathy. An inverted sequence with the same chemical modifications as Sirna-027 was used as control and was shown not to have an effect on any of the parameters analysed (56). The control used in this study demonstrated that the effect of Sirna-027 was sequence specific; this was relevant due to the controversy generated by the results of a study published in 2008 that stated that angiogenesis could be suppressed by 21-nt siRNAs in a sequence-independent manner via TLR3 (57). Further clinical development of Sirna-027 was sponsored by Merck in collaboration with Allergan but, again, clinical trials for this compound were halted because the primary endpoint of efficacy trials was not met.

Using a different strategy Quark developed PF-655, now developed in collaboration with Pfizer, a 19-nt 2'-O-methyl-stabilized siRNA designed against RTP801, a newly discovered target that is rapidly and sharply upregulated in hypoxia and promotes apoptosis of neural cells (58, 59). In addition to its anti-apoptotic effect, this compound cooperates with VEGF inhibitors to reduce retinal neovascularisation. Although full results of the clinical trial have not yet been made public, in March 2011 Quark announced that their compound was not superior to ranibizumab, thus expectations for the phase IIb initiated by Quark alone are somewhat low.

With two of the candidates already halted and a third most likely to be so, the fate of siRNAs in AMD is at least uncertain. Reports supporting that siRNAs 21 nt or longer activate TLR3 have certainly had a say in the matter. Activation of TLR3 in the RPE seems to induce RPE cell death and contribute to development of dry AMD (47), therefore tackling any form of AMD with double stranded RNA has to be achieved with shorter compounds and target specificity should be thoroughly studied.

### 2.3. Diabetic retinopathy

Diabetic retinopathy (DR) is a microvascular complication secondary to diabetes mellitus that leads to structural and functional changes in the retina, this complication is the leading



cause of visual loss in working-age individuals (60). The overall prevalence of this complication among individuals with diabetes is 34.6% (61) and it accounted for 2.4 million cases of blindness worldwide in 2002 (60). Hyperglycemia is a decisive factor in the development of DR because of its damaging effects *per se*; it also serves as a biomarker for control on the dysregulation of metabolism that occurs in diabetes.

In the initial phases of DR the vessels that irrigate the retina show microaneurysms and small leakages, this initial step of the disease is frequently referred to as nonproliferative DR. The changes observed in this phase are result of thickening of the capillary basement membrane as well as apoptosis and migration of pericytes. As the disease progresses the capillaries become permeable due to loss of interaction between endothelial cells and pericytes, and eventually macular edema can develop due to accumulation of fluids in the macula. The progressive occlusion of capillaries in the retina leads to tissue ischemia. As a result of the hypoxia caused by ischemia angiogenic factors such as VEGF are upregulated and capillaries grow into the retina, this stage is also known as proliferative DR (62). The new veins formed in response to the changes seen in the diabetic retina are fragile and permeable and some of them can break through the optic nerve leaking into the vitreous cavity or into the preretinal space. The process of neovascularisation is also associated with accumulation of a fibrous component that when contracted can lead to retinal detachment severely impairing vision (63).

Currently, the gold-standard for treating DR is laser photocoagulation (64); this procedure seeks to mitigate damage but has no effect on the underlying causes of the disease. In addition, steroids can be intravitreally administered into the eye to reduce accumulation of fluids within the retina. Sometimes, the accumulation of blood in the vitreous humour can physically impede laser photocoagulation; in these cases a vitrectomy can be performed in order to remove the blood accumulated in the vitreous prior to laser photocoagulation.

As mentioned previously, the action of angiogenic factors plays an important role in the development of DR. In this sense, several VEGF-inhibitors currently used for the treatment of AMD, are being studied for DR (see section 2.2 for details). Most of them are in late clinical trials and are expected to reach market authorization shortly.

In the oligonucleotide-based field, some of the compounds initially developed for AMD have undergone clinical trials for different forms of diabetic retinopathy as well. Opko Health's bevasiranib is one of these cases. A phase II clinical trial for diabetic macular edema was completed in 2008, and although positive results were reported, further development has not been announced. iCo Therapeutics is using a different approach with their lead compound iCo-007. iCo-007 is a chemically modified ASON inhibiting c-raf; a downstream mediator of several growth factors, including VEGF. In 2011 a phase II clinical trial was started for diabetic retinopathy in which two different doses of iCo-007 are to be assayed, either as a monotherapy or in combination with ranibizumab or laser photocoagulation. Preliminary results of this trial are expected in the second half of 2012. Another antisense alternative is being developed by Antisense Therapeutics, in this case a novel antisense compound ATL1103 which targets growth hormone receptor and has successfully

completed a phase I clinical trial. This compound is being developed for treatment of acromegaly and also diabetic retinopathy (65).

## 2.4. Dry eye pain

Dry eye disease (DED) or keratoconjunctivitis sicca (KCS) is a multifactorial ocular condition resulting from tear film instability that can eventually lead to ocular surface damage. Typical symptoms of DED include ocular discomfort, visual disturbance, itching, burning, sensation of foreign body, light sensitivity; inflammation and pain. Factors contributing to DED are insufficient tear secretion; excessive evaporation and alteration in the composition of the tear film. The tear film has three essential components: aqueous layer, secreted by the lachrymal glands; mucus layer, produced by the goblet cells of the conjunctiva and by epithelial cells of the cornea and conjunctiva and finally a lipid layer, secreted by the meibomian glands. Changes in the tear film can be temporary causing an acute form of DED or long-lasting leading to chronic DED; damage to the ocular surface is usually more severe in the chronic forms than in the acute ones. DED is frequent in some 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. As such, it is not surprising that DED is a very frequent condition; the prevalence varies tremendously depending on the study, and the condition is more frequent in patients with autoimmune diseases, postmenopausal women and elderly population (66).

Treatment of DED depends on the etiology of the condition. The first line treatment is usually use of lubricants such as artificial tears and avoidance of preservatives such as BAK if other eye treatments are required. Other treatment options include procedures that favour tear retention: punctal occlusion, moisture chamber spectacles and contact lenses; pharmacologic agents that stimulate tear secretion or anti-inflammatory therapy.

Although some advances have been made towards alleviating some of the symptoms of DED, pain associated to this condition is not usually addressed. Sylentis is currently developing an siRNA, SYL1001, for the treatment of pain associated to DED. The compound targets TRPV1, a very well known target for pain, which is highly expressed in the cornea and trigeminal ganglion. SYL1001 is applied in eye drops, contains no preservatives and has recently shown favourable local and systemic tolerance results in a phase IA study.

## 2.5. Corneal neovascularitation associated with corneal graft rejection

Optimal vision is contingent upon transparency of the cornea. Corneal neovascularization, trauma and surgical procedures such as photorefractive keratectomy and graft rejection after penetrating keratoplasty (PKP) can lead to corneal opacification (67). Corneal neovascularization, regardless of the underlying cause, leads to decreased vision, recurrent corneal erosion, and incompetent barrier function thus presenting a serious clinical problem for which treatment is poor (68). When transparency of the native cornea cannot be maintained at a functional level corneal transplantation is often the next intervention. Once transplanted, the major cause of corneal graft failure is allograft rejection. Despite this fact,

corneal transplantation has a very high success rate. Over 90% of low-risk corneal transplants retain clarity years after transplantation using only local immunosuppression. Blocking access of the host immune system to the donor cornea is the first line of defense against corneal allograft rejection, of which corneal avascularity is an essential component. Corneal graft rejection is primarily a cell-mediated immune response controlled by T cells (69). Normal corneal immune privilege can be eroded by neovascularisation, especially if it is accompanied by ocular inflammation and increased intraocular pressure. New vessels generated by neovascularisation provide a route of entry for immune-mediating cells to the graft, while the growth of new lymphatic vessels enables the exit of APCs and antigenic material from the graft to regional lymph nodes. The cornea consequently becomes infiltrated with and sensitized to immune reaction mediators. Therefore, neovascularisation may induce an immune response that can lead to immunological corneal graft rejection (70, 71). In normal low risk grafts it is a general practice to avoid exposing suture knots and ends which may stimulate neovascularization, and to treat neovascularization aggressively using topical steroids (67).

Due to the crucial role of VEGF in neovascularisation (see section 2.2), many have suggested that VEGF inhibition may prevent corneal transplant rejection. Despite the efforts made in recent years on the use of anti-VEGF compounds for corneal rejection it is still not clear whether the treatment is adequate (72, 73).

Several nucleic-based therapies are currently being developed with the hope of filling this uncovered therapeutic gap. Although the molecular mechanisms that control neovascularization are not well understood, inflammation has been found to frequently precede corneal neovascularisation. For this reason, many studies have concentrated their efforts on targeting humoral and corneal-derived inflammatory mediators. Among these mediators are arachidonic-acid derived eicosanoids of the cytochrome P450 monooxygenase (CYP) pathways. Seta and collaborators designed specific siRNAs targeting CYP4B1a, CYP4B1b and CYP4B1c and demonstrated that silencing the expression of CYP4B1 diminished corneal vascular response and greatly attenuated VEGF mRNA levels in an animal model of inflammatory neovascularization (68). One of the most advanced nucleic-based therapies is GS-101 (Aganirsen). GS-101, developed by the Swiss company GeneSignal, is a 25-mer phosphorothioate ASON that inhibits the expression of Insulin Receptor Substrate-1 (IRS-1). IRS-1 is a cytoplasmic adapter protein without intrinsic kinase activity. The main function of this protein is to recruit other proteins to their receptors and induce the organization of intracellular signalling cascades (74). IRS-1 interacts with the VEGF-receptor complex in angiogenesis (75) and it promotes lymphangiogenesis by interacting with integrins (76, 77). Downregulation of IRS-1 results in prevention of neovascular growth and has been reported to prevent the angiogenic process in preclinical *in vivo* and *in vitro* experiments (78, 79). Phase I clinical studies demonstrated excellent safety and tolerability of GS-101 when applied as eyedrops three times a day. In April 2007 the EMA granted orphan drug designation for GS-101 for the treatment of corneal graft rejection associated to corneal neovascularisation. After successfully completing a phase II trial GS-101 is about to enter phase III clinical trials (80).

## 2.6. Ophthalmic infections

There are several sight-threatening diseases caused by viral infections. Infections caused by virus imply the use of the cellular machinery by the virus to replicate. Hence, all infected cells will have foreign nucleic acids encoding viral specific proteins inside them. Given that oligonucleotide based therapies pursue downregulating expression of genes it seems obvious to target viral specific genes. In the following section several approaches using this rationale will be explained focusing on antivirals developed for treating viral infections in the eye. The first success case of oligonucleotide-based therapies came precisely in this field, fomivirsen was the first compound based on oligonucleotides to be approved by the FDA.

Citomegalovirus-induced retinitis is among the most common opportunistic infections in severely immunocompromised patients (81). In AIDS patients, CMV infection is associated with gastrointestinal disorders and retinitis. An estimated 15 to 40% of AIDS patients will suffer CMV-induced retinitis. This condition if left untreated leads to blindness within six months (82). Three systemic compounds are available for the treatment of this disease: ganciclovir, foscarnet, and cidofovir. The mechanism of action of these three compounds is inhibition of the CMV DNA polymerase. However, these treatments have some drawbacks such as limited efficacy, poor oral bioavailability, toxicity and emergence of multidrug resistant strains due to mutations in the target gene (83). Because of this, effective therapy usually implies alternation between the three available antivirals or different combinations of them, along with intraocular drug injections. This approach, known as highly active antiretroviral therapy (HAART), reduces 55%-95% the number of new cases of CMV (81). Without acute therapy, retinitis spreads throughout the entire retina causing total retinal destruction and blindness; without chronic suppressive maintenance therapy relapse of the retinitis occurs promptly (84). Looking for an alternative approach Isis Pharmaceuticals Inc. developed an ASON that targets the *immediate-early* (IE) gene of human CMV. Fomivirsen, (Vitravene®) is a phosphorothioate oligonucleotide (ISIS-2922) developed by Isis in partnership with CIBA vision, for the treatment of newly diagnosed and advanced CMV retinitis in AIDS patients and was the first oligonucleotide based drug approved by the FDA in 1998. Administered by intravitreal injection it has shown good clinical activity against this disease (85).

Acute Retinal Necrosis (ARN) is a type of retinitis that affects both healthy and immunocompromised patients (86). This inflammatory disease is caused by several members of the herpesvirus family including herpes simplex virus (HSV-1), varicella zoster virus (VZV) and citomegalovirus (CMV) (87, 88). The disease usually starts with signs of uveitis: red eyes, light sensitivity, eye pain and blurred vision. Detailed examination of the eyes of these patients show infiltration of inflammatory cells in the anterior and posterior segments in all retinal layers (89). As the disease progresses retinal necrosis and occlusive arteriolar retinopathy are found. Current treatment for this condition includes antiviral therapy and topical cortosteroids (89). There is no treatment that can prevent the establishment or persistence of latent infection. Reactivation of HSV-1 infections, are currently controlled clinically with long term administration of acyclovir or its derivatives.

Antiviral drugs can quell symptoms resulting from reactivation outbreaks but cannot eliminate latent virus. Furthermore, long-term usage of antiviral drugs can lead to development of drug-resistant viruses (90). Several authors have designed specific ASON targeting the proinflammatory cytokine tumor necrosis factor alpha (TNF $\alpha$ ) (91, 92). TNF $\alpha$  is known to possess many cell-activating and proinflammatory activities. TNF $\alpha$  mRNA and protein are up-regulated in eyes infected with HSV-1 (93). Moreover, experiments using DNA microarrays have shown that TNF $\alpha$  and its receptors are upregulated in eyes of mice with HSV-1 retinitis (94). The results of these experiments show that either intravitreal or subconjunctival injections of TNF $\alpha$ -ASON provide local therapeutic effects without systemic adverse effects. Another strategy uses morpholino oligomers specifically designed to reduce viral mRNA through steric blocking. Moerdyk-Schauwecker and collaborators have developed five phosphoro- diamidate morpholino oligomers (PMO) that target three HSV-1 genes: *ICP0*, *ICP4* and *ICP27*. Their experimental results demonstrated that PMO targeting HSV-1 mRNAs not only inhibited viral replication in cell cultures but also in mouse models of the disease (90).

HSV-1 not only causes ARN it is also responsible for Herpetic Stromal keratitis (HSK) a well- defined immune mediated blinding corneal disease (95). There is profound evidence that the corneal inflammation in HSK is orchestrated by CD4<sup>+</sup> T cells and is accompanied by uncontrolled development of blood vessels within the eye (96). The corneal infiltration contains abundant polymorphonuclear cells. These cells, although important for controlling virus replication, are also responsible for damage to the cornea (97, 98). Due to the relevant role of TNF- $\alpha$  and IFN $\gamma$  in the progress of HSK, targeting TNF- $\alpha$  and IFN $\gamma$  has been proposed as a therapeutic approach (99). Wasmuth and collaborators demonstrated that topical administration of ASONs targeting either TNF- $\alpha$  or IFN $\gamma$  were capable of reducing their target in mice infected by HSV-1 without altering antiviral immune response (100, 101). In addition to the production of proinflammatory cytokines, neovascularization of the cornea is observed in this pathogenesis. As in other diseases in which neovascularisation plays a role, VEGF seems to be at least in part, responsible for the process. Validation of this theory has been performed by inhibiting HSV induced angiogenesis in mice with either a VEGF neutralizing antibody or an siRNA designed against this target (102).

Acute Hemorrhagic Conjunctivitis (AHC) is a highly contagious eye disease caused primarily by enterovirus 70 (EV70) or coxsackievirus A24 (CVA24) infection. Thus, several authors attempted to develop novel siRNA-based anti-AHC agent effective against both EV70 and CVA24. Resulting siRNAs showed excellent cytoprotective effects and dramatic decreases in viral replication and protein synthesis in primary human conjunctival cells, MRC5 and HeLa cells (103) or in Rhabdomyosarcoma cells (104).

## 2.7. Chronic optic nerve atrophy and ischemic optic neuropathy

Ischemic optic neuropathy is defined as vision loss due to lack of blood supply to the optic disc (infarction). Decreased visual acuity and visual field are usually the only symptoms of



ischemic optic neuropathy regardless of the underlying cause. Ischemic optic neuropathy is primarily of two types: Anterior (AION) and posterior (PION) involving the optic nerve head (ONH) and the rest of the optic nerve, respectively (105). AION can be arteritic (A-AION) and non-arteritic (NA-AION). In the management of AION, it is crucial to identify the form of AION. A-AION is an ophthalmic emergency and requires urgent treatment with high-dose steroid therapy to prevent further visual loss to both the affected eye and the sometimes asymptomatic Contralateral eye. NA-AION is the most common form of the disease and it is usually detected due to unilateral painless visual loss along with edema to the optic nerve. It is more frequent in patients between 40 and 70 years of age and vision loss is usually less severe than in the A-AION. Systemic risk factors particularly nocturnal arterial hypotension, play major roles in the development of NA-AION. NA-AION patients treated with high doses of systemic steroid therapy showed significant improvement in visual acuity and visual field. (106).

Given that the loss of vision in these conditions is due to loss of retinal ganglion cells (RGC) neuroprotection seems a rational approach. Focusing on this line of thought, Quark Pharmaceuticals is currently developing a nucleic-acid based therapy, specifically a siRNA-based therapy: QPI-1007. QPI-1007 is a synthetic siRNA designed to temporarily inhibit expression of the pro-apoptotic protein caspase 2 that is currently in clinical trials for the treatment of NA-AION. Ahmed and colleagues in collaboration with Quark pharmaceuticals have shown that caspase-2 contributes loss of RGC in rat models of the condition and have demonstrated that intravitreal injections of QPI-1007 are effective in protecting against the death of RGC (35). Quark Pharmaceuticals is conducting a Phase I-dose escalation safety study using QPI-1007 in patients suffering from Optic Nerve Atrophy and NA-AION. Caspase 2 isn't the only interesting target that offers a potential avenue for treatment in optic neuropathy. Helen and collaborators have focused on the action of another protein: connexin 43 (Cx43). Preclinical results show that an ASONs designed to specifically reduce upregulated levels of Cx43 in a model of optic ischemia has therapeutic potential (107)

## **2.8. Inherited ocular diseases: Retinitis Pigmentosa (RP) and Ocular Albinism (OA)**

Retinitis pigmentosa (RP) is a class of diseases involving progressive degeneration of the retina and a leading cause of inherited blindness. RP is a heterogeneous disorder that starts in mid-periphery and advances towards the macula and fovea. Typical symptoms include night blindness followed by decreasing visual field, leading to tunnel vision and eventually legal blindness or in many cases complete blindness (108). This disorder involves photoreceptor-cell degeneration and affects ~ 1 in 3000 people (109). On the cellular level the rod photoreceptor system is predominantly affected but in later stages of the disease cone photoreceptors can also be affected (108). More than 40 causative genes have been implicated in RP, although mutations in rhodopsin gene (RHO) account for 15% of all types of RP. Great efforts have been made to explore new gene therapies for RP, but inter and

intragenic heterogeneity represent significant barriers to therapeutic development. For example more than 100 mutations in the human RHO gene, which encodes the photosensitive pigment in rod photoreceptors, have been identified in autosomal dominantly inherited RP (110). Development of therapies for each individual mutation would be technically difficult to achieve and not economically viable; thus a therapeutic approach that circumvents mutational diversity would be of great value. At present, there is one treatment which has reached the clinic for the treatment of Leber's congenital amaurosis form 2 (LCA2), a type of RP, based on adenoviral (AAV) delivered gene therapy. Three years ago, Maguire and colleagues reported results in 12 patients who were treated with gene-replacement therapy. LCA2 is associated with mutations in *RPE65*, which encodes a protein requisite for the isomerohydrolase activity of the retinal pigment epithelium. This activity produces 11-*cis*-retinal, the natural ligand and chromophore of the opsins of rod and cone photoreceptors, the opsins cannot capture light and transduce it into electrical responses to initiate vision. In this study patients received one subretinal injection of AAV2-hRPE65v2 (a viral vector encoding RPE65 protein). Visual improvement was observed in all 12 patients with the greatest gains among younger patients (111).

Still in early research stages are different approaches to the treatment of RP based on the administration of AAV-delivered RNA interfering compounds. Amongst these, both the University of Florida and Smurfit Institute of Genetics have achieved interesting results *in vivo* by silencing the expression of RHO in mouse models (112). More recent studies by Jiang and collaborators show how using an AVV2/8 vector to develop an RNAi-based therapy in a dominant retinal degeneration mouse model expressing bovine GCAP1 (Y99C), significantly improved photoreceptor survival, delaying disease onset and increasing visual function (113). There are also several studies that target inosine 5'-monophosphate dehydrogenase 1 (IMPDH1) with viral delivered siRNA for the treatment of RP10 form of autosomal dominant RP (114). Another interesting approach based on RNAi therapy uses siRNAs to facilitate transplantation of rod photoreceptors, by disrupting junctional proteins and enhance donor cell integration in the retina (115).

Ocular albinism type 1 is a group of X-linked or autosomal genetic disorders characterized by partial or total lack of melanin pigmentation in the eyes due to mutations in genes encoding proteins involved in melanogenesis. Eyes may be severely affected with photophobia and reduced visual acuity. Nystagmus or strabismus, are often associated and the irides and fundus are depigmented. Many forms of albinism, more or less severe, have been described. Some forms also affect skin and hair. As in other forms of albinism, these patients suffer loss of stereoscopic vision due to reduction of the ipsilateral component of the optic tract (116). General prevalence of albinism is 1/15,000 inhabitants. Treatment is exclusively symptomatic. Vetrini and collaborators have described an intronic point mutation in ocular albinism type 1 (OA1) gene in a family with the X-linked form of ocular albinism. Interestingly the mutation creates a new acceptor splice in intron 7 of the OA1 gene leading to aberrant protein expression. In addition to low levels of normally spliced mRNA product of the OA1 gene, patient samples contained aberrant spliced mRNA. OA1 expression was rescued in the patient's melanocytes with an antisense morpholino modified

oligonucleotide (MO) complementary to the mutant sequence. The MO was able to rescue OA1 expression and restore the protein level in the patient's melanocyte by skipping the aberrant inclusion (116).

Compound	Indication	Company	Type of compound	Target	Stage
SYL040012	Glaucoma	Sylentis	Naked siRNA	ADRB2	Ph II
QPI-1007	Glaucoma	Quark Pharmaceuticals	Modified siRNA	Caspase 2	R&D
	NA-ION				Ph I
ARC1905	Dry AMD	Ophthotech Corporation	Pegylated aptamer	Complement component C5	Ph I
	Wet AMD (in combination with ranibizumab)				Ph I
E10030	Wet AMD (in combination with ranibizumab)	Ophthotech Corporation	Aptamer	PDGF	Ph II
Pegaptanib	Wet AMD	Eyetechnology/OSI Eyetechnology/Pfizer	Pegylated aptamer	VEGF	Approved
	Diabetic Macular Edema				Ph III
Bevasiranib	Wet AMD	OPKO Health	Naked siRNA	VEGF	Ph III (Term.)
	Diabetic Macular Edema				Ph II
Sirna-027	Wet AMD	Merck/Allergan	Modified siRNA	VEGF	Ph II (Term.)
PF-655	Wet AMD	Quark/Pfizer	Modified siRNA	RTP801	Ph IIb
iCO-007	Diabetic Macular Edema	iCO Therapeutics	Modified antisense	c-ras	Ph II
ALT1103	Diabetic retinopathy	Antisense Therapeutics	Antisense	GHR	R&D
SYL1001	Dry eye pain	Sylentis	Naked siRNA	TRPV1	Ph I
GS-101	Corneal graft rejection associated to corneal neovascularization	GeneSignal	Phosphorothioate antisense	IRS-1	Ph III
Fomivirsen	Advanced CMV infection in AIDS patients	Isis/CIBA	Phosphorothioate antisense	IE gene CMV	Approved

**Table 1.** Compounds based on oligonucleotides under development for the treatment of ocular diseases.

### 3. Safety issues

#### 3.1. Immunotoxicity

As part of the defensive duties of the innate immune system it needs to discriminate between foreign and endogenous genetic material. Destruction of exogenous genetic

material is an essential part of protection against microorganisms, but can be a hurdle when trying to introduce synthetic genetic material for therapeutic purposes. The immune system responds to microbial RNA and DNA by producing type I interferon and proinflammatory cytokines. The chain reaction leading to production of these immune mediators is initiated by detecting conserved motifs of pathogens; this job is performed by several cell membrane and cytoplasmic receptors present at low levels in most cells. Toll-like receptors (TLR) are present in either the cell surface or in intracellular endocytic organelles and activation of these receptors leads to increase in the presenting capacity of dendritic cells and production of type I interferon. The TLRs responsible for detecting oligonucleotides are present exclusively in endosomes and are TLR3 that senses dsRNA (117), TLR7/8 that sense ssRNA (118) and TLR9 that senses ssDNA (119). TLRs are mainly expressed in immune cells; but foreign material has to be detected in non-immune cells as well; this task is performed by cytosolic sensor proteins. The dsRNA-dependent protein kinase (PKR) is a cytoplasmic sensor of RNA. Upon activation by binding to dsRNA, PKR forms a homodimer that acquires phosphorylation activity. Among the phosphorylation targets of PKR is eIF-2 $\alpha$ ; phosphorylation of this transcription factor leads translation inhibition and apoptosis (120). A second cytosolic sensor of dsRNAs is 2'-5' oligoadenylate synthetase (OAS). Activation of this protein promotes the activation of ribonucleases that degrade endogenous and exogenous RNA, again leading the cell to apoptosis (121). Other cytosolic sensors of dsRNA are the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) (122). RLRs are DExD/H RNA helicases. Two members of this family, RIG-I and MDA-5, have in addition to the helicase domain, two caspase activation and recruiting domains. Binding of dsRNA to RIG-I or MDA-5 activates a complex signalling cascade that ultimately leads to interferon- $\beta$  production and apoptosis (123). As reviewed above, the immune system is prepared to react when foreign biological material is detected inside the body; several strategies can be used to bypass or modulate the response of the immune system:

- Avoidance of sequence motifs or structures that are specifically recognized by the oligonucleotide sensors of the innate immunity. Lowering the number of uridines in a given RNA seems to lower the likelihood of an immune response; completely replacing the uridines by adenosines completely abrogates immune activation (124). Also, avoiding blunt ends on siRNAs could help reducing the immune response. RIG-I recognizes siRNAs with or without 2-base 3' overhangs, but only those with blunt ends are able to trigger its ATPase activity and subsequent downstream signalling (125).
- Introducing chemical modifications in nucleotides and/or their backbones to avoid activation of the immune response. 2'OMe and pseudouridine are modifications frequently present in mammalian RNAs; these modifications help avoiding triggering the immune response. Other modifications such as 2'F and LNA can also help evade immune detection (124). Whenever modifications are introduced into an oligonucleotide the modified molecule should be tested in order to make sure that the silencing activity has not been abolished.
- Use targeted delivery of oligonucleotides (126) or delivery techniques that avoid the endosome routes for sequences that could activate TLRs. Use of inhibitors of

endosomal maturation such as chloroquine and bafilomycin A1 can also abrogate the immune response (125).

- Use of immunomodulators to impair or abolish the immune response.

Some of the first therapeutic approaches using oligonucleotides were made in the field of ophthalmology. The eye is an immune privileged site, and this fact is one of the rationales behind the election of this site for the first trials. Several overlapping mechanisms contribute to establishing and maintaining immune privilege: the eye has a high content of immunosuppressive factors; it has a low expression of MHCII, hence limiting antigen presentation; stromal cells of the iris, ciliary body and retina are able to convert immune T cells to regulatory (Treg) cells, and retinal epithelial pigment cells are able to inhibit primed T cells; and finally death receptors such as FasL and PDL-1 are expressed by stromal cells that are able to induce apoptosis in immune cells that enter the eye (127). These advantages should not be interpreted as a free pass in terms of immunity for oligonucleotide therapies in the eye. In this regard, it has been shown that some siRNAs are able to activate TLR3 in a sequence-independent manner. Activation of this receptor by dsRNA has been found to suppress angiogenesis (57) and to induce retinal cell death; thus promoting atrophic AMD (46).

### 3.2. Non immune off target effects

The interaction of therapeutic oligonucleotides with their intended target RNAs is, with the exception of aptamers, highly sequence-specific. However, binding of one of these molecules to a non-target sequence requires only homology with a few base pairs. The result of this undesired interaction could be the inhibition of an unintended gene or an *off target effect* (OTE). Off-targeting is mostly mediated by the interaction between certain regions of the therapeutic molecule and complementary sites in the unintended target rather than overall homology between the two molecules (128). Careful comparison of candidate sequences with the entire transcriptome, attempting to avoid long stretches of homology, still remain necessary but are inadequate on their own to predict the actual risk of OTEs. Use of chemical modifications and improvement in oligonucleotide design has been successfully employed to reduce the likelihood of OTEs.

The specificity of a sequence can be improved taking into account some important designing parameters such as thermodynamic stability of the duplex and 5' and 3'-ends, the  $T_m$  value of the interaction region between the therapeutic oligonucleotide and possible off-targets (129). When dealing with knock down of mRNAs using siRNAs or antisense oligonucleotides the target position should be selected choosing regions that are as far away as possible from the initiation codon (130). The silencing effect of oligonucleotides is in general concentration-dependent, considerable success in reducing siRNA/antisense off-targeting has been achieved optimizing doses of the therapeutic oligonucleotide (131). Improvements in specificity achieved by altering sequences and/or introducing chemical modifications as well as in delivery of the molecule have also shown to have an impact on reducing potential off-target effects. Placing a 2'OMe at position 2 of



the guide strand of a siRNA (132), or incorporating a destabilizing UNA at position 7 are examples of modifications that reduce OTEs (131).

### 3.3. Oversaturation of endogenous RNAi-silencing complex (RISC)

Bioactive drugs that rely on cellular processes to exert their functions face the risk of saturating endogenous pathways. This may be the case with RNAi-based drugs. Naturally occurring, small RNAs have undergone the process of evolution because of selection pressure and they exist in a perfect balance with their precursors and targets, as well as with the associated machinery involved in this process. Gene Silencing is performed by introducing artificially synthesized small RNAs into the cell or by expressing siRNAs/shRNAs within the cell, which enter the endogenous RNAi pathway at different levels. shRNAs and siRNAs are very similar to miRNA precursors before and after Dicer processing, respectively, relying on endogenous miRNA machinery to achieve target silencing. If the siRNA design parameters are not optimal they might cause an imbalance of the endogenous small RNA mediated pathways resulting in various and deleterious unwanted effects in the cells. It becomes therefore crucial to optimize the siRNA/shRNA design parameters and work at the lowest possible concentrations to mitigate the potential of side effects.

## 4. Advantages of RNAi-based therapeutics and challenges ahead

This new revolutionary technology presents many advantages for therapeutic development with respect to classical compounds, mainly:

- They are based on an endogenous mechanism, thus involving the administration of a type of molecule already present in the cells, and hence, in principle, less hazardous: cells should have the capacity to handle resulting breakdown products.
- As with antisense molecules before them, RNAi molecules can potentially address any pathological target, this means that even diverse intracellular molecules can be the object of these therapies. However, one must bear in mind that in practice, some genes are harder to target than others, and further more sophisticated algorithms must be developed to address this issue.
- Also in comparison to antisense, RNAi is considerably more potent (133) and consequently may be used at lower concentrations. It is believed also to have a more persistent pharmacological action than traditional drugs because it blocks protein synthesis and hence, the cell will have to re-synthesise the protein from scratch to return to its previous state. Thus allowing the use of lower or less frequent doses.
- Given the selectivity and specificity of these compounds, coupled with the fact of their *in silico* design and analysis they will have potentially less harmful side effects.
- Another main advantage from an industry perspective is their much shorter pharmacological development, the estimate being 2-3 years vs 4-6 years from proof of concept. This can be attributed among other things, to the fact that the compound can be designed against homologous regions of the human gene sequence between the

different animal models used for preclinical studies, thus simplifying toxicology studies.

- Finally, despite being novel entities they are easy to manufacture using a nucleotide synthesiser which simplifies large scale production. Additionally, this means the compounds are chemical entities rather than biological products, greatly helping the regulatory process for approval of these medicaments.

Nevertheless, RNAi still has certain hurdles to overcome before its full potential can be exploited. The main obstacle is delivery of siRNAs to the desired tissue, and although many advances have been made in this area, much work still remains for therapeutic possibilities to be fully exploited. On the other hand, in a clinical setting, RNAi can only be used to treat pathologies caused by expression or overexpression of a given protein or by the presence of exogenous organisms, as its mechanism works through suppression of protein expression; i.e. it is only of use when the therapeutic option requires a loss of function. Furthermore, although any gene is a potential target for RNAi, in practice not all mRNAs are as easily silenced and new more sophisticated algorithms will need to be developed to overcome this issue. And last but not least, as discussed in the previous section the issue of off-target effects resulting from siRNAs silencing unwanted genes can lead to important safety considerations when developing new medicaments.

From the perspective of ocular disorders, the eye is a relatively isolated compartment which makes it an ideal target organ for gene silencing. Local delivery of the compound to the eye limits exposure to the rest of the body and reduces the amount that is needed. siRNA injected into the vitreous cavity readily diffuses throughout the eye and is detectable for at least five days (56), the amounts used for intraocular injections are small compared to those used for systemic application and so as siRNA gets out of the eye it is diluted and is difficult to detect. This allows local silencing of a gene with little chance for an effect on the same gene outside the eye, reducing concern of remote effects in other tissues complicating observations in the eye. The sequence specificity of siRNA resulting in targeting of a single gene combined with local administration in the relatively isolated confines of the eye provides an ideal way to study eye-specific effects of gene disruption (34). While the development of siRNA-based therapies has promise for all tissues, the unique characteristics of the eye provide advantages which explain why the first siRNA compounds to advance through clinical trials were for the treatment of wet AMD, a disease affecting the back of the eye.

## 5. Prospects for current therapies based on nucleic-based strategies

The clinical progress of oligonucleotide drugs has been slow because realizing them requires inventing a new model for pharmaceutical development that allows large, highly charged molecules to be synthesized economically, distribute to target tissues, enter cells, and function within acceptable limits for toxicity. Oligonucleotides are large, unlike traditional small molecule drugs (<500-700 molecular weight), and much effort has been required to understand their properties and optimize them. However, considering RNAi was

discovered just over a decade ago, this technology has advanced towards clinical trials with amazing speed. Both for antisense molecules and RNAi compounds initial most advanced therapies were developed taking advantage of the environment within the interior of the eye. One antisense oligonucleotide has been approved by the FDA in 1998: Vitravene® or fomivirsen developed by Isis Pharmaceuticals for the treatment of cytomegalovirus retinitis in immunocompromised patients. Initial most advanced siRNAs were designed to treat wet age-related macular degeneration (AMD) taking advantage of the relatively RNase free environment of the interior of the eye. These therapies were based on intravitreal injection, a form of administration which allows bypassing one of the main rate-limiting issues for therapeutic oligonucleotides, that of delivery to the required target tissue.

Delivering oligonucleotides in whole organisms requires crossing many barriers. Degradation by serum nucleases, clearance by the kidney, or inappropriate biodistribution can prevent the oligonucleotide from ever reaching its target organ. Generally, the oligonucleotide must pass through the blood vessel wall and navigate the interstitial space and extracellular matrix. Finally, if the oligonucleotide succeeds in reaching the appropriate cell membrane, it will usually be taken up into an endosome, from which it must escape to be active. Antisense oligonucleotides are usually delivered in saline and rely on chemical modifications to enable uptake. Their phosphorothioate backbone binds to serum proteins, slowing excretion by the kidney (134). The aromatic nucleobases also interact with other hydrophobic molecules in serum and on cell surfaces; many types of cells *in vivo* express surface receptors that actively take up oligonucleotides.

Delivery is even more challenging for siRNAs, where all the aromatic nucleobases are on the inside, leaving heavily hydrated phosphates on the outside of the duplex. This hydrated surface interacts poorly with cell surfaces and is rapidly excreted in the urine. Hence, except for direct injection in targets such as the eye where they are also delivered in saline, researchers have invested heavily in the development of delivery vehicles for siRNA. Various delivery strategies include nanoparticles, cationic lipids, antibodies, cholesterol, aptamers and viral vectors for short hairpin RNAs. In this case, the delivery system most clinically validated is that based on SNALP (stable nucleic acid lipid particle) technology developed by Tekmira for systemic administration of siRNAs. However, although it is being used to formulate at least 4 different siRNAs currently undergoing clinical trials, it has yet to be used more extensively before victory can be claimed on the battle of delivery.

The field of oligonucleotide therapeutics has often swung from irrational optimism to irrational despair. This is true for industry, but in the laboratory as well, gene knockdown experiments have fallen in and out of favour with researchers. In reality, oligonucleotides are useful tools with strengths and weaknesses (135). The promise of RNAi as a powerful new approach for therapeutic treatment of diseases has propelled early stage clinical testing of siRNAs for a variety of diseases. However, it is still too soon to evaluate whether or not RNAi based therapeutics will live up to their expectations. Given the mood swings in this area, with big pharma investing large sums in the technology at one point and then backing out only two or three years later, the industry has been in a turmoil which is now beginning to even out. This relative calm should bring about maturity in the field with the science

becoming more solid as we gain more knowledge of the biology of these compounds. From an industry perspective, it is foreseeable that Big Pharma's involvement will continue the later trend of product-specific licensing and co-development rather than the purchase of platform technologies from smaller biotech experts performed in the early days of the technology. This is also in line with structural changes in the industry towards the outsourcing of research (136).

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