

Antisense Oligonucleotide Tool in Therapy for Polyglutamine Neurodegenerative Disorders

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INTRODUCTION

Antisense oligonucleotides (**AONs**) are synthetic single stranded strings of nucleic acids nitrogenous bases, between 7 and 40 nucleotides in length, that bind to RNA through the gold standard Watson–Crick base pairing rule. AONs interfere with gene expression ultimately results in specific gene knockdown. This genomic tool offered useful opportunities to study development because it allowed timed gene knockdown in early or later stages of development, as well as therapeutic opportunities to knockdown genes involved in cancer, inflammatory diseases, and viral infections. Currently, an AON to treat cytomegalovirus retinitis (Vitracene) has been registered as a drug, and other AONs to treat cancer and inflammatory diseases are in phase II and III clinical trials [1,2]. Additionally, there has been a recent improvement of interest in the use of AONs to cure human intractable polyglutamine (polyQ) neurodegenerative disorders. Depending

on sequence and modifications, AONs can alter RNA role through several distinct mechanisms, making them a diverse genomic engineering tool. AONs can be used to restore protein expression, reduce expression of a toxic protein, or modify mutant proteins to reduce their toxicity. In 1978, antisense-mediated gene inhibition was first introduced by Stephenson and Zamecnik [3]. It has been showed that inhibition of replication and cell transformation of the Rous sarcoma virus, using a DNA molecule of 13 nucleotides in length with modifications at the 3' and 5' OH moieties. Since then, modifications to the backbone and sugar component have improved stability, binding strength and specificity which has made AONs suitable for therapeutic application [4]. For several polyQ neurodegenerative disorders, AONs therapy has now moved from the preclinical to the clinical stage, facilitated by the remarkable widespread distribution and cellular uptake of AONs once delivered into the brain [5,6]. There are many other types of nucleic acid molecules that can interfere at the RNA level using the RNA-induced Silencing Complex (**RISC**) - (ribonucleoprotein complex involved in mRNA degradation)- [7]) that promote selective degradation of homologous cellular mRNAs, but these will not be our focus in this chapter. RNA interference (**RNAi**) was the another main tool to be used as a potential therapeutic way to reduce expression of target transcripts causing nature knockdown for the targeted gene product [8,9]. RNAi is an endogenous cellular defense mechanism against exogenous viral components and is also involved in transcriptional regulation [10]. Specific knock down of target sequences is achieved by introducing exogenously modified oligonucleotides (e.g. short hairpin RNA (**shRNA**) and short interfering RNA (**siRNA**)) that bind to the target transcript, which is subsequently degraded or its translation blocked.

In this chapter we will outline the key characteristics of AONs that make them very suitable for treating intractable polyQ neurological disorders. We will discuss the design and the available chemical modifications of AONs that have been applied to polyQ neurodegenerative conditions. Additionally, delivery of AONs to the nervous system will be mentioned. Finally different functional mechanisms to alter RNA function in the nervous system will be discussed, as well as several polyQ neurodegenerative disorders where these different AONs mechanisms are being applied.

DESIGN AND CHEMICAL MODIFICATIONS

Various characteristics for AONs that determined by the nucleotide sequence are important for its activity. The huge relevant information was gathered for siRNAs and used to set up guidelines for effective siRNA design [11]. The rules for siRNAs involve their nucleotide composition, their duplex thermodynamics and the structure of the duplex ends [12,13]. The stable structure formed by the siRNA guide strand or AONs [14], as well as the stable structure of the target sequence [15-17], reduces the efficiency of transcript inhibition. However, some AONs were shown to be more sensitive to target structure than siRNAs [18]. The siRNA hybridization with the target is facilitated by RNA-induced Silencing Complex (**RISC**), which contains helicase activity, but it is likely that the ability of AON to find the target sequence is also stimulated by yet unknown proteins [19].

Another point of AONs design is the selection of the chemical modification design. Initially, AONs were used in the form of synthetic unmodified single strand of RNA [20]. Though successful, these types of oligonucleotides proved very susceptible to degradation by endo- and exonucleases. It quickly became apparent that if AONs were to be used for clinical applications, their pharmacological pattern would have to be amplified. Oligonucleotides have since benefited from technical advances in chemical modifications leading to significantly making better characteristics. The AONs need suitable chemical modifications for activity improvement. Chemical modification not only increases the biological stability of the AONs and the efficiency of its hybridization with the target sequence, but may also be critical for its effective delivery and low toxicity. These issues will be discussed in the following subsections. AONs are available with a range of different chemical modifications on the phosphate backbone and ribose sugar group. The modifications used in studies of poly Q neurodegenerative disorders and their mode of actions will be discussed in the paragraphs below.

Backbone Modifications: Counteracting Nucleases

Rapid degradation by endo- and exonucleases for AONs is one of the main factors that halt its efficacy. Within 30 min in serum, 3' to 5' exonuclease is able to degrade unprotected AONs [21], whilst intracellular exo- and endonucleases can cause degradation in an even shorter time period [22]. In CSF, brain associated α -exonuclease is likely responsible for degradation of AONs [6]. The Phosphorothioate (**PS**) backbone is one of the first successful modifications providing a good degree of protection from these nucleases, termed the first generation of oligonucleotide modifications. The PS backbone is achieved by substitution of one of the non-bridging oxygen atoms in the backbone with a sulphur atom [23]. This SP chemical modification makes AONs more stable with reported half-lives of 9 h in human serum and 19 h in rat CSF [24]. Furthermore, the negative charge of PS DNA allows for good uptake into numerous cell types *in vitro* [25] and into many brain cell types after microinjection into brain [26]. Another important feature of the PS backbone is its ability to activate Ribonuclease H (**RNase H**), allowing for use in applications where target RNA down regulation is required [27]. RNase H recognizes an RNA–DNA heteroduplex, and then cleaves the RNA strand, resulting in a 5-phosphate on the product and release of the intact DNA strand [28,29]. A less beneficial characteristic of the PS backbone is that it destabilizes duplexes, leading to a decrease in melting temperature [30]. It has been reported that the cytotoxic effects of PS at high concentrations [31]. This undesired effect is considered to be due to protein binding [32,33] or complement activation [34]. PS modified antisense oligonucleotides have extensive pharmacokinetic testing aimed at peripheral administration. In this report, it was found that PS oligonucleotides bind serum proteins, leading to reduced renal clearance and an increased circulation time [35,36]. These favourable pharmacokinetic characteristics in the periphery are clash with the poor ability of PS oligonucleotides to cross the Blood Brain Barrier (**BBB**) [37].

Sugar Group Modifications: Improving Affinity and Reducing Toxicity

The 2' position of the ribose sugar for RNA oligonucleotides can be further modified, in addition to the PS backbone. The second generation oligonucleotide modification is the term used for these types of modifications. This modification in combination with the PS backbone, leads to a great importance for advancing oligonucleotide safety and pharmacologic properties. In this category of modifications, the 2'-O-methyl (**2OMe**) and 2'-O-Methoxy-ethyl (**MOE**) have proven most successful until now. These modifications increase hybridization affinity to their target RNA sequence in contrast to the unmodified phosphorothioates [38–40] as well as increase resistance towards nuclease degradation [41,42]. An additional significant advantage of the 2' modifications is their ability to reduce sequence independent toxicity arising from the PS backbone [43], which also holds true in the CNS [44]. An important feature for 2'-modifications is their inability to recruit RNase H [45]. AONs that are fully modified in this fashion cannot induce RNase H-mediated target RNA down regulation. Very mild toxicity for 2'-O-modified-PS antisense oligonucleotides only has been reported, which did not interfere with their desired effects after ICV delivery in rodent brain [46], or in cultured neuronal cells [47]. Although it has been shown that PS antisense oligonucleotides can have an immunostimulatory effect via toll-like receptors, appropriate 2'-O modifications, such as 2OMe and MOE, can suppress these effects [48–50]. It is important to mention that possible toxic and immunostimulatory effects of 2OMe-PS antisense oligonucleotides may also be due to dosage, concentration, or duration of treatment [51].

Locked Nucleic Acids (**LNA**) are a 2'-modification where the 4'-carbon has been tied to the 2'-hydroxyl group. LNAs present resistance to nucleases [52] and show much enhanced hybridization compared to the other 2'-modifications [53,54]. LNAs are also unable to induce RNase H-mediated target RNA down regulation [55,56]. Although LNAs provide a better RNA binding affinity than most other 2'-modifications, there appear to be more severe toxicological problems with these oligonucleotides in systemic treatment [57]. Whether this also holds true for the central nervous system will remain to be determined, though one study reports that LNAs are tolerated in rat brain [58]. Additionally, the high affinity of LNAs can lead to a reduction in target specificity [54,59]. For these reasons, a chimera design where LNA modified nucleosides are placed between unmodified or differently 2'-O-modified nucleosides can be implemented [53]. LNAs can be further modified by addition of oligospermine nucleobases, thus this resulting antisense oligonucleotide–oligospermine conjugates termed Zip Nucleic Acids (**ZNA**) [60]. These conjugates famine the electrostatic repulsion of the negatively charged phosphate backbones and polyanionic nature, leading to increased binding affinities and improved cellular uptake. ZNA conjugates were reported to reduce huntingtin expression in Huntington Disease (**HD**) patient-derived cells [61]. Although ZNA conjugates are used firstly as probes for real-time PCR, they have power of some therapeutically interesting good effects.

Tricyclo-DNA (**tc-DNA**) is considered as another conformationally unnatural oligonucleotide modification available. This modification is aimed at making less the flexibility around the C3'–C4' and C4'–C5' bonds by addition of an ethylene bridge fused with a cyclopropane unit. The result in a more stable duplex formation that is not compatible with RNase H [62]. Tc-DNA has been shown to be stable in serum and resulted in more potent splicing correction compared to a 2OMe-PS oligonucleotide when tested in cells [63]. Very few studies have been performed making use of tc-DNA, but there has been one study reported splicing modulation take place in the brain after peripheral administration of tc-DNA in a Duchenne muscular dystrophy mouse model [64]. Interestingly, dystrophin restoration in the mouse brain resulted in complete reversal of its behavioural phenotype [64].

The 2'-fluoro and S-constrained-ethyl (cEt) oligonucleotides are other sugar modifications less frequently used in CNS studies. The 2'-fluoro is very close to OMe and MOE modifications, replaces the 2'-hydroxy with a fluoro group. This simple chemical difference leading to a higher affinity than most other 2'-modifications [45][65], in addition allowing resistance to nucleases degradation [65]. The cEt modified AONs supply similar binding affinity as LNA, yet appear to have a more toxicity profile [66], and have recently shown good promise in a mouse model for HD [67].

In general, we can assume that the second generation of oligonucleotide modifications has provided a good degree of improvement on the PS backbone, with enhancements in nuclease resistance, binding affinity and reduction of PS-induced toxicity. However, in light of the fact that most CNS research utilising oligonucleotides aims at down regulation of target RNA, the fact that these modifications are not compatible with RNase H cleavage is an important consideration. For this reason, a RNase H compatible gapmer strategy was developed, in which a PS oligonucleotide is not modified with 2'-O-modifications over its whole length.

Other Oligonucleotide Modifications

Besides the PS backbone and several ribose sugar modifications described above, a lot of attempts have been made with newer modifications that combine backbone, ribose and nucleosides modifications.

Peptide Nucleic Acids (**PNA**) are produced by substitution of the entire sugar phosphate backbone with polyamide linkages [68], but are still able to hybridize through Watson–Crick binding pairing rule [69]. PNAs are uncharged, provide a high resistance to nuclease and protease degradation [70], and show high binding affinity for the target RNA sequence [71]. PNAs are mostly execute splicing modulation methods or translation inhibition due to their inability to activate RNase H. A clear drawbacks for PNAs is their water insolubility and poor cellular uptake [72, 73]. However, both water solubility and uptake can be improved using peptide conjugates [74,75]. It has been reported that the uptake in neuronal cells of unmodified PNA *in vivo* [76] but the use of PNA antisense oligonucleotides in polyQ neurodegenerative disorders remains

somewhat limited. PNAs are rapidly cleared, when administered peripherally, [77] and these poor pharmacokinetic properties likely in part explain their limited *in vivo* use until now.

Phosphorodiamidate Morpholino Oligomers (**PMOs**) have a morpholine ring instead of the ribose ring, and have phosphoroamidate intersubunit linkages. PMO backbone is neutrally charged and is not compatible with RNase H, similarly with PNAs [78]. PMOs are highly resistant to protease and nuclease degradation [79]. In a phase 1 clinical trial using IV administration, PMOs were well tolerated, but were shown to suffer from the same pharmacokinetic drawbacks as PNAs since they are rapidly cleared from the blood [80]. Peptides can be conjugated to the PMOs for enhancing pharmacokinetics properties and cellular uptake [81]. In addition, PMOs has been shown successfully ability to modify splicing when administered directly in mouse brain regardless there chemistry [82], though toxicity at higher doses may appear [83]. Another interesting conjugated PMO is the vivo-morpholino, which features a terminal octaguanidinium dendrimer aiming to improve cell permeability and thus tissue uptake when administered peripherally [84]. Unfortunately, there have been studies showed the severe toxicity following IV administration of vivo-morpholino in mice [85]. Crossing of the BBB does not appear to occur efficiently for vivo-morpholinos [86], and, though microinjection in rat brain was effective, target protein down regulation lasted for only 14 days [87].

Off-Target Effects

The required efficiency of gene inhibition is usually achieved by an excess of AON over the target. This approach raises specificity problems because in human transcriptomes, there are multiple sequences that are partially complementary to the AON sequence. Some of these unintended interactions are productive, causing sequence-specific off-target effects. The AONs are considered more selective than RNAi tools. The RNase H cleavage mechanism is very sensitive to base mismatches, and only one, two or at most three mismatches are tolerated. The Argonaute (**AGO**)-mediated silencing mechanism is more tolerant for siRNA-target mismatches, as these are typically present in miRNA-mRNA interactions. For most miRNAs, base-pairing in the seed region (nucleotides 2–8 from the 5' end of short RNA) is crucial for activity [88,89], and therefore, complementarity in this region is mainly used for predictions of potential off-targeting by siRNA [90,91]. The siRNA designs aimed at reducing off-target effects [92,93] included shortening the passenger strand [94,95] and introducing chemical modifications into the sense strand of the siRNA to render it inactive. Another issue is off-targeting, which is independent of the AON hybridization to transcripts. The competition of exogenous RNAi triggers with endogenous miRNAs for RISC charging may cause alterations in natural miRNA regulatory networks [96,97]. Moreover, AONs of various types may interact with other cellular and extracellular proteins and induce toxic effects. Many proteins are involved in the activation of the immunological response to exogenous nucleic acids [98,99]. Furthermore, specific immunostimulatory sequence motifs were identified, which should be avoided in the AON design [100-103]. This issue is the subject of several reviews [104-106], and relevant information is gathered in the RNAimmuno database [103].

DELIVERY TO THE NERVOUS SYSTEM

Antisense oligonucleotide modifications will largely determine the most efficient route of delivery. In drug discovery, the aim is to find a substance which is potent, selective, and preferably bioavailable that needs to reach its target at sufficient concentrations [107]. For drugs to reach the nervous system they first have to cross the vascular barrier, which is made up of the BBB or the Blood–Spinal Cord Barrier (**BSCB**). These vascular barriers prevent most molecules from entering the nervous system from the blood circulation. Despite this gate-controlling system, essential nutrients, such as glucose, are permitted to pass [108]. The vascular barriers of the nervous system are comprised of a monolayer of endothelial cells forming tight junctions through interactions of cell adhesion molecules [109]. Other structural components are astrocytes that surround the endothelial cells with their processes, pericytes located between the endothelial cells and astrocytes, macrophages, and finally the basement membrane. The endothelial cells of the BBB are characterised by only few fenestrae and pinocytotic vesicles, limiting transport to and from the brain. The BBB thus also largely separates the peripheral immune system from the brain. Although the BSCB is largely made up of the same components as the BBB, there are some functional and morphological differences [110]. For instance in the BSCB, the equivalent of the BBB endothelial cells is the choroid plexus epithelial cells [111] and the permeability of the two barriers is different probably due to differences in tight junction protein expression [112]. In neurodegenerative diseases, disruption of the vascular barrier is common [109,113,114] and it was shown in animal models that a compromised BBB barrier in itself can lead to neurodegeneration [113]. However, for most diseases of the nervous system that could be treated with antisense oligonucleotides, the antisense oligonucleotides will not be able to cross the vascular barrier when delivered systemically. Several methods have been used to deliver antisense oligonucleotides to the nervous system (see Figure. 1).

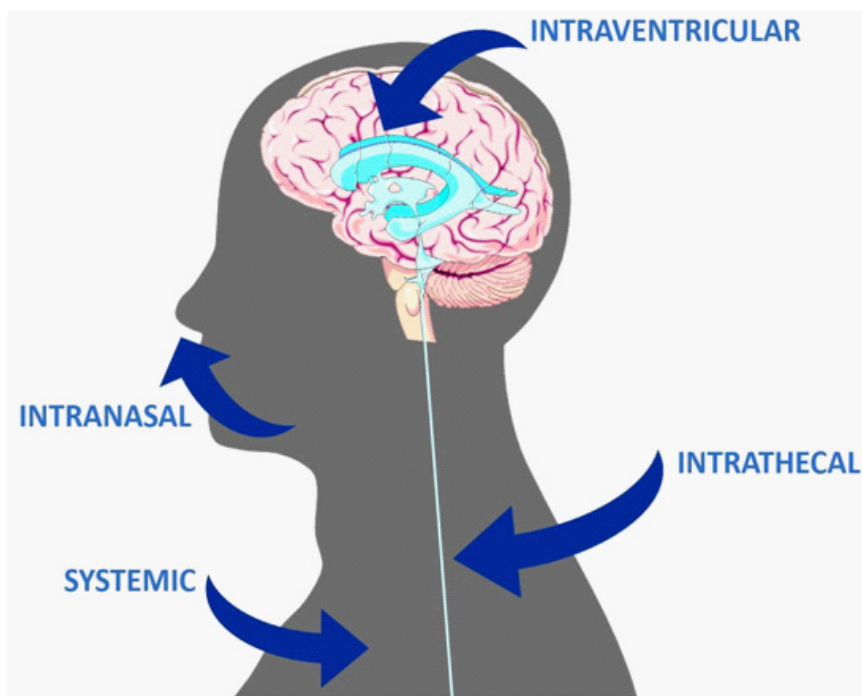


Figure 1. Routes for delivery of AONs to the central nervous system. Following systemic delivery as the first way, AONs can be directly delivered into the CSF through intracerebroventricular or intrathecal infusion using an implanted reservoir that is connected to the ventricles within the brain or spinal cord via an outlet catheter. Another alternative less invasive route of delivery of AONs could be possible via intranasal administration.

Peripheral Delivery

Endeavors are developing to deliver ASOs to the nervous system via the systemic route. Basically, when a drug is administered systemically, a fraction will be bound to blood proteins (e.g. serum albumin, lipoprotein etc.) and a fraction will be unbound. The bound fraction is the pharmacologically applicable fraction, since it is able to cross the vascular barrier [109] depending on its physicochemical properties. Simple diffusion is the first mechanism by which AONs could apply to cross the vascular barrier. Only molecules with a molecular weight smaller than 400 Da are able to cross the vascular barrier [115]. In addition, small lipophilic substances which have a hydrogen bond are more likely to pass the vascular barrier [116] than compounds without hydrogen bonds. In general, AONs have a molecular weight of approximately 6000 to 10,000 Da and thus are too large to cross the vascular barrier by simple diffusion and reach a fruitful concentration in the nervous system. Actually, some early studies reported that less than 1% of the injected radioactive oligonucleotide is measured in brain [117-120] that showing the limited brain uptake of peripherally administered AONs. The saturable oligonucleotide transport system is the term given to this mechanism by which this AON employed to cross the BBB [118].

Receptor-mediated endocytosis is another mechanism to cross the vascular barrier, which allows macromolecules, such as transferrin, insulin, leptin, and insulin-like growth factor 1, to enter the nervous system [121]. Studies used biotinylated AONs captured with a streptavidin conjugated radioactive labelled monoclonal antibody to the mouse transferrin receptor to quantify gene expression *in vivo* [122]. This radioactive labelled AON conjugate reached the brain through the endogenous transferrin transport pathway (receptor-mediated endocytosis) in a transgenic mouse model [122]. This same transferrin transport pathway was used to transport nanoparticles carrying AONs targeting aquaporin 4 into the brain parenchyma [123].

Cell-Penetrating Peptide (**CPP**)-based delivery systems had taken great attention in recent researches [124]. These have reported that strong transmembrane capacity and great potential for therapeutic approaches for polyQ neurodegenerative disorders [125]. The length of CPPs can be up to 30 amino acids and can carry a wide variety of cargos. Depending on cell types and cargos, different CPPs use distinct cellular translocation pathways [126]. Systemically delivered AONs tagged with arginine-rich CPPs were widely distributed throughout the brain of wild-type mice via its ability to cross the BBB [127]. Considering, not all types of AON modifications are suitable for coupling with CPPs. Although two promising CPP antisense oligonucleotides were having been deserted as therapeutic agents since Repetitive Intravenous (**IV**) bolus injections of CPP antisense oligonucleotides caused lethargy and weight loss in rats [128] and tubular degeneration in the kidneys of monkeys [129], CPP systems are still a promising delivery system.

AONs encapsulated in exosomes would theoretically also be able to cross the vascular barrier after IV injection, although it is not tested until now. Exosomes are a well-studied groups of extracellular vesicles known to function well in the communication process between cells via transfer of proteins and nucleic acids [130,131]. IV injection of exosomes transduced with short viral peptides derived from Rabies Virus Glycoprotein (**RVG**) resulted in crossing of the BBB and small interfering RNA (**siRNA**) delivery to the brain [132]. These IV injected RVG-targeted exosomes bound to neurons, microglia and oligodendrocytes, resulting in a specific gene knockdown in the brain [133].

Direct Delivery to the Nervous System

Besides mechanisms to cross the vascular barriers, there are also techniques that bypass them through direct infusion into the Cerebrospinal Fluid (**CSF**). AONs can be infused Intra Cerebro Ventricularly (**ICV**) after which the ASOs would have to pass the ependymal cell layer that lines the ventricular system to enter the parenchyma. Intrathecal (**IT**) delivery means delivery of the ASOs into the subarachnoid space of the spinal cord. From here it will have to pass the pia mater to enter the parenchyma. ASOs can be delivered ICT or IT through an outlet catheter that is connected to an implanted reservoir. Drugs can be injected into the reservoir and delivered directly to the CSF.

This route of delivery has several advantages over peripheral administration. It results in immediate high drug concentrations in the CSF meaning that a smaller dose can be used, potentially minimizing toxicity. Also, because there is free exchange between the CSF and brain parenchyma, and the BBB prevents transport of the AONs into the peripheral circulation, direct delivery into the nervous system can relatively rapidly result in therapeutic drug concentrations. This infusion technique was used in antisense oligonucleotide applications in rodent models of polyQ neurodegenerative disorders as well as non-human primates [133]. Moreover, IT drug therapy has been applied to a wide variety of neurological conditions [134,135]. To date there have been two phase I clinical trials completed using IT infusion of AONs in Amyotrophic Lateral Sclerosis (**ALS**) and Spinal Muscular Atrophy (**SMA**) patients [136,137] without any major adverse side effects [136].

Intranasal Delivery to the Brain

Intranasal administration is a very promising alternative route of delivery of AONs to the brain, although there have been very few studies showing this kind of delivery so far. Molecules can be transported along the olfactory and trigeminal nerve pathway and the rostral migratory stream, after intranasal administration [138]. It is less invasive method for AONs delivery compared to the above mentioned delivery routes. Additionally, this method was used successfully in clinical trials showing improved cognition after intranasal insulin application in Alzheimer disease patients [139]. Also intranasal delivery of small interfering RNA (**siRNA**) was shown to be very efficient. CPP conjugated to glycol-polycaprolactone copolymers was able to target siRNAs to the brain via the intranasal route [140]. Intranasal delivery of AONs was able to slow down intracerebral tumour growth and lead to good distribution throughout the brain in rats [141]. Accordingly, these studies point out that intranasal delivery may become a practicable choice for AON delivery in the future.

FUNCTIONAL MECHANISMS

AONs can be used in many different pathways to modulate gene expression, depending on the chemistry and target site. Below we will discuss the major functional mechanisms that can be used in PolyQ neurodegenerative disorders (for a schematic diagram see Figure 2) where either the RNA is altered or broken down with the use of AONs.

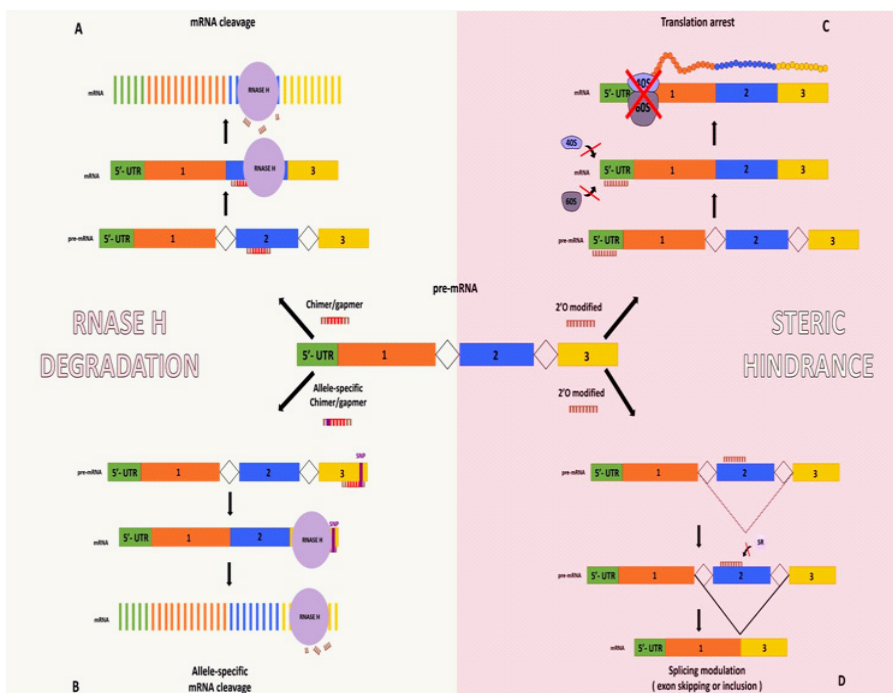


Figure 2: Functional mechanisms of AONs for CNS polyQ neurodegenerative disorders. Depending on the chemistry and target site: AONs can be used in different ways to adjust gene expression, A. AON gapmers induce RNase H-mediated breakdown of target RNA. B. RNase H-mediated breakdown by AON gapmers targeting specific point mutations, a Single Nucleotide Polymorphism (**SNP**), structural differences between wild-type and mutant mRNA or that is unique to the mutant RNA. C. Suppressing RNA translation by AONs sterically blocking the binding of RNA binding protein complexes, such as ribosomal subunits or targeting the RNA translation start site. D. AONs binding to targeting exonic/intronic inclusion signals or splice sites that will lead to skipping or inclusion of the targeted exon. SR, pre-mRNA splicing machinery.

RNase H-Mediated Degradation

As described previously, 2'-modifications improve safety and pharmacologic properties of AONs. However, RNase H requires a free 2'-oxygen and oligonucleotides that are fully 2'-modified cannot induce RNase H-mediated target RNA down regulation [45]. An alternative strategy using gapmers has been developed to induce gene knockdown through RNase H (Figure. 2A and B). A gapmer AON composed of a central DNA region with flanking 2'-modified nucleosides. It only binds to two or three residues at the 3'- or 5'-end because the active site of an exonuclease and a short stretch of 2'-modified RNA nucleosides at both ends is sufficient for the protection against exonucleases [142]. Thus, the gapmer molecule benefits from nuclease resistance and improved uptake from the wings, whilst activating RNase H through the gap region protection [45]. In fact,

general down regulation for a protein has important cellular functions would be undesirable. In such demonstration, specific lowering of the mutant protein is most strongly wished. Selective RNase H-mediated degradation can be achieved using AON gapmers targeting (1) a Single Nucleotide Polymorphism (**SNP**) that is unique to the mutant RNA, (2) structural differences between wild-type and mutant mRNA or (3) specific point mutations [143-147] (Figure. 2B).

Steric Hindrance

Preventing translation, next to RNase H-mediated breakdown of mRNA, can also be considered as tool to reduce the expression of the target protein levels (Figure. 2C). Here, all nucleotides of the AON have sugar modifications rendering them RNase H resistant. AONs selecting the RNA translation start site or sterically blocking the binding of RNA binding protein complexes, such as ribosomal subunits could be the way for achieving suppressing RNA translation to reduce protein levels [148] (Figure. 2C).

The manipulation of splicing is gaining more interest as the best-known other AON applications that do not induce the lowering of transcript levels [149] (Figure. 2D). Via alternative splicing, an important mechanism for gene regulation, most human genes expresses more than one mRNA [150]. A very high level of alternative splicing is presented in the brain, so any disturbance of normal splicing patterns can cause or modify human disease [151]. AONs can be used to disrupt this naturally occurring regulatory process by targeting splice sites or exonic/intronic inclusion signals. Hence, this pathway will result in skipping or inclusion of the targeted exon and altering the RNA and protein sequence (Figure. 2D). For polyQ neurodegenerative disorders this can have multiple applications, e.g., restoring the reading-frame by removing an exon with a mutation, skipping an aberrantly included exon to restore the normal transcript, removing disease-causing mutations from genes or switching from a harmful isoform to a less harmful isoform [152-155].

ANTISENSE OLIGONUCLEOTIDE APPROACHES FOR POLYGLUTAMINE (POLYQ) NEURODEGENERATIVE DISORDERS

Several AON therapies for non-polyQ neurodegenerative disorders are under development. The most famous example is for Duchenne muscular dystrophy the closest to clinical application via antisense-mediated exon skipping approach [156,157]. Moreover, myotonic dystrophy type 1, one of the first repeat expansion diseases, where AON mediated RNase H dependent degradation of mutant RNA was developed [158]. However, reaching sufficient concentrations of AONs in the organ of interest and establishing sufficient high cellular uptake is a major issue. PolyQ neurodegenerative disorders have the advantage as most AONs are readily taken up by neurons and glia [5,6] after reaching the nervous system. The exact mechanism of cellular uptake is still poorly understood [159-161] but it has been suggested that uptake occurs through nucleic acid channels [162]. Once the AON has been delivered into the nervous system, it is easier to reach clinically effective concentrations. Because the vascular barriers will prevent it from entering the periphery and there will be no rapid excretion or break down by the kidney and liver. In rodents, it

was reported that the tissue half-life of MOE-PS antisense oligonucleotides was around of 71–206 days in the brain and 145–191 days in the spinal cord [163] after ICV injection. Whereas, 2OMe-PS antisense oligonucleotides IV infused can have a much shorter tissue half-life to be 10–65 days in peripheral tissue [164,165]. This noticeable difference in tissue half-life between peripheral and neuronal tissues could be explained by differences in endosome handling between neuronal and peripheral cell types [166] or by lower intrinsic nuclease activity in neuronal tissue compared to peripheral tissues [5]. Although AONs have a long half-life they are degraded finally, offering the possibility to terminate the therapy process [167]. For clinical trials with long term administration it would be perfect to have the option to stop the treatment if unwanted side effects happened. In Spinal Muscular Atrophy (**SMA**) mice, administration of a fully complementary oligonucleotide three weeks after ICV injection of a therapeutic MOE-PS antisense oligonucleotide was reported to reverse the antisense oligonucleotide-mediated Survival Motor Neuron 2 (**SMN2**) exon 7 inclusion [166].

Polyglutamine Neurodegenerative Disorders

Polyglutamine (**polyQ**) neurodegenerative disorders are a group of neurodegenerative diseases caused by CAG triplet repeat expansions in the sequence of specific functionally unrelated genes. These disorders include Spinal Bulbar Muscular Atrophy (**SBMA**), Dentatorubral-Pallidoluysian Atrophy (**DRPLA**), Huntington's Disease (**HD**), and Spino Cerebellar Ataxia (**SCA**) types 1, 2, 3, 6, 7 and 17. In addition, SCA8 shares some characteristics with polyQ disorders due to the antisense transcription of non-protein-coding gene containing CTG expansion and the translation of antisense transcripts to polyQ proteins. Of these polyQ disorders, HD and SCA3 have the highest prevalence worldwide [168]. The disease causing proteins in these polyQ diseases are very different, but in each case the expanded stretch of glutamines results in a toxic-gain-of function of the protein causing aggregates and this leads to neurodegeneration [169,170]. Protein aggregates are found in the nucleus and cytoplasm of cells, indicating that protein misfolding is a common feature of these disorders. The expanded repeats in these polyQ neurodegenerative diseases are unstable resulting in anticipation; a more severe and earlier onset of disease in following generations [171]. There is an inverse correlation of disease onset and polyQ neurodegenerative diseases length in the protein. The longer the CAG repeat, the earlier the age of onset of the disease [169]. All are progressive, ultimately fatal disorders that typically begin in adulthood and progress over 10 to 30 years. So, the common feature of polyQ diseases is their late onset, as initial symptoms usually appear in affected subjects in their 30s or 40s. Typically, normal alleles of polyQ disease genes contain 10–30 CAG repeats, and mutant alleles contain 40–60 repeated units. However, repeats as short as 21 CAG tracts in the *CACNA1A* gene can cause SCA6 and expansions reaching more than 100 repeated units may occur in HD and SCA7. PolyQ diseases also share some pathogenic pathways leading to neurodegeneration. The mutant genes are ubiquitously expressed in the central nervous system and peripheral tissues [168], but the pathology develops primarily in distinct brain areas characteristic of each disorder. Interestingly,

the expression of the mutant gene is usually not much higher in the brain areas mainly affected by the disease than in other brain areas and peripheral tissues. This result suggests that additional factors are required to stimulate pathogenesis. Currently no treatment is available to delay onset or even slow progression of polyQ diseases. Although the polyQ disorders remain incurable and only symptomatic treatment is offered to patients, many drugs are currently being tested to reverse the disease or to slow its progress. The most direct therapeutic strategy is silencing the casual gene expression. For some of these approaches, pre-clinical testing in rodent and cells models are well advanced, and small-scale clinical testing is just beginning. In this chapter, we briefly refer to the pathogenesis of polyQ neurodegenerative diseases and present the current status of therapeutic strategies, especially the AONs-based techniques.

Pathogenesis of Polyq Neurodegenerative Diseases

In the traditional point of view for gene expression, the expanded CAG repeats present in mutant polyQ neurodegenerative disease genes are transcribed into CAG repeats in RNA and translated into polyQ neurodegenerative diseases tracts in the encoded proteins. The abnormal protein–protein interactions triggered by mutant proteins were long thought to be the only factor responsible for the pathogenesis of polyQ neurodegenerative diseases. In recent years, however, several examples of mutant transcript toxicity were demonstrated, and additional toxic RNA and protein entities were identified.

Protein Toxicity

Proteins implicated in polyQ neurodegenerative diseases differ in size and cellular function and are mainly involved in the regulation of transcription [172]. The toxicity of mutant proteins in polyQ neurodegenerative diseases are thought to result primarily from a gain-of-function mechanism triggered by protein misfolding and aggregation [173]. The nuclear ubiquitin-positive aggregates of mutant proteins are the hallmark of polyQ neurodegenerative disorders [174-177]. There is no clear answer yet as to whether Neuronal Intranuclear Inclusions (**NIIs**) play an essential role in the pathogenesis or partially protect cells from cytotoxicity. Several studies on cellular and animal models showed no clear correlation between inclusion formation and cell death [178-180]. Importantly, mutant protein fragments containing polyQ tracts are more toxic than full-length proteins and might be crucial for pathogenesis [181]. Such fragments, which are mainly the products of cleavages by caspases, were identified for huntingtin, atrophin-1, ataxin-1, ataxin-2, ataxin-3 and ataxin-7 [182-184]. In agreement with these observations, more severe phenotypes are observed in animal models of polyQ neurodegenerative diseases that express fragments of mutant genes than in models expressing full-length proteins [185].

Post-translational modifications are also significant contributors to polyQ neurodegenerative diseases protein toxicity [186]. The phosphorylation of the polyQ neurodegenerative diseases protein may affect its proteolytic cleavage, as well as influence SUMOylation and ubiquitylation. Interestingly, specific post-translational modifications can affect the toxicity of polyQ proteins in

opposite ways, e.g. huntingtin phosphorylation at Ser421 results in a decrease in NII formation and reduces toxicity [187], whereas phosphorylation of ataxin-1 at Ser776 is required for SCA1 pathology [188]. In the case of ataxin-1, the importance of Ser776 phosphorylation comes from the fact that it triggers a structural change in the region in which several functional signals are localized [189-190].

Not only new protein-protein interactions are triggered by mutant proteins, interactions characteristic of wild-type proteins can also be either enhanced or attenuated in case of mutant polyQ neurodegenerative diseases proteins [191]. The beta-sheet conformation and coiled-coil structure are thought to mediate aggregation, as well as interactions with other proteins [192-196]. Such altered protein-protein interactions were characterized very extensively for huntingtin [197-200], and they are thought to be crucial for the vulnerability of specific types of neurons to degeneration. The other examples include the following: ataxin-1 association with translational repressor Capicua [201] and transcription factor RORalpha [202] and ataxin-7 interactions with transcription coactivator complex STAGA [203,204]. The formation of additional toxic protein products may be triggered by the expanded CAG repeat tracts in transcripts. These extra proteins include frame-shifted polypeptides [205, 207] and products of Repeat-Associated Non-AUG (RAN) translation [208,209].

RNA Toxicity

Growing evidence supports the importance of mutant transcript toxicity in the pathogenesis of polyQ neurodegenerative diseases. Expanded CAG repeats in transcripts form hairpin structures containing periodic A-A mismatches [210- 215]. A similar hairpin structure, formed by CUG repeats [216], triggers the major pathogenic mechanism in Myotonic Dystrophy Type 1 (**DM1**) [217], the disease caused by a CTG expansion in the 3' UTR of the *DMPK* gene [218]. The hallmark of CUG repeat toxicity is the formation of nuclear foci by mutant transcripts and sequestered MBNL1 protein [219]. Ribonucleoprotein foci formation and MBNL1-dependent deregulation of alternative splicing were also observed in HD and SCA3 cells [220]. The toxicity caused by expanded CAG repeat RNA was demonstrated using genetic constructs containing mutant CAG repeat tracts expressed in different model organisms [221]. Experiments performed in *Drosophila* compared the effects of transcripts that were translated with those that were not and contained pure or CAA-interrupted CAG repeats encoding polyQ neurodegenerative diseases tracts [222-224]. Significant toxicity was reported for translated and untranslated CAG repeat tracts, but it was not observed for untranslated CAA-interrupted tracts, which do not form stable hairpin structures [225]. Pathogenic features were also observed in a transgenic mouse model in which the expression of an expanded untranslated CAG repeat tract was directed to muscle [226]. A comparison of two HD mouse models, which contained different patterns of CAA-interrupted CAG repeat tracts, supported the contribution of CAG RNA toxicity to the pathogenesis of polyQ neurodegenerative diseases [227]. Using a *Drosophila* SCA3 model and a HD mouse model, the involvement of the NXF1/U2AF65 RNA export pathway in RNA-mediated toxicity was

demonstrated [228]. The interaction of mutant CAG repeats with nucleolin was shown to induce nucleolar stress, leading to apoptosis in *Drosophila* and human cellular models of SCA3 as well as in HD mouse model [229]. Helicase p68 was also shown to colocalize with expanded CAG repeats and increase MBNL1 binding to mutant transcripts [230]. Moreover, splicing factor SRSF6 was reported to interact with expanded tracts in HTT transcripts, which results in short HTT sense transcripts being translated into toxic peptides [231]. Furthermore, other toxic RNA entities were identified: short CAG repeat RNAs [232,233] and antisense transcripts [234].

Targeting of Mutant Genes and their Expression Products—Therapeutic Avenues for PolyQ Neurodegenerative Diseases

Taking into consideration the fact that each polyQ neurodegenerative disease is monogenic, a rational therapeutic plan could be outlined to lower the causative gene expression. As proof of the concept, the inducible expression of a mutant transgene was turned off in rodent models of HD and SCA1, and recovery from the disease could be seen [235- 238]. This result included the reversal of aggregate formation and improvement in the motor phenotype.

The possible ways to block the pathogenic pathway in polyQ neurodegenerative diseases include the following: genome editing, transcription inhibition, transcript degradation or modification, translation arrest, and inhibition or degradation of toxic protein. To prevent the activation of pathogenic pathways, the mutation should be eliminated or blocked at the earliest step of mutant gene expression, i.e. in the DNA sequence. The technologies designed for DNA editing are developing rapidly [239-241] and were already tested for therapeutic use [242- 247]. In the case of polyQ neurodegenerative diseases, gene editing tools can be directly targeted to expanded CAG sequences in genomic DNA. Zinc finger nucleases that recognize and cleave CAG repeat sequences were tested in mammalian cells [248]. More recently, the CAG repeat-targeting Zinc Finger Proteins (**ZFPs**) were used in a R6/2 HD mouse model to achieve efficient and specific repression of mutant Huntingtin Gene (**HTT**) [249]. The ZFP-mediated mutant transgene repression likely involved steric blockade formation for RNA polymerase during transcription and resulted in a substantial correction of the molecular and behavioral pathogenic hallmarks. In attempts to therapeutically regulate gene transcription, the promoter sequence may be targeted with short RNA duplexes [250- 253]. The feasibility of this approach is supported by growing evidence that core RNA interference (**RNAi**) proteins are present and operate in the nucleus [254-257]. It is not clear whether the observed changes in the histone code are caused by short RNAs targeting DNA or RNA (sense or antisense) covering the promoter sequence, but clearly Argonaute proteins are involved in these processes [258,259]. In the case of polyQ neurodegenerative diseases, this strategy would likely result in silencing both alleles of the gene. The prevailing strategy for the therapeutic regulation of gene expression is transcript targeting with AON-based tools. This regulation takes advantage of various mechanisms, which are determined by the AON type, its features such as structure, chemical modification and target localization and its dependence on specific cellular proteins [260- 262].

AONs may be designed to target pre-mRNA for its correction. Specific AONs can modulate splicing, e.g. exclusion of exon containing mutation or skipping transcript region encoding protein portion implicated in pathogenesis [263- 265]. This strategy was tested for SCA3 and HD in cell culture and initially assessed *in vivo* with AONs modified with 2'-*O*-methyl (2'OMe) and containing a Phosphorothioate (**PS**) backbone. The AONs directed the exclusion of two *ATXN3* exons that contained the CAG repeat tract while the reading frame and functional domains of ataxin-3 were preserved [266]. In the HD model, the AONs induced the skipping of the exon 12 fragment of the HTT transcript that encodes a crucial cleavage site [267]. This resulted in a protein resistant to caspase-6 cleavage, which is responsible for generating more toxic truncated huntingtin.

AON-based approaches that have been developed to promote specific mRNA cleavage include mainly RNase H-activating AONs and RNAi-based tools. AONs directing transcript degradation by RNase H now constitute the majority of AON-based drugs in clinical trials [268]. RNase H localizes to the nucleus and cytoplasm and mediates the cleavage of the RNA in an RNA-DNA heteroduplex [269]. In mammalian cells, the 15–20 nt long chemically modified AONs are used for RNase H1 activation, and the cleavage site is located between the 8th and 12th nucleotides from the 3' DNA terminus of the heteroduplex substrate [270- 272].

Another type of regulatory AONs is this that does not induce mRNA degradation but are transcript blockers and may affect translation, the local structure or interactions with proteins. One possibility is that translation arrest by AONs targets translation initiation, which results in the formation of a steric blockade to mRNA scanning [273,274]. Translational inhibition may also be induced by miRNA-like siRNAs, in a RISC-dependent process, which was tested for polyQ neurodegenerative diseases will be described in detail in the next section ('CAG repeat-targeting'). Moreover, the AON-based blockers may be designed to inhibit toxic interactions of specific proteins with mutant transcripts. Various tools targeting the expanded CUG repeat tract in DMPK transcripts were tested. Morpholino oligomers and AONs containing 2'OMe and a PS backbone or locked nucleic acid (LNA) residues caused the reversion of splicing defects and myotonia in DM1 mouse models [275- 277]. This reversion was achieved by blocking the expanded CUG repeat interactions with the splicing factor MBNL1.

In another strategy, structure-specific small-molecule drugs are designed or selected for targeting expanded repeats in transcripts [278]. An extensive high-throughput search was performed for CUG repeat-binding drugs as potential therapeutics for DM1, taking advantage of the stable hairpin structure formed by the expanded CUG repeats [279- 282]. The crystal structures of the CUG and CAG repeat duplexes [283- 286] were very useful in the design of this type of drugs. The bis-benzimidazole-derivatives were shown to efficiently bind to expanded CAG repeats in the transcript [287]. More recently, the benzylguanidine-containing small molecule was described as an efficient inhibitor of the expanded CAG repeat RNA interaction with MBNL1, which resulted in the reversal of the alternative splicing defects in cell culture [288]. Nevertheless,

it seems that transcript targeting by small-molecule drugs could only inhibit RNA toxicity in polyQ neurodegenerative diseases.

Another way of interfering with the expression of a mutant gene is targeting specific protein factors involved in the transcription and translation of the mutant entities. In the case of polyQ neurodegenerative diseases, the transcription elongation factor Supt4h was shown to be required for the transcription of long CAG repeat tracts [289]. The down regulation of Supt4h by siRNAs in cultured neuronal cells resulted in a decrease in the mutant huntingtin level. In another recent study, the translation regulatory protein complex containing the MID1 protein was demonstrated to stimulate the translation of CAG repeat expansions and was suggested to be a potential therapeutic target [290].

Some other therapeutic strategies, which will be only briefly mentioned here, are focused on the elimination of protein toxicity by targeting mutant proteins. These approaches include (i) inducing protein degradation and (ii) inhibiting processes of aggregation, proteolytic cleavage and post-translational modifications of mutant protein [291,292]. In spite of the inconsistent data regarding the toxicity of polyQ neurodegenerative diseases aggregates, the therapeutic potential of decreasing polyQ neurodegenerative diseases aggregation was shown upon increasing chaperone activity [293] or using specific inhibitors [294-296]. Small molecule drugs were screened for altering polyQ neurodegenerative diseases aggregation and enhancing the clearance pathway [297]. Direct polyQ neurodegenerative diseases protein degradation may be enhanced by stimulating the ubiquitin–proteasome system [298] or autophagy [299]. For the degradation of mutant huntingtin, the polyglutamine binding peptide 1 (QBP1) was fused with the binding motif of heat shock cognate protein 70 (HSC70) [300]. The fusion protein was intrastrially delivered in an Recombinant Adeno-Associated Virus (**rAAV**) vector and ameliorated the disease phenotype in the HD mouse model. Recently, the NUB1 factor was shown to lower the mutant huntingtin level by enhancing its proteasomal degradation [301]. The stimulation of NUB1 was achieved by the treatment of cell culture with interferon. Structural motifs such as beta-sheet conformation of the polyQ neurodegenerative diseases tract or coiled-coil structure, which are common in polyQ neurodegenerative diseases proteins, could also be targeted to diminish polyQ neurodegenerative diseases pathology. In the case of particular polyQ neurodegenerative diseases proteins, specific domains or structural motifs could also be blocked to prevent abnormal interactions, e.g. blocking the AXH domain of ataxin-1 [302,303]. Apart from targeting mutant transcript and protein in polyQ neurodegenerative diseases, it can be advantageous to over express the normal allele of a mutant gene as was shown for ataxin-1-like (Atxn-1l) over expression in SCA1 mouse models [304,305]. Elevated Atxn1l levels suppressed SCA1 neuropathology likely by displacing mutant ataxin-1 from its native complex with Capicua [135]. Therapeutic approaches that focus on the inhibition of the main downstream pathogenic pathways in polyQ neurodegenerative diseases are described elsewhere [306-310]. These neuroprotective strategies include the correction of mitochondrial dysfunction, calcium dysregulation or neuroinflammation.

Aon-Based Tools for Silencing Mutant Genes Causing Polyq Neurodegenerative Diseases

CAG repeat-targeting

The differences in the length of the repeat tracts in normal and mutant alleles are advantage of allele-selective CAG repeat-targeting tool. However, this tool is challenging because typical AON-based silencing tools are relatively short, and their binding sites are present not only in the mutant transcript but also in the normal allele and numerous other transcripts containing CAG repeat tracts. Furthermore, transcripts containing CUG repeats may be targeted by the sense strand of siRNA duplexes composed of CAG and CUG repeat strands. There are ~200 and ~100 mRNAs in human cells that contain repeat tracts composed of at least six units of CAG and CUG repeats, respectively [311]. Due to this fact, it was thought that CAG repeat-targeting strategy cannot be selective enough to be used for therapeutic purposes. This not good scenario was noticed when RNAi triggers composed of fully complementary CAG/CUG repeats, shRNA and siRNA [312,313] were used, which lead to non-selective down regulation of mutant and normal transcripts. These AONs activated the typical RNAi pathway, i.e. the mechanism of specific transcript cleavage, which turned out to be biased for longer and shorter repeat tracts. However, recent studies have shown that high selectivity for mutant allele silencing can be achieved, and this tool is now considered applicable.

The allele-selective silencing of polyQ genes by repeat-targeting was achieved by different types of AONs, which reduced the mutant protein level without transcript degradation. The Corey group described the first inhibitors of this type were chemically modified translation blockers [314]. CTG peptide nucleic acid (PNA) oligomers were the most efficient inhibitors know. It's conjugation with lysine residues, showed >6-fold selectivity for mutant huntingtin and ataxin-3 inhibition. Further studies reported similar effects were obtained with AONs modified with MOE-2'F-PS, 2'OMe-PS, LNA, cEt, and morpholino oligomers [315]. These wide chemically modified oligomers are thought to act as efficient blockers for the translation of the mutant polyQ neurodegenerative diseases protein.

Furthermore, various siRNAs targeting CAG repeat expansion were described, and some of them showed significant preference in the silencing of mutant alleles. The idea of Corey's group was to weaken the siRNA-target interaction by introducing base substitutions to the siRNA, resulting in one or more base mismatches with the target sequence [312]. The substitutions were of different types, and in the most selective siRNAs, they were located in the central region. Krzyzosiak's group reported that the same effect could be achieved by placing base substitutions in the 3' portions of the CUG repeat strands [313]. The wobble base pairs were formed in this design to make the siRNA antisense strand interact with its CAG repeat target. The formation of mismatches with the target resulted in miRNA-like activity of these siRNAs, in both siRNA designs [312,313, 316]. The CAG/CUG miRNA-like siRNAs were first described as an allele-selective tool for HD [312, 313] and soon after for SCA3 [317].

Ionis (Isis; previously) Pharmaceuticals' researchers developed a pattern of chemical modifications that was introduced to single-stranded siRNAs (ss-siRNAs) to ensure their high stability and activity *in vivo* [318]. Corey et al. in collaboration with Ionis showed efficient inhibition of huntingtin expression by using the same pattern of siRNA [319]. The ss-siRNAs composed of CUG repeats contained both base substitutions in the central region of AON, generating mismatches with targeted CAG repeats, as well as a specific pattern of 2'F, 2'OMe, PS and 2'MOE modifications. Some ss-siRNAs showed high allele selectivity in silencing mutant huntingtin expression in HD fibroblasts and were successfully tested in a HD mouse model [319]. Recently, similar AONs were tested for ATXN3 silencing in SCA3 fibroblasts, and allele selectivity was also achieved, in addition [320]. Interestingly, the CAG repeat-targeting ss-siRNAs tested as RNAi-activating inhibitors of ataxin-3 translation were also shown to be RNAi-independent activators of the alternative splicing of the ATXN3 transcript, resulting in CAG repeat skipping.

Recently, it has been developed self-duplexing siRNAs (sd-siRNAs) as another type of allele-selective CAG repeat-targeting AONs for HD [321]. The CUG repeat single strands to which one, two or three U to A or U to G substitutions were introduced in either the central or 3' region formed guide-strand-only duplexes functioning as miRNAs. In that study, the first repeat-targeting shRNA showing allele selectivity was also described [321].

CAG repeat-targeting siRNAs are not typical RNAi triggers, and their silencing mechanism, as well as basis for allele selectivity, needs further investigation. The involvement of the AGO2 and GW182 proteins in the silencing mechanism has already been established [316]. The hairpin structure likely formed by the expanded CAG repeats in cells is not stable enough to resist the attack by RISC [312,313,322]. The molecular basis of allele selectivity is thought to result from the cooperative action of multiple RISCs residing on expanded CAG repeats [316]. The single RISC bound to shorter repeats is easily stripped by translating ribosomes. This result also explains the minimal sequence-specific off-target effects in repeat-targeting, which was previously considered a serious point.

Allele-selective versus non-allele-selective tools

Targeting both alleles of the gene and only the mutant allele may be designed as therapeutic strategies for suppressing causative gene expression in polyQ neurodegenerative diseases. The cellular function of the wild-type protein is an important factor considering the requirement of allele selectivity in therapeutic gene down regulation. The main question is whether partial loss of the wild-type protein could be tolerated in adult life? Studies employing conditional knock-out mouse model of HTT shed some light on this point. Using this model, an essential role of huntingtin, mainly in the CNS, was reported [323,324]. Thus, a risk to patients may be happened due to decreasing the level of wild-type protein. In addition, the wild-type protein function may decrease the toxicity of the mutant protein, as indicated for SCA3 [325] and for HD [326]. In the study of HD mouse model, loss of wild-type *Htt* was shown to negatively affect neuropathology,

motor dysfunction and lifespan of mice [326]. These results could explain the disease phenotype in rare homozygous SCA3 and HD patients with two mutant alleles, whose phenotype is more severe than that of patients with a single mutant allele [327,328]. However, the over expression of wild-type ataxin-3 in SCA3 rat model was found not to mitigate the pathology [329].

Non-allele-selective targeting

The pre-clinical testing of various AON-based therapeutic tools, performed in rodent models of polyQ neurodegenerative diseases, was the most extensive testing for the non-allele-selective strategy, until now. The mutant human transgene was targeted and silenced, taking advantage of sequence differences between mouse and human homologues in most cases. In these studies, much attention was given to indicate the reversal of the molecular and behavioral pathogenic hallmarks.

A study in the SCA1 mouse model provided the first *in vivo* evidence for efficient gene silencing in a polyQ neurodegenerative disorder [330]. A few studies were performed using transgenic HD models and *HTT* transgene-specific RNAi triggers: shRNAs delivered in rAAV vectors [331-334] or siRNAs [335,336]. Locally delivered RNAi triggers caused a substantial reduction in transgene expression, which resulted in reduced neuropathy and positively affected the behavioral phenotypes of the mice. In a very extensive study in three mouse models of HD, the mutant *HTT* transgene was silenced with AONs [337]. A reduction in the huntingtin level throughout the central nervous system up to ~25% is shown after treatment with a 2'-O-methoxyethyl- (2'MOE-) modified gapmer and resulted in the improvement of motor coordination. Recently, artificial miRNA mimics targeting the 3' UTR of the mutant *ATXN3* transgene and delivered in AAV vectors were tested in a transgenic SCA3 model [338,339]. Constructs based on miRNA precursors, which are considered safer, were effective in transgene silencing in the cerebellum. However, no correction of the phenotype was reported, likely due to the insufficient distribution of the inhibitor across the brain [338].

The silencing of the normal endogenous *HTT* and *ATXN3* was investigated in several experiments to test the non-allele-selective strategy. In rodent models, normal *Htt* or *Atxn3* was inhibited usually together with the mutant human transgene, while only endogenous *HTT* was silenced in non-human primates. Studies conducted in HD mice treated with RNAi triggers indicated that lowering the wild-type protein to 30–40% of control level was well tolerated [334,340,341]. Similarly, in a study performed in SCA3 rat model, silencing of endogenous *Atxn3* to ~30%, together with mutant *ATXN3*, was shown to reduce neurodegeneration and suggested feasibility of inhibition of both alleles [329]. Moreover, it was shown that silencing of endogenous *Atxn3* in wild-type and SCA3 rats did not cause additional neuropathology [329]. Three studies investigated the RNAi-mediated silencing of endogenous *HTT* in non-human primates [342-344]. A partial reduction in huntingtin after the injection of shRNA in the rAAV vector to putamen was well tolerated, as shown by the analyses performed up to 6 weeks after treatment [342].

siRNA delivery for a period of 4 weeks to the *Rhesus* brain by infusion pump, resulting in a 50% decrease in the huntingtin level, was shown not to cause any significant histopathological changes [343]. shRNA delivered in the rAAV vector caused a 45% reduction in *HTT* expression, while no histopathological changes occurred in the brain and no motor abnormalities were detected even 6 months after the treatment [344]. *HTT* silencing was also performed in non-human primates by AON administration. Huntingtin downregulation up to 30% of the control level was achieved, but the long-term safety of this suppression was not analyzed [337]. In spite of the lack of adverse effects on the phenotype observed after silencing the endogenous *HTT* in animal models, a serious concern remains regarding human cases in which longer-lasting gene inhibition will be required.

The silencing of both alleles of the causative gene is combined with the exogenous expression of a version of the normal allele that has been engineered to be resistant to the silencing agent as another strategy [345]. This gene-knockdown and replacement strategy was tested for SCA6 and SCA3 in cell culture models [346,347]. In these studies, the expression of both alleles of the *CACNA1A* and *ATXN3* endogenes was silenced in human cultured cells using siRNAs. For other disorders, such as retinitis pigmentosa, there are reports of testing the replacement strategy *in vivo* [348]. In principle, with the use of this tool, a similar effect could be achieved as with the allele-selective tools, i.e. the elimination of the mutant allele only. However, the replacement strategy is more demanding as both the silencing agent and the expression vector for the normal allele must be efficiently delivered, and the exogenous protein has to be expressed at an appropriate level.

Single nucleotide polymorphism- (SNP-) targeting

AONs targeting transcript regions harboring SNP variants that distinguish between the normal and mutant alleles of the causative gene may be achieved via allele-selective silencing tool. It is advantageous if the SNP is highly heterozygous in the population and/or is associated with the expansion mutation. This configuration allows the design of a single AON inhibitor for a group of patients carrying such a variant. The therapeutic AON is designed to form a canonical base pair with the SNP variant present in the mutant allele, which triggers transcript cleavage and degradation, whereas the mismatch present in the interaction with the normal allele should render it resistant to cleavage.

Design rules for the SNP-targeting AONs that were described for siRNAs refer mainly to mismatch type and localization [349,350]. The central positioning of a purine–purine mismatch is recommended in the interaction of the antisense strand of the siRNA with the normal transcript. However, the sequence flanking the SNP variant may strongly influence the silencing efficiency and selectivity. Therefore, the design rules are rather rough guidelines, and often a set of siRNAs needs to be tested to find efficient and allele-selective inhibitors.

In the majority of studies that used SNP-targeting to silence the polyQ neurodegenerative disease genes, the AONs were designed to function using the RNAi pathway. The first studies of this type were performed using cellular models of SCA3 [351,352]. The targeted SNP is located at

the 3' end of the CAG repeat tract, and its G and C variants are strongly associated with the normal and mutant *ATXN3* alleles, respectively [353]. The designed siRNAs included strong G-C pairing in the interaction with the mutant allele and a strong G-G mismatch in the interaction with the normal allele, which allowed for highly preferential mutant allele silencing [347,351,352]. The SNP-targeting strategy was also tested using shRNA in rat and mouse models of SCA3 [354,355]. shRNAs were delivered in Lentiviral (**LV**) vectors by injection to the striatum. Two months after the injections, the correction of neuronal dysfunction and a significant decrease in the formation of disease-associated inclusions were observed and in the rat model [354]. The substantial reversal of the neuropathological phenotype was also observed in a severely affected mouse model [355]. The SNP site was also targeted in a cellular model of SCA7 [356]. The exogenous *ATXN7* transcripts containing different SNP variants were selectively targeted by siRNAs expressed from pri-miRNA-based hairpin constructs.

SNP-targeting was most extensively investigated for HD, for which approximately 10 different SNPs were targeted to silence mutant *HTT* expression. The relatively long sequence of the *HTT* gene gives the opportunity to identify many SNP variants in patients' cells [357]. High allele-discriminatory properties of siRNA were observed for two localizations of mismatch with the target: the central and 16th nt positions (counting from the 5'-end of the antisense strand) [347,358- 362]. In a comprehensive study, five allele-selective siRNAs were designed for common variants of three SNPs to cover approximately three-quarters of the HD population [361]. Furthermore, RNase H-activating SNP-targeting AONs were described by the Hayden group [363] and the Seth group [364] as selective inhibitors of mutant *HTT* expression. In the first study, the most efficient AONs were PS substituted 19-mers with nine DNA residues in the gap and five S-constrained-ethyl (S-cEt) motifs on each wing [363]. The AONs were injected into the striatum of animals from two HD mouse models, in which different SNP variants were identified in the transgenes. The expression of mutant human *HTT* was efficiently silenced only in the BACHD model, in which the targeted SNP variant was present and the selectivity of silencing was assessed to be ~5-fold [363]. More recently, the researchers from Ionis Pharmaceuticals in collaboration with the Hayden group demonstrated the power of chemistry in designing an AON that targeted the same SNP variant with >100-fold selectivity in suppressing mutant huntingtin [364]. Such a dramatic improvement in allele selectivity was achieved by iterative redesigning of the specific chemical modification pattern in AON, which included the base, backbone and sugar modifications. The best selectivity was achieved with a 15-mer containing 2'MOE, S-cEt and PS modifications. This AON was tested in a new humanized mouse model of HD [365], and efficient *HTT* silencing across brain tissue was reported, together with high tolerability for AON administration.

Cells and Animal Models Used to Assess the Aon-Based Therapeutic Tools

Cellular models of polyQ neurodegenerative diseases are of great value in the initial screening of AONs. Fibroblast cells derived from polyQ neurodegenerative diseases patients are a very convenient model that is extensively used in evaluating the allele-selective approaches. These cells

contain endogenous mutations, express both normal and mutant alleles and are relatively easy to culture and transfect. Even in testing the therapeutic reversal of the polyQ neurodegenerative diseases pathogenic hallmarks, fibroblasts may be a useful model, as some pathogenic events occur in these cells [366,367]. Nevertheless, in recent trend, cultured neuronal cells are being used. These cells are more adequate, as they better represent brain cells, which should be mainly targeted in therapeutic treatment. The relevant neuronal cells may be derived from brain tissue of polyQ neurodegenerative diseases rodent models. Human neuronal cells may be obtained from patient fibroblasts by their reprogramming to induced pluripotent stem cells (iPSCs), followed by differentiation to neurons. The first example of the use of iPSC-derived neurons for modeling polyQ neurodegenerative diseases was described in the study of SCA3 pathogenesis [368]. In the most extensive study published to date, a set of iPSCs obtained from HD patient-derived fibroblast cells was generated and analyzed in the context of pathogenesis [369]. Several studies on polyQ neurodegenerative disorders were performed to characterize the iPSC models and the neuronal cells derived from them [370-375]. With regard to testing therapeutic approaches, the cultured mouse neurons, as well as human neuronal cell models derived from embryonic stem cells and iPSCs, were used for the validation of specific polyQ neurodegenerative diseases protein-targeting strategy for HD [301]. These cells were also used in cell-replacement strategies after their genetic correction and differentiation into neuronal cells [376-379]. The use of neuronal cell culture models is anticipated to increase rapidly in the coming years, both for the studies of pathogenesis, as well as for testing therapeutic drugs.

The *in vivo* testing of AONs targeting polyQ neurodegenerative diseases was performed mainly in transgenic rodent models. The experience gathered in testing AONs in rodent models of HD, SCA3 and SCA1 can be useful in the design of strategies for other polyQ neurodegenerative diseases. There is a variety of models available that differ in their genetic and phenotypic characteristics and are used based on their validity for different approaches [380,381]. PolyQ neurodegenerative diseases rodent models differ in the severity of their phenotypes, which depend on the specific features of the mutant exogene, mainly the length of the whole exogene and its repeated sequence, as well as its expression level. There are important issues to consider in testing the allele-selective silencing of causative genes *in vivo*. Due to sequence differences between rodent and human orthologs, the designed ONs are often specific only for human transgenes [354,355]. A model best suited for testing the allele selectivity of targeting should contain two alleles of full-length human sequences, the normal version and the mutant with the pathogenic repeat tracts, as well as the relevant SNP variants. However, such a model might present a mild phenotype; therefore, the simultaneous use of a model with a faster and stronger phenotype may be required. Most of the full-length HD models contain only mutant transgenes and include knock-in types with chimeric sequences under mouse promoters or transgenic types with full-length human HTT [118]. Recently, a transgenic mouse model of HD with two human HTT alleles (containing 18/97 CAG repeats and a human promoter) was created [365] and used in the successful testing of AON in an

allele-selective strategy [364]. The development of new cellular and mouse models is underway for better evaluation of ON-based therapeutic strategies, and models of larger mammals are also under investigation [382,383].

Comparison of Different Aon-Based Plans

A number of important issues still need to be addressed in utilizing AON-based tools for therapy in human. The high efficiency of AONs is required, which depends on their delivery, uptake and stability. Low doses and appropriate delivery are needed to prevent toxicity. Two strategies are being tested to ensure long-lasting activity of AONs: (i) incorporation of chemical modifications to increase AON stability and (ii) delivery of silencing reagents in genetic vectors. These strategies differ in the design of the relevant therapeutic tools and in the obstacles that need to be overcome in pre-clinical testing.

Clinical Features

Still, some limitations must be overcome for therapeutic use of AONs. They are mainly due to shortcomings in the efficiency and safety of AONs in clinical trials. This comprises problems regarding efficient AON delivery to targeted tissues without causing any adverse effects. There are additional issues to be considered while taking AONs from pre-clinical testing to human trials for the development of treatments available to patients. The basic evaluation of AON potency as a therapeutic agent is the analysis of targeted gene down regulation. For a more complete assessment of AON properties *in vivo*, specific analyses need to be performed to observe the potential reversal of selected markers of the molecular pathogenesis of polyQ neurodegenerative diseases. From a clinical point of view, it is challenging to develop methods for the validation of therapeutic benefits that would not be harmful and could be used in human clinical trials [384,385].

The question remains whether it is sufficient to suppress mutant gene expression locally in the regions where the disease primarily develops or whether it is necessary to target either the whole central nervous system or also the peripheral tissues. As significant dysfunctions in the periphery were reported in patients and in mouse models of HD and other polyQ neurodegenerative disorders [386-391], systemic delivery seems to be the best option, provided it also successfully delivers AONs to the central nervous system. Another important question is at what stage of disease is it necessary to intervene and silence the gene expression so the pathology will completely, or at least partially, reverse? Treatment at the late stage of disease poses the risk that some toxic events will become irreversible. Results from an inducible mouse model of SCA1 indicate that the pathogenic effects of the mutation in ataxin-1 accumulate starting from postnatal development [202]. The recovery from disease was observed in several inducible rodent models when the mutant transgene was turned off at an early or middle stage of the polyQ neurodegenerative disease [235,236,392,393]. Nevertheless, full recovery was not achieved when the transgene expression was halted at a late stage of the disease [236,393]. Similar

results were obtained with AON-based silencing of the mutant transgenes in rodents, but partial recovery even from a severe phenotype was reported [355]. Considering the human lifespan, it is preferable that a single administration of a drug provides long-term mutant gene silencing. In this context, the result obtained by Cleveland et al. is very promising. Using HD mouse models, the authors demonstrated that AONs intraventricularly infused into the CSF caused phenotypic recovery lasting much longer than mutant gene down regulation [337]. ‘Huntingtin holiday’ is term described this finding [394], is one of the major breakthroughs in HD research and a very good news for HD patients.

Conclusions and Future Views

In this chapter, we gathered the existing information and discussed the progress of research aimed at developing therapeutic approaches for polyQ neurodegenerative diseases. We presented the most advanced gene inhibition strategies, AONs, including their therapeutic potential with some shedding light on RNAi methods.

The AONs directing transcript degradation by RNase H have been explored as potential drugs since the late 1970s, whereas RNAi has been used for post-transcriptional gene silencing since the early 2000s. Therefore, AON tools are more advanced in pre-clinical and clinical testing, but the experience gathered in testing AONs is in many respects useful for the development of RNAi-based strategies [395]. This advantage refers to the general administration, distribution, metabolism and excretion characteristics of AONs, which can be relevant in many ways for both the AONs and siRNAs [396]. As a result, rapid progress in advancing siRNAs to clinical trials is expected in coming years.

At this point, it is difficult to say which AON-based therapeutic strategy is best suited for polyQ neurodegenerative diseases. The existing strategies offer great opportunities for therapy but also have limitations. The allele-selective SNP- and CAG repeat-targeting strategies are regarded as more safe. CAG repeat-targeting strategy, which was considered some years ago a ‘mission impossible’, turned out to be not only feasible but also very promising according to the first *in vivo* studies in mouse models of HD [319]. However, a full assessment of the CAG repeat-targeting strategy needs to be accomplished. The potential advantage of this strategy is its universality, as one drug could possibly be used to treat most of the polyQ neurodegenerative diseases.

The AON-based strategies are more advanced than other strategies capable of interfering with mutant gene expression. AONs may be designed for any transcript and may function by many mechanisms. Other strategies, such as genome editing, that are aimed at repairing a mutant gene or preventing its transcription are very attractive but still far from clinical applications. The clinical application seems to be still more distant for the cell therapy of polyQ neurodegenerative diseases.

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