Effect of 2'-O-methyl/thiophosphonoacetate-modified antisense oligonucleotides on huntingtin expression in patient-derived cells

Masayuki Matsui¹, Richard N Threlfall², Marvin H Caruthers², and David R Corey^{1,*}

¹Department of Pharmacology; UT Southwestern Medical Center; Dallas, TX USA; ²Department of Chemistry and Biochemistry; University of Colorado at Boulder; Boulder, CO USA

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Optimizing oligonucleotides as therapeutics will require exploring how chemistry can be used to enhance their effects inside cells. To achieve this goal it will be necessary to fully explore chemical space around the native DNA/RNA framework to define the potential of diverse chemical modifications. In this report we examine the potential of thiophosphonoacetate (thioPACE)-modified 2'-O-methyl oligoribonucleotides as inhibitors of human huntingtin (*HTT*) expression. Inhibition occurred, but was less than with analogous locked nucleic acid (LNA)-substituted oligomers lacking the thioPACE modification. These data suggest that thioPACE oligonucleotides have the potential to control gene expression inside cells. However, advantages relative to other modifications were not demonstrated. Additional modifications are likely to be necessary to fully explore any potential advantages of thioPACE substitutions.

Introduction

The development of nucleic acid therapeutics is challenging because of the need to optimize efficacy, potency, pharmacokinetics, and cellular delivery while minimizing potentially toxic off-target effects.¹ The difficulty of overcoming these challenges explains why only three oligonucleotide-based drugs have been approved after approximately 25 years of commercial development. Progress will require fully exploring the potential of chemical modifications to improve their properties.

Thiophosphonoacetate (thioPACE)-modified oligonucleotides contain an acetate group in place of a non-bridging oxygen within the phosphate linkage, as well as a phosphorothioate substitution.^{2–4} These modifications are a substantial departure from normal phosphodiester linkages and have the potential to significantly alter hybridization and cell uptake. Previous work has shown that thioPACE oligonucleotides can activate RNase H1 *in vitro*,^{2,3} are stable to nuclease digestion,^{2,3} are more easily taken up into cells,⁴ and improve the inhibition of microRNA-122 in Huh7 cells compared with phosphonoacetate (PACE) or 2'-OMe phosphorothioate oligonucleotides.⁴ Recently, thioPACE modified CRISPR RNA has been shown to stabilize the CRISPR-Cas system when transfected into primary cells.⁵

Huntington's disease (HD)⁶ is a currently incurable genetic disease caused by an expansion of the trinucleotide CAG within

the huntingtin (*HTT*) gene. Other diseases caused by CAG expansions include dentatorubral-pallidoluysian atrophy (DRPLA,⁷ within the atrophin-1 gene) and Machado-Joseph disease⁸ (within the gene encoding ataxin-3). Because expanded CAG repeats are the cause of these diseases, an agent complementary to CAG repeats would have the potential to treat multiple neurological conditions.

We have previously shown that synthetic nucleic acids complementary to CAG repeats can inhibit the expression of mutant proteins including Htt,^{9–13,15} ataxin-3,^{9,14–16} and atrophin-1.¹⁷ Inhibition is allele selective, with anti-CAG oligomers demonstrating up to 40-fold selectivity for blocking expression of mutant protein versus wild-type.¹¹ Oligomers that were capable of allele-selective inhibition included peptide nucleic acids,⁹ locked nucleic acids,^{9,10} carba-locked nucleic acids,¹⁰ 2',4'-constrained ethylene-bridged nucleic acids,¹⁰ single-stranded silencing RNA,^{12,13,15–17} mismatch-containing duplex RNA,^{11,17} abasic-substituted duplex RNA,¹⁴ and unlocked nucleic acids.¹⁶

These studies have shown that expanded CAG repeats are an excellent model for evaluating the potential of chemically modified nucleic acids for the recognition of challenging cellular RNA targets. In this study we test the potential of thioPACE oligonucleotides for recognition of the expanded repeat within mutant *HTT* mRNA.

*Correspondence to: David R Corey; Email: david.corey@utsouthwestern.edu

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Figure 2. Inhibition of HTT expression by chemically modified single-stranded antisense oligonucleotides targeting CAG repeats of *HTT* mRNA. Top: western blot analysis; bottom: quantification of triplicate independent experiments. Oligonucleotides were transfected into HD patient-derived fibroblast cells (GM04281, **A**) or striatal neuron-derived cells (STHdhQ7/Q111, **B**) at 100 nM by using Lipofectamine RNAiMAX. Four days after transfection, cells were harvested for protein gel blot. NT: No treatment. Error bars are mean $\pm$ SD (n=3). *, $P < 0.05$; **, $P < 0.01$ (paired t-test).

Discussion

Our experiments provide significant insights into the potential of thioPACE oligonucleotides to recognize a challenging RNA target. We find that thioPACE oligonucleotides can enter cells when delivered with cationic lipid and block expression of HTT. Potent and allele-selective inhibition was achieved in striatal-derived cell line *STHdhQ7/Q111*, possibly aided by the large number of CAG repeats within the mutant allele. Allele-selective inhibition was much less apparent in GM04281 cells.

While our experiments demonstrate that thioPACE oligonucleotides can achieve inhibition of HTT protein expression, it is also clear that further refinement is necessary to explore the full potential of the modification. Although oligonucleotide 1, which contained four 2'-O-methyl/thioPACE modifications, retained a similar T_m value relative to the positive control LNA(T)-PS, inhibition of mutant HTT by oligonucleotide 1 was not as strong and allele selectivity was reduced, suggesting that chemical structure is a key factor in determining potency and selectivity for HTT inhibition.

It also appears that overall thermodynamic stability of repeat-targeting antisense oligonucleotides to complementary targets is important. Indeed, inhibition and selectivity was better with LNA(T)-PS and oligonucleotide 1, which had lower T_m values, in *STHdhQ7/Q111* cells compared with oligonucleotides 2 and 3, which had higher T_m values. These results suggest that a higher T_m of oligonucleotides may contribute to the lack of allele-selective inhibition in *STHdhQ7/Q111* and GM04281 cells by overpowering the subtle differences in recognition between the mutant and wild-type alleles.

The compounds tested in these studies contained only four thioPACE substitutions. It will be interesting in the future to examine compounds with more extensive thioPACE modification in different modification patterns. Additional thioPACE substitutions might affect cellular uptake, lysosomal release, or cytoplasmic localization. The possession of better developed structure activity relationships through testing additional compounds might also lead to identification of optimized compounds with superior properties.

Materials and Methods

Measurement of melting temperature by differential scanning calorimetry

Melting temperatures (T_m) for antisense oligonucleotide:RNA (CAGCAGCAGCAGCAGCAGC) duplexes were measured on a MicroCal VP-DSC capillary cell microcalorimeter (Malvern Instruments). Duplexes (10 μ M) in phosphate buffer (10 mM Na₂HPO₄/NaH₂PO₄, 150 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA); pH 7.2) were annealed by heating to 95°C and then gradually cooling to room temperature. Samples were scanned under pressure from 40–130°C at a scan rate of 90°C/h with two rescans. The temperature corresponding to the maximum peak in heat capacity (C_p) was taken as the T_m .

Cell culture and transfection

HD patient-derived fibroblast cells GM04281 (Coriell) were cultured at 37°C and 5% CO₂ in Minimum Essential Medium Eagle (Sigma) supplemented with 0.5% MEM nonessential

Figure 3. Dose-response profiles of CAG-repeat-targeting single-stranded antisense oligonucleotides (LNA(T)-PS (B), Oligo1 (C), Oligo2 (D), Oligo3 (E), and negative control (–Ctrl (A)) for HTT inhibition. Each oligomer was transfected into GM04281 fibroblast cells at 0, 6.25, 12.5, 25, 50, or 100 nM by using Lipofectamine RNAiMAX. Four days after transfection, cells were harvested for western blot. Error bars are mean±SD (n=3–5). *, $P < 0.05$; **, $P < 0.01$ (unpaired t-test).

amino acids (Sigma) and 10% fetal bovine serum (FBS). Mouse striatal neuron-derived cells *STHdhQ7/Q111* (Coriell) were cultured at 37°C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% FBS. Cells were seeded in six-well plates at 60,000 cells/well two days before transfection. Cells were transfected with oligonucleotides by using Lipofectamine RNAiMAX (Invitrogen). Cells were harvested for protein gel blot analysis four days after transfection.

Western blot analysis

Cells were lysed using lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM dithiothreitol (DTT), and protease inhibitor (Calbiochem). Protein concentrations were determined by using a BCA assay kit (Thermo Scientific). SDS-PAGE was performed using 5% Tris-acetate gel (2.8% crosslinker) for Htt or 7.5% Tris-HCl gel (3.8% crosslinker, Bio-Rad) for β-actin. Gels were run at 100 V for 5 h (Htt) or 1 h (β-actin). After gel electrophoresis, proteins were transferred to a nitrocellulose membrane (Hybond C-Extra, GE Healthcare). Membranes were blocked using 5% non-fat dry milk/PBST and then incubated with primary antibodies specific for Htt (MAB2166, 1:10,000, EMD Millipore) or β-actin

(A5441, 1:20,000, Sigma). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (715–035–150, Jackson ImmunoResearch) was used for visualizing proteins by using Super-signal West Pico Chemiluminescent Substrate (Thermo Scientific). Protein bands were quantified by using Image J software. Data plots from dose response experiments were fitted to the following equation: $y = 100(1-x^m/(n^m+x^m))$, where y is percent expression of HTT and x is concentration of synthetic oligomers. m and n are fitting parameters, where n is taken as the IC₅₀ value ($\pm$ SE from the fit).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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