

Silencing of genes responsible for polyQ diseases using chemically modified single-stranded siRNAs

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Polyglutamine (polyQ) diseases comprise a group of nine genetic disorders that are caused by the expansion of the CAG triplet repeat, which encodes glutamine, in unrelated single genes. Various oligonucleotide (ON)-based therapeutic approaches have been considered for polyQ diseases. The very attractive CAG repeat-targeting strategy offers selective silencing of the mutant allele by directly targeting the mutation site. CAG repeat-targeting miRNA-like siRNAs have been shown to specifically inhibit the mutant gene expression, and their characteristic feature is the formation of mismatches in their interactions with the target site. Here, we designed novel single-stranded siRNAs that contain base substitutions and chemical modifications, in order to develop improved therapeutic tools with universal properties for several polyQ diseases. We tested these ONs in cellular models of Huntington's disease (HD), spinocerebellar ataxia type 3 (SCA3) and dentatorubral-pallidoluysian atrophy (DRPLA). Selected siRNAs caused the efficient and selective downregulation of the mutant huntingtin, ataxin-3 and atrophin-1 levels in cultured human fibroblasts. We also prove the efficiency of novel ONs, with chemical modification pattern mainly containing 2'-fluoro (2'F), in HD mouse striatal cells.

Key words: siRNA, CAG repeats, polyglutamine diseases, Huntington's disease

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INTRODUCTION

For fifteen years, RNAi-based strategies designed for mutant gene down-regulation, have been rapidly being developed. A variety of oligonucleotides (ONs) have been tested for polyglutamine (polyQ) diseases (Fiszera & Krzyzosiak, 2014). This group of neurological disorders is caused by ORF-located CAG repeat expansion in specific genes, and includes HD, DRPLA, spinal bulbar muscular atrophy (SBMA), and SCA types 1, 2, 3, 6, 7 and 17 (Fan *et al.*, 2014).

Although polyQ disorders remain incurable and only symptomatic treatment is offered to patients, many drugs are currently in testing with the aim to reverse the disease or to slow its progress (Kumar *et al.*, 2015). The pathogenesis of polyQ diseases is caused by the disruption of cellular pathways by the expression products of the mutant gene, i.e., proteins containing polyQ tracts and mutant transcripts. The pathology develops in the brain areas characteristic for each disorder, e.g. *striatum* in HD, *cerebellum* in SCA3 (Fiszera & Krzyzosiak, 2013; Evers *et al.*, 2013; Labbadia & Morimoto, 2013). The

most direct and beneficial therapeutic strategy for polyQ diseases is silencing the causal gene expression, and the prevailing strategy is transcript targeting using ON-based tools (Bennett & Swayze, 2010; Sibley *et al.*, 2010; Kole *et al.*, 2012). For several polyQ diseases, allele-selective strategies using ONs targeting regions containing SNP variants or expanded CAG repeat tracts, have been considered (Fiszera & Krzyzosiak, 2014).

Short-interfering RNAs (siRNAs) are common tools used for post-transcriptional gene silencing in potential therapeutic strategies. In the RNAi pathway, one strand from the siRNA duplex, the guide strand, is loaded into RNA-induced silencing complex (RISC) with the core Argonaute 2 (AGO2) protein. The use of single-stranded siRNAs (guide-only siRNAs) is desired due to potentially lower off-target effects, but it was initially considered invalid due to the requirement for ON delivery to cells at very high concentrations (Holen *et al.*, 2003). In cells, AGO2 loading is generally less efficient for single-strands than it is for duplexes (Martinez *et al.*, 2002; Xu *et al.*, 2004). A specific chemical modification pattern enables high activity of guide-only siRNAs and miRNAs (Haringma *et al.*, 2012; Chorn *et al.*, 2012; Lima *et al.*, 2012). A variety of ON chemical modifications is mainly introduced in the internucleotide bond (e.g., phosphothioate – PS) or sugar unit (e.g., 2'-O-methyl – 2'OMe, 2'-O-methoxyethyl – 2'OMOE, 2'F). The efficiency of siRNAs has significantly increased as a result of their appropriate chemical modification (Amarzguoui *et al.*, 2003; Sipa *et al.*, 2007; Bramsen *et al.*, 2009; Engels, 2013). Another option for activating guide-only siRNAs in cells is to design antisense strands that are able to form a self-duplex (Hossbach *et al.*, 2006; Fiszera *et al.*, 2013).

CAG repeat-targeting strategy, developed for several polyQ disorders, was proven effective and allele-selective in studies carried out by David Corey's group (Hu *et al.*, 2009; Hu *et al.*, 2010; J Hu *et al.*, 2011; Yu *et al.*, 2012; Aiba *et al.*, 2013; Liu *et al.*, 2013) and by us (Fiszera *et al.*, 2011; Fiszera *et al.*, 2012; Fiszera *et al.*, 2013). The CAG repeat-targeting approach is based on multiple binding sites in the expanded repeat tract, and the degree of selectivity depends on the difference between the repeat tract length in the normal and mutant alleles. In this approach, miRNA-like siRNAs are designed to contain base substitutions resulting in the formation of mis-

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Abbreviations: DRPLA (dentatorubral-pallidoluysian atrophy), HD (Huntington's disease), ONs (oligonucleotides), polyQ (polyglutamine), sd-siRNAs (self-duplexing siRNAs), PS (phosphothioate modification), RISC (RNA-induced silencing complex), SCA3 (spinocerebellar ataxia type 3), ss-siRNAs (single-stranded siRNAs), 2'OMe (2'-O-methyl modification), 2'F (2'-fluoro modification)

matches with the target (i.e., an expanded CAG repeat tract). Different types of siRNAs have been used: siRNA duplexes (Hu *et al.*, 2010; Fiszer *et al.*, 2011; Hu *et al.*, 2011; Liu *et al.*, 2013; Hu *et al.*, 2014a), chemically modified single-stranded siRNAs (ss-siRNAs) (Yu *et al.*, 2012; Aiba *et al.*, 2013; Liu *et al.*, 2013; Hu *et al.*, 2014b) and self-duplexing siRNAs (sd-siRNAs) (Fiszer *et al.*, 2013).

In this study, our aim was to further consolidate the beneficial effects induced by siRNAs by modifying siRNA strands with both, nucleotide substitutions and chemical modifications. These CAG repeat-targeting siRNAs were tested in cellular models of polyQ diseases.

MATERIALS AND METHODS

Cell lines. Fibroblasts from patients with polyQ diseases (HD: GM04281 – 17/68 CAG in *HTT* gene, SCA3: GM06153 – 18/69 CAG in *ATXN3* gene, DR-PLA: GM13716 – 16/68 CAG in *ATN1* gene), obtained from the Coriell Cell Repositories, were grown in a minimal essential medium (Lonza) supplemented with 8–15% fetal bovine serum (Sigma-Aldrich), antibiotics (Sigma-Aldrich) and non-essential amino acids (Sigma-Aldrich); the incubator conditions included 5% CO₂ and a temperature of 37°C. Mouse striatal cell lines (*STHdb*) (Trettel *et al.*, 2000), were purchased from the Coriell Cell Repositories and grown in a medium containing DMEM (Gibco), FBS (Sigma-Aldrich), G418 and penicillin/streptomycin, with incubator conditions of 5% CO₂ and 33°C. For storage in liquid nitrogen, these cell lines were cryopreserved in the Recovery Cell Culture Freezing Medium (Gibco).

Oligonucleotides and cell transfection. RNA ONs and chemically modified ONs were synthesized by FutureSynthesis or IDT. The sequences of oligonucleotides used in this study are presented in Fig. 1. Cell transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Transfection efficiency was monitored using a BlockIT fluorescent siRNA (Invitrogen).

Western blot. Western blots for huntingtin and ataxin-3 proteins were performed as described previously (Fiszer *et al.*, 2011). A western blot protocol for atrophin-1 protein analysis was adapted from (Hu *et al.*, 2014a). Briefly, 25 µg of the total protein was run on a Tris-HCl SDS-polyacrylamide gel (4% stacking gel, 6% resolving gel, acrylamide:bis-acrylamide ratio 37.5:1) in Laemmli buffer, at 125 V, or NuPAGE 3–8% Tris acetate gels (Invitrogen) in XT Tricine buffer (Bio-Rad), in an ice-water bath. The immunoreaction was performed using the following antibodies: anti-atrophin-1 (Bethyl Laboratories), anti-vinculin (Cell Signaling Technology) and anti-rabbit HRP-conjugate (Jackson ImmunoResearch), and detected using the WesternBright Quantum HRP Substrate (Advanta). The protein bands were directly scanned from the membrane using a camera and were quantified using the Gel-Pro Analyzer.

Northern blot. The detection of siRNA pools present in cells after transfection was performed using northern blotting, as previously described (Fiszer *et al.*, 2013). Briefly, total RNA (2 µg) and 2 pmoles of synthetic ON (in the "S" lane) were resolved on a denaturing polyacrylamide gel. The RNAs were transferred onto a Gene-Screen Plus hybridization membrane (PerkinElmer) using semi-dry electroblotting (Sigma-Aldrich). The membrane was probed with a specific DNA probe complementary to the A2 sequence and labeled with [³²P] ATP (5000 Ci/mmol, Hartmann Analytics). The hybridization was

performed overnight at 37°C, in a buffer containing 5× SSC, 1% SDS and 1X Denhardt's solution. The radioactive signals were quantified by phosphorimaging (Multi Gauge v3.0, Fujifilm).

Statistical analysis. The statistical significance of silencing was assessed using a one-sample t-test, with an arbitrary value of 1 assigned to cells treated with control siRNA (C). Selected data were compared using an unpaired t-test with Welch's correction to assess the allele-selectivity of silencing (normal *vs.* mutant allele silencing). Two-tailed *p* values less than 0.05 were considered to be significant. All experiments for which statistical analyses were done, were repeated three times.

RESULTS

Design of chemically modified CAG repeat-targeting ss-siRNAs

We designed several CAG repeat-targeting ONs based on (I) the nucleotide sequences of miRNA-like siRNAs that were found to be effective and allele-selective in the silencing of mutant polyQ gene expression and (II) the chemical modification patterns that were described as being suitable for ss-siRNA activity (Fig. 1). A2F, WF and PF were based on the sequences of previously described RNA ONs (A2 (Fiszer *et al.*, 2013), W13/16 (Fiszer *et al.*, 2011) and PM3 (Hu *et al.*, 2010), respectively) and a chemical modification pattern mainly containing 2'F and two 2'OMe nucleotides from the 3' end (Chorn *et al.*, 2012; Haringsma *et al.*, 2012). Additionally, the WF and PF ONs contained A-substitution, forming an additional bulge in the region of interaction with the target sequence, which has been described as potentially increasing the specificity of siRNA (Dua *et al.*, 2011). A2M was based on both, the A2 sequence and a chemical modification pattern very close to the one developed by Ionis Pharmaceuticals (Lima *et al.*, 2012). A 5' phosphate was included in all of the ONs because it is required for activity within RISC, whereas chemically modified ONs may not be good substrates for endogenous kinases.

Silencing of different mutant polyQ genes (*HTT*, *ATXN3*, *ATN1*) with selected chemically modified CAG repeat-targeting siRNAs in fibroblast cell models

The activity of a set of chemically modified ONs was initially assessed in HD fibroblasts (Fig. 2A). The ONs were transfected at 100 nM, and 72 h later, the cells were lysed for protein isolation and huntingtin level assessment. In this HD cellular model, ONs with chemical modifications A2F, WF and PF were tested and did not demonstrate any significantly better silencing properties when compared to unmodified ONs A2 and W1316 (marked as "W" in the figure), which were used as a reference. Nevertheless, under the tested conditions,

A2F	5'P	GCUGCUGCAGCUGCUGCUGCU	5'P - 5'-Phosphorylation
WF	5'P	CAUGCUGCUGCUGUUGUUGCUG	N - 2'-Fluoro
PF	5'P	GACUGCUGCAAUGCUGCUGCU	N - 2'-O-Methyl
A2M	5'P	G*<u>C</u>*UG*CU*GC*AG*CU*GC*U*G*C*U*G*C*U	* - Phosphorothioate

Figure 1. Nucleotide sequences and chemical modifications of the tested ONs.

Nucleotides that are base substitutions resulting in mismatch formation with the target sequence are marked in bold. An additional base substitution at position 2 is underlined.

A2F and WF caused the huntingtin levels to decrease to ~50% of the control level (Fig. 2A).

The same set of ONs was tested in SCA3 fibroblasts (Fig. 2B). A2F showed very similar efficiency and selectivity in mutant *ATXN3* silencing when compared to unmodified A2, e.g., the mutant ataxin-3 level was decreased to ~25% of the control level, but silencing of the normal allele was also considerable. WF's silencing properties were better than the effect observed in this model for unmodified W1316: a significant allele selectivity was observed as a consequence of the mutant protein decreasing to ~40% of the control level, and no changes in normal ataxin-3 levels were observed (Fig. 2B, left panel). A2F and A2M activity was also analyzed in more detail after transfection at 1, 5, 20 or 50 nM (Fig. 2B, right panel). Both ONs caused a significant downregulation of mutant ataxin-3, already at 5 nM. A2F showed better allele-selective properties, but A2M also caused alterations in splicing of *ATXN3* transcript, and as a result a lower molecular weight protein product, lacking the exon containing polyQ tract, is produced in SCA3 cells (Liu *et al.*, 2013).

The activity of A2F and A2M was also analyzed in a DRPLA fibroblast model (Fig. 2C). Both ONs silenced the expression of *ATN1* efficiently, causing atrophin-1 downregulation to ~25% of the control level after transfection at 50 nM concentration. A2F showed better allele-selective properties, as 60% of the normal protein level remained in the cells (and less than 50% using A2M) (Fig. 2C).

Evaluation of desired properties of chemically modified siRNAs

Taking A2 and A2F ONs as an example, we wanted to verify how chemical modifications, mainly 2'F, influence ON stability in fibroblast cell culture. We transfected fibroblast cell line SCA3 with 50 nM A2 or A2F, and isolated total RNA and protein at specific time points. We then performed northern blotting with an A2-specific probe to detect transfected ONs (Fig. 3A). A2 and A2F displayed a similar pattern of detection with a gradual decrease in their levels at the analyzed time points. It is worth emphasizing that northern blots show the total pool of ONs present in cells, which does not directly

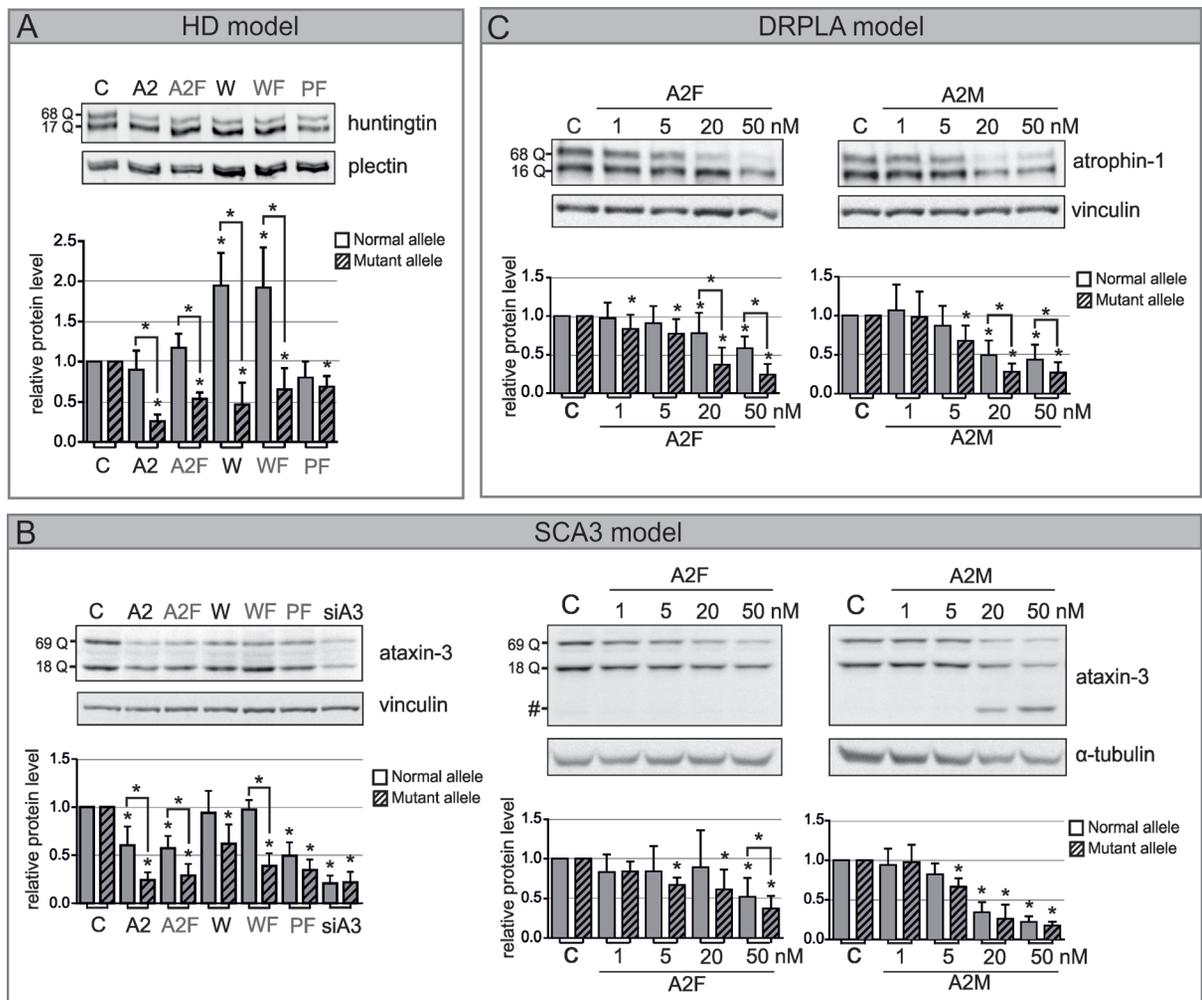


Figure 2. Efficiency and selectivity of selected ONs assessed in human fibroblasts by western blot analysis of polyQ protein levels. (A) Western blot analysis of huntingtin levels in HD fibroblasts (GM04281, 17/68 Q), at 72 h after transfection with 200 nM of the indicated ON. (B) Western blot analysis of ataxin-3 levels in SCA3 fibroblasts (GM06153, 18/69 Q), at 72 h after transfection with 100 nM siRNA (left panel), and after transfection with 1, 5, 20 or 50 nM ON A2F or A2M (right panels) “#” – additional ataxin-3 protein product. (C) Western blot analysis of atrophin-1 levels in DRPLA fibroblasts (GM13716, 16/68 Q), at 48 h after transfection with 1, 5, 20 or 50 nM ON A2F or A2M; “C” – control line, total protein from fibroblasts transfected with BlockIT siRNA. For semi-quantitation, signal intensities were normalized to reference protein levels: plectin, α -tubulin or vinculin. The error bars represent standard deviations. The p-value is indicated with an asterisk ($*p < 0.05$).

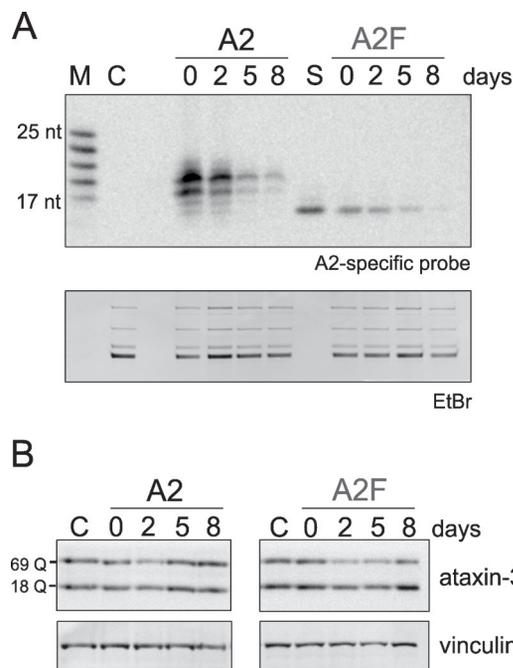


Figure 3. Time span of ON stability and silencing effects.

(A) Northern blot analysis of cellular levels of A2 and A2F. ONs were detected in the total RNA fraction from SCA3 fibroblasts (GM06153) lysed at different time points: 0, 2, 5 and 8 days post-transfection, with 50 nM ONs. Ethidium bromide (EtBr) staining was used as a loading control. "0" – 3 h post-transfection, "5" – synthetic A2F loading, "C" – control line, total RNA from fibroblasts transfected with BlockIT siRNA. (B) Western blot analysis of ataxin-3 levels after the same transfections as in (A). Representative blots are shown. "C" – control line, total protein from fibroblasts transfected with BlockIT siRNA.

correspond to the active pool, functioning within RISC. After 8 days, the signals were very weak, and these results are in agreement with the western blot analysis, where the most significant downregulation of ataxin-3 was observed on the second day and was later less prominent (Fig. 3B). In both cases, the cells are growing during the analyzed time post-transfection, so the effect of ON concentration in a single cell is somewhat diluted. Because neurons are not dividing cells, in this cell type, the stability of an ON and the duration of its silencing effects are only dependent on its resistance to degradation.

Silencing of different mutant HTT gene with selected chemically modified CAG repeat-targeting siRNAs in *StHdh* striatal cells

Cultured neuronal cells are the preferred model for testing ONs because they better represent brain cells, which should be the main cell target in therapeutic treatment for polyQ diseases. The relevant neuronal cells may be derived from brain tissue of polyQ rodent models or differentiated from induced human pluripotent stem cells (iPSCs). We have used mouse striatal cell lines (Trettel *et al.*, 2000) as a model that is more relevant to HD. First, we optimized transfection in these cells to efficiently deliver ONs (Supplementary Fig. 1 at www.actabp.pl). To evaluate mutant *Htt* silencing, we used two cell lines, heterozygous *STHdh* 7/111Q and homozygous *STHdh* 111/111Q, and selected ONs, A2, A2F and WF, were transfected at 100 nM concentration (Fig. 4). After 72 h, the cells were lysed, and the huntingtin levels were assessed using western blot. All of the tested ONs silenced the expression of the *Htt* mutant allele encoding 111 Qs

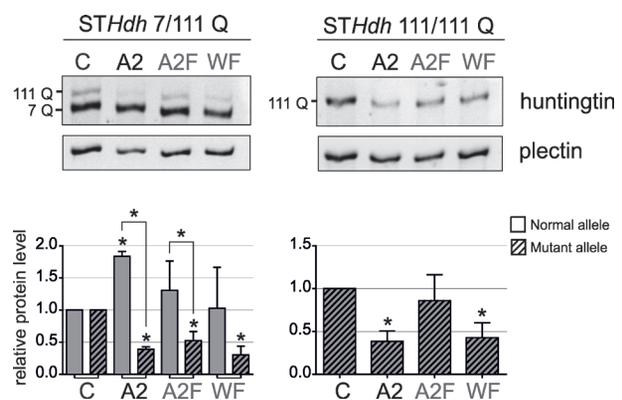


Figure 4. Western blot analysis of huntingtin levels in mouse striatal cells derived from an HD model (*STHdh* 7/111Q and *STHdh* 111/111Q) at 72 h after transfection with 100 nM ONs: A2, A2F and WF.

"C" – control line, total protein from fibroblasts transfected with BlockIT siRNA. For semi-quantitation, signal intensities were normalized to plectin protein levels. The error bars represent standard deviations. The *p*-value is indicated with an asterisk (**p*<0.05).

in the heterozygous cell line, with protein downregulation reaching 30% of the control level. The expression of the normal allele encoding 7 Qs remained at the control level or was upregulated by A2. In the homozygous cell line containing two mutant *Htt* alleles encoding 111 Qs, A2 and WF showed an efficient downregulation of huntingtin, reaching 40–45% of the control level (Fig. 4).

DISCUSSION

Currently, two strategies are being tested to ensure a long-lasting activity of ONs: (I) the incorporation of chemical modifications to increase ON 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 preclinical testing. The drawbacks of genetic vectors are as follows: applicability to RNAi tools only, problematic dosage control, and possible mutagenicity and immunogenicity of the viral vectors. Synthetic ONs offer an exciting alternative because both, the sequence and the chemical modification pattern of the ON can be optimized to achieve high *in vivo* efficiency together with low toxicity. Synthetic ONs may require repetitive administration, but their transient activity may be considered an advantage with respect to safety owing to direct dosage control. In addition, synthetic ONs have been found to be more equally and broadly dispersed in the brain because free uptake by neuronal cells is observed for ONs delivered *via* an intra-CNS route. In several cases of ONs testing for polyQ diseases, synthetic siRNAs or AONs were either locally injected or infused into the CNS, which caused an efficient downregulation of the targeted gene (Wang *et al.*, 2005; DiFiglia *et al.*, 2007; Stiles *et al.*, 2011; Kordasiewicz *et al.*, 2012; Yu *et al.*, 2012; Ostergaard *et al.*, 2013).

An important aspect of ON design is the selection of a chemical modification pattern that is suitable for its *in vivo* activity. Chemical modification not only increases the biological stability of the ON and the efficiency of its hybridization with the target sequence, but may also be critical for its effective delivery and low toxicity. The PS, 2'F and 2'OMe modifications are well tolerated by RISC. Additionally, the use of one strand of siRNA may

be considered more straightforward and safer due to the lack of potential unwanted activity of a sense strand.

We designed RNAi-based ONs that contained specific base substitutions and chemical modification patterns. By these means, we wanted to achieve an effective and specific silencing of selected mutant genes. Generally, the activity of the tested oligonucleotides was not significantly changed when compared to the pure RNA versions. Nevertheless, the use of chemically modified versions is expected to be more beneficial in *in vivo* experiments conducted in mouse models. We also tested a modified version of sd-siRNA A6, which originally did not induce silencing of *HTT* expression (Fiszler *et al.*, 2013). Its modified version, A6M (analogous chemical modification pattern to A2M), was also inactive (data not shown), which implicates the dominant role of base-substitution patterns for activity of this type of ONs.

At this point, it is difficult to say which ON-based therapeutic strategy is best suited for polyQ diseases. The existing strategies offer great opportunities for therapy but also have limitations. The allele-selective strategies are considered to be safer. The CAG repeat-targeting strategy, which was considered some years ago to be 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 (Yu *et al.*, 2012). The potential advantage of this strategy is its universal scope as one drug could possibly be used to treat most of the polyQ disorders.

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