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 (DR-PLA). 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 (Fiszer & 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 (Fiszer & 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 (Fiszer & 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 (Haring 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, 2'0'-methoxyethyl – 2′OMOE, 2′F). The efficiency of siRNAs has significantly increased as a result of their appropriate chemical modification (Amarzguioui 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; Fiszer 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; Alba et al., 2013; Liu et al., 2013) and by us (Fiszer et al., 2011; Fiszer et al., 2012; Fiszer 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-
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, DRPLA: 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 (STH2B) (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 (Nisshin Pharmaceuticals, Tokyo, Japan). 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., 2012; Haringsma et al., 2011). 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 Isis 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.

**RESULTS**

**Design of chemically modified CAG repeat-targeting ss-siRNAs**

We designed several CAG repeat-targeting ONs based on (I) the nucleotide sequences of mRNA-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 Isis 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,

![Figure 1. Nucleotide sequences and chemical modifications of the tested ONs.](image)

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). 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...
对应到活性池，功能在RISC之内。8天后，信号非常弱，这些结果与 western blot分析结果一致，其中最显著的降解发生在atxin-3中，在第二天被观察到，之后稍微降低（Fig. 3B）。在两种情况下，细胞在时间点后增长，因此对ON浓度的影响在单个细胞中是轻微的。因为神经元不包括细胞，在这种细胞类型中，ON的稳定性以及其降解效果只依赖于其对降解的抵抗力。

不同突变HTT基因与化学修饰CAG重复靶向siRNAs在StHdh小脑细胞中

培养的神经细胞是用于测试ONs的首选模型，因为它们更好代表大脑细胞，这应该是主要目标细胞在治疗性治疗中对polyQ疾病。相关神经元细胞可以由大脑组织中polyQ模型或从诱导的人类诱导多能干细胞（iPSCs）中获得。我们使用小鼠小脑细胞系（Trettel et al., 2000）作为更相关于HD的模型。首先，我们优化了转染，通过Western blot分析，发现有效降解的ONs（Supplementary Fig. 1 at www.actabp.pl）。为了评估mutant Htt沉默，我们使用了两种细胞系，杂合子StHdh细胞（7/111Q）和等位子StHdh细胞（111/111Q），并选择了ONs A2、A2F和WF，在转染后100 nM浓度（Fig. 4）。72 h后，细胞被裂解， huntingtin水平被测。所有检测的ONs沉默了表达的Htt mutant allele编码111 Qs

![Figure 3](image)

**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.

DISCUSSION

目前，两种策略正在被测试，以确保ONs具有中长期活动：(I) 将化学修饰整合到ONs中，以提高其稳定性；以及 (II) 将沉默试剂通过基因载体的递送。这些策略区别在于它们的递送工具和在障碍中需要克服的问题。目前的研究主要集中在活跃期内，可能在疗效方面考虑一个优势，因为ONs的化学修饰模式可以优化以达到高活性。合成ON们提供一种令人兴奋的替代品，因为它们的活性和化学修饰模式的ONs可能被优化以达到高活性。合成ONs在在体内/在体效率方面具有低毒性。合成ONs可能需要反复给药，但其性能可能被视为一个优势，因为ONs被神经元细胞递送是被观察到的。ONs递送经 CNS后。在某些情况下，ONs在polyQ疾病中，合成siRNAs或AONs在等位子StHdh 111/111Q中被选择，ONs A2、A2F和WF，在转染后100 nM浓度（Fig. 4）。72 h后，细胞被裂解， huntingtin水平被测。所有检测的ONs沉默了表达的Htt mutant allele编码111 Qs

![Figure 4](image)

**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-quantification, 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).

在杂合子细胞系中，与polyQ疾病的降解相比，huntingtin的降解会减少30%。正常7 Qs在控制水平中保持不变。在杂合子细胞中，huntingtin的降解为30%。应考虑ONs的化学修饰模式，因为ONs的化学修饰模式可以优化以达到高活性。合成ONs在在体内/在体效率方面具有低毒性。合成ONs可能需要反复给药，但其性能可能被视为一个优势，因为ONs被神经元细胞递送是被观察到的。ONs递送经 CNS后。在某些情况下，ONs在polyQ疾病中，合成siRNAs或AONs在等位子StHdh 111/111Q中被选择，ONs A2、A2F和WF，在转染后100 nM浓度（Fig. 4）。72 h后，细胞被裂解， huntingtin水平被测。所有检测的ONs沉默了表达的Htt mutant allele编码111 Qs

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 degrade 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

![Diagram](image)
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 (Fiszter 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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