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Limits of Using Oligonucleotides for Allele-Selective Inhibition at Trinucleotide Repeat Sequences –Targeting the CAG Repeat within Ataxin-1

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Abstract

Trinucleotide repeats are responsible for many genetic diseases. Previous studies have shown that duplex RNAs (dsRNAs) can be used to target expression of a mutant repeat allele while leaving expression of the wild-type allele untouched, creating opportunities for allele-selective inhibition and better therapeutic outcomes. In contrast to successes with other genes, we report here that we cannot achieve allele-selective inhibition when targeting the expanded CAG repeat within Ataxin-1 (ATXN1), the cause of spinal cerebellar ataxia-1 (SCA1). The most likely explanation for this unfavorable outcome is that the mean CAG repeat number within wild-type ATXN1 is relatively high compared to other trinucleotide repeat diseases. Because the wild-type repeat number is high, it is likely that there is poor discrimination between the mutant and wild-type repeat and less opportunity for allele-selective inhibition across the entire spectrum of mutations found in SCA1 patients. Our data support the conclusion that the potential for multiple binding cooperative binding interactions is a critical factor governing allele-selective recognition of trinucleotide repeat genes by duplex RNAs. These results should be helpful in predicting which diseases and which patients are most likely to benefit from allele-selective targeting of expanded repeats.

Keywords

Duplex RNA; RNAi; Trinucleotide repeat; Allele-selective inhibition; spinal cerebellar ataxia-1 (SCA1); Ataxin 1 (ATXN1)

Over forty diseases are caused by mutations consisting of expansions of simple repetitive nucleotide sequences^[1]. Repeats vary in their sequence (CAG, GUC, AAG, GGGGCC etc.), length (from dozens to over a thousand), whether they are within exons or noncoding regions, and whether they act primarily in the nucleus or in the cytoplasm. Agents that successfully block gene expression would lower production of disease-causing mutant proteins and is one approach to therapy.

Many of these mutant expansions are dominant, with patients having one mutant and one wild-type copy of the affected genes. In many cases expression of the wild-type protein will

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be necessary for normal cellular function. It may, therefore, be necessary to develop allele-selective agents that block expression of the mutant gene while retaining expression of the wild-type gene^[2].

We have previously used duplex RNAs (dsRNAs) to target expanded CAG repeats and achieve allele-selective inhibition of huntingtin (Huntington's disease)^[3], ataxin-3 (ATXN3) (Machado Joseph Disease)^[4], and atrophin-1 (ATN1) (Dentatorubral-pallidoluysian atrophy)^[5]. The relative allele-selective inhibition was between ten and thirty-fold for mutant versus wild-type protein expression depending on the compound used and the target gene^[5]. Inhibition was robust and was achieved by many different designs of dsRNAs.

These successes led us to test duplex RNAs as a strategy for inhibiting another gene responsible for CAG trinucleotide expansion disease – ataxin-1 (ATXN1). ATXN1 is responsible for spinocerebellar ataxia type 1 (SCA1) and has a CAG repeat tract within a coding exon^[6,7]. SCA1 is a neurodegenerative disease with progressive worsening of symptoms with death ten to thirty years after diagnosis. Homozygous knock-out mice that do not express ATXN1 gave significant defects, suggesting that allele-selective inhibition may be important^[8].

We first established an assay for monitoring protein expression for both alleles. For this assay, we obtained GM06927 SCA1 patient-derived fibroblast cells. These cells have 29 CAG repeats within the wild-type allele and 52 repeats in the mutant allele. Unaffected individuals have 19-36 repeats, while patients have 43-81 repeats^[9].

Establishing an assay for monitoring the expression of the mutant and wild-type alleles of a trinucleotide repeat gene is challenging. The difference in repeat length leads to proteins that differ by a relatively few amino acids. In this case, mutant ATXN1 is 23 repeats longer than its wild-type counterpart. Successful assays require optimized electrophoresis conditions capable of separating the variant proteins and an antibody capable of detecting both proteins by western analysis. Our first goal was to develop an assay for identifying both mutant and wild-type ATXN-3 proteins by western blot.

We obtained a polyclonal antibody from Bethyl labs and compared detection of ATXN1 in GM06927 cells to detection in several cultured cell lines that are homozygous for wild-type ATXN1. The uncropped gels revealed multiple bands (Figure 1A). ATXN1 is approximated 90 kDa and two bands in GM06927 protein extract at near the 100 kDa marker appear likely corresponding to the wild-type and mutant ATXN1 proteins. The top, presumably mutant, band does not appear in extracts from any of the other cell lines. We also used an antibody against the poly-glutamine tract, which should only detect proteins with expanded poly-glutamine repeats (Figure 1B). This antibody also detected the higher molecular weight band corresponding to mutant ATXN1 protein in protein extracted from GM06927 cells but not the six other cell lines that were homozygous for wild-type ATXN1.

After establishing an assay, we selected duplex RNAs for testing. Duplex RNA (dsRNA) siREP was fully complementary to the CAG repeat within exon 8 (Figure 2AB). Previous work targeting HTT, ATN1, and ATXN3 had shown that siREP was not allele-selective^[2-5]. When dsRNAs are fully complementary to cellular RNAs they induce cleavage of their

targets by argonaute 2 (AGO2). Our previous experience suggests that fully complementary RNAs that function through AGO2 cleavage cannot discriminate between mRNAs with differing repeat lengths. Cleavage by fully complementary RNA/AGO2 complexes is a powerful mechanism for gene silencing and the potential for cleaving the expanded repeat likely overwhelms any subtle differences in length or structure between the wild-type and mutant CAG repeat sequences.

We also tested dsRNAs with central mismatches relative to the repeat target (Figure 2AB). When mismatches are introduced into the central region of a duplex RNA, cleavage by AGO2 is prevented but recognition of a complementary RNA target by the RNAi guide strand can still occur^[10]. Such mismatched RNAs have been shown to achieve potent and allele-selective inhibition of protein expression^[3-5,11]. The fact that the mismatched RNAs can bind, but not cleave targets, leads to more selectivity when mutant repeats are relatively long and wild-type repeats relatively short.

RNA P9 has one mismatch at position 9 and PM4 has four central mismatches. ss775 is a single-stranded silencing RNA (ss-siRNA) that is complementary to the CAG repeat region except for a single mismatch relative to the CAG repeat target at position 9. ss-siRNAs are single-stranded RNAs that have been modified to be stable inside cells and function through the RNAi pathway^[12,13]. CM is a noncomplementary negative control RNA. siATX1 is complementary to a region of the mRNA outside the CAG repeat and serves as a positive control for confirming successful transfections. In previous studies, P9, PM4, and ss775 have all demonstrated strong allele selectivity for inhibiting the expression of other trinucleotide repeat genes.^[3,4,5,12]

Initial testing showed that, four days after transfection, both siREP and siATXN1 inhibit expression of both the mutant and the wild-type alleles of ATXN1 (Figure 2C). This transfection protocol was used in subsequent experiments and cells were harvested for testing after four days. These experiments showing the loss of predicted ATXN1 bands using silencing dsRNAs confirmed the identification of ATXN1 protein.

We then tested P9 and PM4 at varying concentrations from 0 to 100 nM. Both compounds inhibited expression of ATXN1 but reduced levels of both the wild-type and mutant alleles equally. P9 (one mismatch) was more potent than PM4 (four mismatches, consistent with higher complementarity to the target CAG repeat. ss775 also inhibited both mutant and wild-type ATXN1 equally.

These data stand in stark contrast to robust allele-selective inhibition of mutant HTT, ATN1, or ATXN3 by these same dsRNAs or ss-siRNAs^[3-5,13]. One hypothesis to explain why allele-selective inhibition can be achieved for HTT, ATN1, or ATXN3 repeat genes is that the expanded repeat is much longer than the wild-type repeat. Longer repeats can accommodate the binding of more anti-CAG RNAs, increasing the strength of the protein roadblock needed to decrease translation.

For example, for Huntington's Disease mutant cell line GM04281 the wild type allele has 17 repeats containing 51 bases that have the capacity to bind no more than one or two anti-CAG

Materials and Methods

siRNAs

Duplex RNAs were purchased from IDT (Newark, NJ). Duplex RNAs were annealed in 2.5x Dulbecco's phosphate-buffered saline (Sigma Aldrich). Single-stranded ss-siRNA was provided by Ionis Pharmaceuticals (Carlsbad, CA) and reconstituted in nuclease-free water.

Cell culture and transfection

Patient-derived fibroblast cell lines SCA1 (GM06927), SCA7 (GM03561), DRPLA (GM13717), HTT (GM04281) were obtained from the Coriell Institute (Camden, NJ). Other cell lines were from ATCC (Gaithersburg, MD). The fibroblast cells were maintained at 37°C and 5% CO₂ in Minimal Essential Media Eagle (MEM) (Sigma, M4655) supplemented with 10% heat inactivated fetal bovine serum (Sigma) and 0.5% MEM nonessential amino acids (Sigma). Cells were plated at a density of 80,000 per well of a 6-well plate 48 h before transfection. Duplex RNAs or ss-siRNAs were transfected into cells with lipid RNAiMAX (Invitrogen) as previously described^[3]. Cells were typically harvested 4 days after transfection for analysis of protein levels.

Western blot analysis

Standard SDS-PAGE protocol was used for analysis of ataxin 1 protein expression. Twenty to thirty milligrams of total protein were denatured, run on 7.5% Tris-HCl precast gel (Bio-Rad) and blotted on nitrocellulose membrane (Amersham Protran 0.45 µM, GE Healthcare). Membranes were blocked for 1 hour by 5% milk in PBST, and then incubated with primary antibodies overnight at 4 °C. After three times wash with PBST, the membrane was incubated with anti-mouse or anti-rabbit HRP conjugate antibody (Jackson ImmunoResearch Laboratories) for 30 minutes at room temperature. The membrane was soaked in SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and then expose to film. Primary antibodies were used as indicated: anti-ataxin 1 (A302-292A, 1:2000; Bethyl labs), anti-PolyQ (MAB1574, 1:10,000, Millipore), and anti-β-actin (1:10,000; Sigma).

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Figure 1.

Detection of wild-type and mutant ATXN1 protein in GM06927 patient-derived cells by western blot. Protein extracts were separated by 7.5% SDS-PAGE gel and probed with a) anti-ATXN1 antibody, or b) anti-poly glutamine PolyQ antibody that detects the expanded repeat sequence. Mut = mutant ATXN1 protein; WT = wild-type protein. Mutant and wild-type protein in GM06927 cells are shown boxed.

Figure 2.

Repeat-targeting siRNA/REP reduces both wild-type and mutant ATXN1 expression in SCA1 patient derived GM06927 fibroblast cells. **A)** Scheme of *ATXN1* transcript coding region showing the CAG repeat within exon 8. **B)** List of siRNAs tested in the study. **C)** Inhibition of ATXN1 expression by siRNA siATXN1 and siRNA/REP. CM is a noncomplementary dsRNA control.

Figure 3. Effects of dsRNAs **A**) P9 and **B**) PM4 or **C**) ss-siRNA ss775 on ATXN1 expression in SCA1 patient-derived GM06927 fibroblast cells. CM is a noncomplementary dsRNA control.

Figure 4.

Mutant HTT mRNA has sufficient CAG repeats to allow the binding of several anti-CAG duplex RNAs or ASOs. A) Wild type HTT mRNA has a much shorter CAG tract that allows binding of only one or two compounds. The mean CAG repeat (29 CAG repeats) within mutant ATXN1 mRNA can bind three or four anti-CAG duplex RNAs or ASOs. The HTT alleles are based on the repeats found in benchmark GM04281 patient-derived human fibroblast cells (17 wild-type, 74 mutant). B) The complex of AGO2 and TNRC6 can form cooperative interactions that increases binding of RNAs that have two or more adjacent binding sites relative to RNAs that have just one binding site.