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Allele-Selective Inhibition of Expression of Huntingtin and Ataxin-3 by RNA Duplexes Containing Unlocked Nucleic Acid Substitutions

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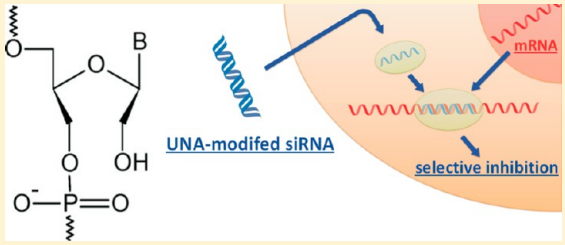
Supporting Information

ABSTRACT: Unlocked nucleic acid (UNA) is an acyclic analogue of RNA that can be introduced into RNA or DNA oligonucleotides. The increased flexibility conferred by the acyclic structure fundamentally affects the strength of base pairing, creating opportunities for improved applications and new insights into molecular recognition. Here we test how UNA substitutions affect allele-selective inhibition of expression of trinucleotide repeat genes Huntingtin (*HTT*) and Ataxin-3 (*ATX-3*). We find that the either the combination of mismatched bases and UNA substitutions or UNA substitutions alone can improve potency and selectivity. Inhibition is potent, and selectivities of >40-fold for inhibiting mutant versus wild-type expression can be achieved. Surprisingly, even though UNA preserves the potential for complete base pairing, the introduction of UNA substitutions at central positions within fully complementary duplexes leads to >19-fold selectivity. Like mismatched bases, the introduction of central UNA bases disrupts the potential for cleavage of substrate by argonaute 2 (AGO2) during gene silencing. UNA-substituted duplexes are as effective as other strategies for allele-selective silencing of trinucleotide repeat disease genes. Modulation of AGO2 activity by the introduction of UNA substitutions demonstrates that backbone flexibility is as important as base pairing for catalysis of fully complementary duplex substrates. UNA can be used to tailor RNA silencing for optimal properties and allele-selective action.



Synthetic nucleic acids are widely used for research, medical diagnosis, and drug development. Nucleic acids are a promising approach for clinical therapy,[1] and a systemically administered antisense oligonucleotide (ASO) has recently been approved by the Food and Drug Administration (FDA) as a therapy for familial hypercholesterolemia.[2] The clinical success of oligonucleotides after many years of slow progress has led to optimism that nucleic acids may become a major class of therapeutics.

Many different chemically modified nucleosides are available, and their wide range of properties encourages development.[3] Upon introduction into an oligonucleotide, each type of nucleoside has a unique potential to alter the oligonucleotide's properties. When carefully chosen, the introduced nucleosides can tailor the function of the oligonucleotide for the demands of a given application. This engineering improves the robustness of protocols that use nucleic acids and the likelihood that they will be widely adopted.

Locked nucleic acid (LNA) nucleosides[4,5] have a methylene bridge connecting the 2′-ribose with the 4′-carbon. This bridge increases rigidity and reduces the entropic penalty paid upon binding a complementary sequence. The reduced entropic cost of binding leads to a higher affinity, with LNA substitutions improving melting temperatures (T_m) by as much as 3−8 °C per substitution.[6] LNA has become one of the most successful modified nucleosides, with applications in the clinical development of ASOs,[7] as a research tool for inhibiting the action of miRNAs,[8] and as a molecular probe.[9] Similar bridged nucleic acids (BNAs) that contain alternate ribose constraints add to the potential for using increased rigidity to improve applications.[10,11]

In contrast to the increased rigidity of LNA nucleosides, unlocked nucleic acid (UNA) substitutions[12−14] permit researchers to explore decreased rigidity as a variable for improving the function of oligonucleotides. UNA nucleosides are acyclic and lack a connection between the C2′ and C3′ atoms (Figure 1). In contrast to that of LNA, the introduction of UNA nucleosides allows a tailored decrease in the affinity of

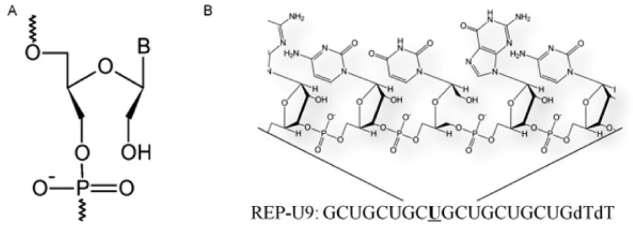


Figure 1. (A) UNA structure. (B) Representative chemical structure of UNA-modified RNA.

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oligonucleotide binding. For duplex RNAs, UNA substitutions can reduce the extent of off-target gene silencing, possibly by destabilizing seed sequence interactions at off-target genes,[15,16] and can improve function in combination with other types of chemical modifications.[17] Acyclic oligonucleotides with 2′,3′-secouridine substitutions have also been studied to investigate the interplay of backbone flexibility and enzyme activity.[18] When introduced into antisense oligonucleotides, the acyclic substitutions increased the rate of cleavage of the substrate by RNase H, suggesting that strategically placed modifications can affect catalysis.

In this report, we test the hypothesis that UNA substitutions can improve allele-selective gene silencing of mutant huntingtin (*HTT*) and ataxin-3 (*ATX-3*). Mutant *HTT* causes Huntington's disease (HD),[19,20] and mutant *ATX-3* causes Machado-Joseph disease (MJD).[21,22] Relative to the wild-type *HTT* or *ATX-3* genes, the mutated alleles contain longer CAG repeats within their mRNA coding regions. For *HTT*, the CAG repeats are close to the 5′ terminus. For *ATX-3* mRNA, the repeats are located at its 3′ end. HD patients have an average of 45 CAG repeats, while the number of wild-type *HTT* genes is almost always fewer than 26. For MJD, the mean repeat length can vary from 73 to 80 repeats in different populations, and in most unaffected individuals, the CAG tract has fewer than 31 repeats. HD and MJD are representative of a large family of neuromuscular diseases caused by CAG expansions, and anti-CAG therapeutic strategies have the potential to treat multiple pathologies.

Antisense oligonucleotide and duplex RNA inhibitors of HTT and ATX-3 expression have been intensively studied.[23−33] We and others have previously shown that the allele-selective inhibition of gene expression can be achieved by duplex RNAs or single-stranded silencing RNAs (ss-siRNAs) containing central mismatches.[34−42] These mismatches prevent argonaute 2 (AGO2) from cleaving the target mRNA and shift the mechanism of action toward one that resembles the mechanism of miRNAs. Allele selectivity can also be achieved by duplexes containing abasic substitutions that, like mismatches, remove the potential for normal base pairing.[41]

While mismatched and abasic duplexes provide a substantial pool of promising compounds for therapeutic discovery, meeting the challenges of the clinical development of inhibition candidates will benefit from the identification of a wider number of potent and allele-selective agents. Exploring the limits for applying chemical modification to gene silencing by duplex RNA also provides insights into substrate recognition by AGO2 during catalysis.

Here we find that duplexes that contain both central mismatches and UNA substitutions possess improved potencies and selectivities. Even though UNA substitutions preserve base paring with the target mRNA, UNA substitutions within fully complementary duplexes also yield allele-selective inhibition. These results expand the range of therapeutic leads for allele-selective inhibition of CAG repeat disease genes and introduce UNA as a strategy for tailoring the properties of allele-selective duplexes.

MATERIALS AND METHODS

RNA Synthesis. UNA-modified antisense RNAs and unmodified sense RNAs were synthesized and characterized using electrospray ionization mass spectrometry by Sigma Custom Products and reconstituted in nuclease-free water. Double-stranded RNAs were prepared by mixing the two RNA strands and annealing them in 2.5× PBS solutions. Stock solutions (20 μM) were prepared for transfection in cell cultures.

Thermal Denaturing by UV Melt Analysis. Thermal denaturation analysis of UNA-containing RNA duplexes was conducted using a CARY Varian model 3 UV−vis spectrophotometer (Agilent Technologies, Santa Clara, CA). In a 1 cm quartz cuvette, the absorbance was monitored at 260 nm. UNA-modified antisense RNAs (1 μM) were mixed with an equimolar sense RNA strand (5′-CAGCAGCAGCAGCAGCAGCdTdT-3′) in 0.1 M phosphate buffer (pH 7.4) and melted three times from 15 to 95 °C at a ramp rate of 1 °C/min. The melting temperature (T_m) was calculated using CARY WinUV Thermal Application software using a baseline fitting method.

Cell Culture and Transfection. Patient-derived fibroblast cell lines GM04281 (*HTT*; 69 CAG repeats) and GM06151 (*ATX-3*; 74 CAG repeats) were obtained from the Coriell Institute (Camden, NJ). The fibroblasts were maintained at 37 °C and 5% CO_2 in minimal essential eagle's medium (MEM) (Sigma, catalog no. 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 60000 (HTT) or 70000 (ATX-3) per well of a six-well plate 48 h before transfection. siRNAs were transfected into cells with lipid RNAiMAX (Life Technologies) as previously described.[33,34] Cells were typically harvested 3 days after transfection for quantitative polymerase chain reaction (qPCR) and RNA immunoprecipitation (RIP), or 4 days for protein assay.

Western Blot and qPCR Analysis. HTT and ATX-3 expression was analyzed by Western blot analysis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis was used to separate HTT isoforms as described previously,[34] and ATX-3 protein was separated with 4 to 20% acrylamide precast gels (Bio-Rad). The following primary antibodies were used: anti-HTT (MAB2166, Millipore), anti-ATX-3 (MAB5360, Millipore), and anti-β-actin (Sigma). Protein bands were quantified using ImageJ. The percentage of inhibition was calculated as a relative value to a control sample. The dose fitting curve was generated using GraphPad Prism 6 with the equation $y = 100[1 - x^m/(n^m + x^m)]$, where y is percentage of inhibition, x is the siRNA concentration, n is the IC_{50} value, and m is the Hill coefficient value.

Total RNA was extracted using TRIzol (Life Technologies), and 2 μg of RNA was subjected to DNase I (Worthington Biochemical Corp.) treatment. cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). After an appropriate dilution of the cDNA sample, qPCR was performed on a CFX96 real-time PCR system (Bio-Rad) using iTaq SYBR Green Supermix (Bio-Rad). Data were normalized relative to levels of GAPDH mRNA. The following qPCR primer sets were used for HTT: 5′-CGACAGCGAGTCAGTGAATG-3′ (forward) and 5′-ATCCTGAGCCTCTGATACTC-3′ (reverse). GAPDH primers were obtained from Applied Biosystems. The qPCR cycles are as follows: 50 °C for 2 min, 95 °C for 3 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

RNA Immunoprecipitation (RIP). HTT fibroblast cells were seeded at a density of 1400K cells in 150 cm^2 dishes. Duplex RNAs were transfected with RNAiMAX on the next day. Cells were harvested 72 h later and were lysed in a buffer {20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$, 0.5% NP-40, 0.5 mM DTT, protease inhibitor [EDTA-free (Roche)], and RNase inhibitor [final concentration of 50 units/mL (Promega)]} with

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A

siRNA	Sequence	Position of UNA modification	Tm[a] (°C)	mut IC_{50} (nM)	wt IC_{50} (nM)	Selectivity (fold)
P9	GCUGCUGC*A*GCUGCUGCUGdTdT	-	79.7	3.2±0.9	>100	>31
REP	GCUGCUGCUGCUGCUGCUGdTdT	-	85.9	5.0±3.0	13±5.0	3
REPU9	GCUGCUGC**U**GCUGCUGCUGdTdT	9	78.1	2.6±0.5	>50	>19
REPU10	GCUGCUGCU**G**CUGCUGCUGdTdT	10	78.4	1.8±0.3	34±10	19
REPU11	GCUGCUGCUG**C**UGCUGCUGdTdT	11	74.2	2.7±0.5	>50	>18
REPU91011	GCUGCUGC**UGC**UGCUGCUGdTdT	9, 10, 11	69.4	37±13	>50	>1.3

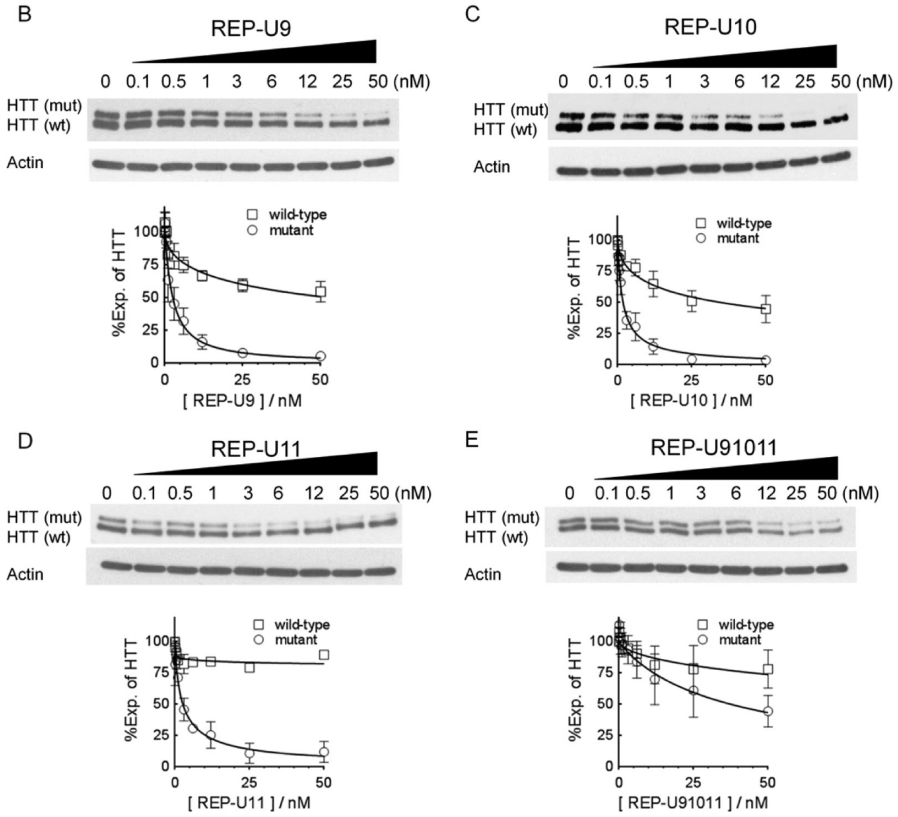

Figure 2. Effects of UNA substitutions on allele-selective inhibition of HTT by RNA duplexes that are fully complementary to the CAG repeat. Data are from Western analyses of HTT protein expression in GM04281 patient-derived fibroblast cells. (A) Sequences of duplex RNAs. REP is a duplex with two fully complementary strands that targets the CAG repeat and has no UNA substitutions. P9 is a duplex that has a mismatch at position 9 and no UNA substitution. For other RNAs, UNA nucleosides are shown in boldface and underlined. Gels and dose response curves showing effect of adding (B) REPU9, (C) REPU10, (D) REPU11, or (E) REPU91011 at increasing concentrations. Dose curves are averaged data from three independent experiments.

a volume ~3 times the cell pellet size. The mixture was placed on ice for 10 min after being thoroughly mixed. After centrifugation, the supernatant was isolated and stored at −80 °C. After 60 μL of Protein A/G agarose Plus had been washed twice with lysis buffer, beads were incubated with 4 μL of antibodies [anti-AGO2, 4G8, 011-22033 (Wako) or normal mouse IgG, 12-371 (Millipore)] and cell lysate in lysis buffer at 4 °C with gentle agitation for 3 h.[43] The beads were further washed three times with the lysis buffer described above. The beads were finally eluted with elution buffer (1% SDS, 20 mM $NaHCO_3$, and RNase inhibitor). After proteinase K treatment, RNA extraction, and precipitation, samples were treated with recombinant DNase I, followed by reverse transcription. The mRNA levels were quantified by qPCR. Results were normalized first by GAPDH levels and second by IgG levels.

***In Vitro* Cleavage Assay.** The RNA substrate-containing fragment of *HTT* exon 1 with 17 CAG repeats was prepared as previously reported.[44] This transcript was gel purified, dephosphorylated, and 5′-phosphorylated with [γ-^{32}P]ATP. Purified recombinant human Ago2 protein (a gift from Q. Liu) was preincubated with 5′-phosphorylated antisense RNA with or without UNA modification at room temperature for 1.5 h. Then the 5′-radiolabeled RNA substrate was added and the solution further incubated at 37 °C for 1.5 h. The final reaction conditions are as follows: 50 nM 5′-phosphorylated antisense RNA, 10 units of Superase-IN (Ambion), 50 mM Tris (pH7.4), 2 mM $MgCl_2$, 0.5 mM DTT, 0.25 mM ATP, 100 mM KCl, and 50 mM NaCl. The reaction was stopped by addition of 2% $LiClO_4$ in acetone, and RNA was precipitated by centrifugation. After being washed with acetone, the RNA was reconstituted in 90% formaldehyde and 1× TBE with dye and separated with a 12% acrylamide−7 M urea gel.

RESULTS

Design and Synthesis of UNA Oligonucleotides. In our initial studies, we observed that RNA duplexes that were fully complementary to CAG repeats were potent but non-allele-selective inhibitors of HTT and ATX-3 expression.[35,37] We subsequently observed that RNA duplexes containing centrally mismatched bases were allele-selective inhibitors of expression

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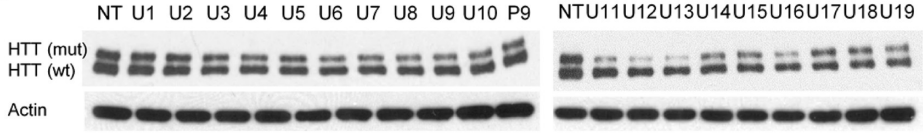
A

siRNA	Sequence	Position of UNA modification	Tm[a] (°C)	mut IC_{50} (nM)	wt IC_{50} (nM)	Selectivity (fold)
P9U1	**G**CUGCUGCAGCUGCUGCUGdTdT	1	77.0	>50	>50	-
P9U2	G**C**UGCUGCAGCUGCUGCUGdTdT	2	75.7	>50	>50	-
P9U3	GC**U**GCUGCAGCUGCUGCUGdTdT	3	75.2	>50	>50	-
P9U4	GCU**G**CUGCAGCUGCUGCUGdTdT	4	71.7	>50	>50	-
P9U5	GCUG**C**UGCAGCUGCUGCUGdTdT	5	68.0	>50	>50	-
P9U6	GCUGC**U**GCAGCUGCUGCUGdTdT	6	71.1	14±4.6	>50	>3.5
P9U7	GCUGCU**G**CAGCUGCUGCUGdTdT	7	71.7	5.2±0.7	>50	>9.6
P9U8	GCUGCUG**C**AGCUGCUGCUGdTdT	8	72.4	6.3±0.9	>50	>8.0
P9U9	GCUGCUGC**A**GCUGCUGCUGdTdT	9	77.0	2.3 ± 0.4	>100	>43
P9U10	GCUGCUGCA**G**CUGCUGCUGdTdT	10	74.6	2.6±0.2	>50	>19
P9U11	GCUGCUGCAG**C**UGCUGCUGdTdT	11	71.5	7.3±2.9	>50	>6.9
P9U12	GCUGCUGCAGC**U**GCUGCUGdTdT	12	71.9	3.2±1.2	>50	>15
P9U13	GCUGCUGCAGCU**G**CUGCUGdTdT	13	70.9	2.2±0.5	>100	>44
P9U14	GCUGCUGCAGCUG**C**UGCUGdTdT	14	66.5	28±10	>50	>1.8
P9U15	GCUGCUGCAGCUGC**U**GCUGdTdT	15	71.9	14±10	>50	>3.4
P9U16	GCUGCUGCAGCUGCU**G**CUGdTdT	16	73.1	8.2±2.7	>50	>6.1
P9U17	GCUGCUGCAGCUGCUG**C**UGdTdT	17	75.3	>50	>50	-
P9U18	GCUGCUGCAGCUGCUGC**U**GdTdT	18	76.9	>50	>50	-
P9U19	GCUGCUGCAGCUGCUGC**G**dTdT	19	78.2	>50	>50	-

B



C

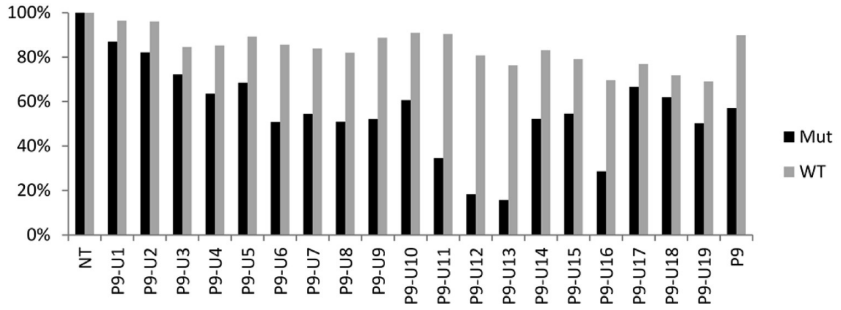


Figure 3. Effects of UNA substitutions within duplex RNAs that also have a mismatch at position 9 (P9). (A) Sequences of UNAs. UNA nucleosides are shown in boldface and underlined. (B) Effect of 25 nM UNA on inhibition of HTT expression, analyzed by Western analysis. (C) Quantitation of inhibition of wild-type or mutant HTT expression shown in panel B. NT means no treatment and no duplex added. The T_m value of the corresponding mismatched duplex at position 9 (P9) that lacks a UNA substitution is 79.7 °C.

for both genes.[34,35,37] Our goal in this study was to test the effect of UNA substitutions (Figure 1) within duplexes that were fully complementary relative to the target mRNAs or into duplexes that contained a mismatched base at position 9 of the antisense strand. These compounds test whether preserving base pairing while "unlocking" the backbone of key nucleosides can affect allele selectivity.

Effect of UNA Substitutions on the Inhibition of HTT by Fully Complementary Duplexes. We synthesized duplexes that were fully complementary to the CAG repeat and contained single UNA substitutions at position 9, 10, or 11 from the 5′ termini of the guide (antisense) strand (Figure 2A). We also synthesized a duplex that was substituted at positions 9, 10, and 11 with three UNA nucleosides. The UNA nucleosides were introduced by standard oligonucleotide coupling protocols.

We found that a single UNA substitution reduced the melting temperature (T_m) of the duplex by ∼8−12 °C compared with that of the fully complementary RNA duplex (REP). Three of the UNA-substituted duplexes (REPU9, REPU10, and REPU11) contained a single UNA, and the T_m values of these RNAs were 1−2 °C lower than those of analogous RNAs containing mismatched bases. For the duplex with three UNA substitutions (REPU91011), T_m decreases 16.5 °C compared with that of duplex REP. This value is ∼4 °C lower than that of the triply mismatched analogue.

We transfected the RNA duplexes into patient-derived fibroblast cells using cationic lipid and monitored inhibition of HTT protein expression by Western analysis. Duplexes containing single substitutions at position 9, 10, or 11 were potent and selective inhibitors of mutant HTT expression, demonstrating that unlocking the ribose could yield good allele selectivity even though the potential for full base pairing is maintained. Potencies were 1.8−2.7 nM, and selectivities for inhibition of mutant versus wild-type expression were >18-fold.

By contrast to the potent and allele-selective duplexes with single UNA substitutions, duplex REPU91011 substituted with three UNA nucleobases was less potent and had low selectivity. REPU91011 had a T_m value 5−9 °C lower than those of RNAs with one UNA substitution. This weakened ability to recognize a complementary target is a likely cause of its reduced potency and selectivity.

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Effect of UNA Substitutions on the Inhibition of HTT by Mismatched Duplexes. We also introduced UNA substitutions into duplexes containing a mismatched base at position 9 (Figure 3A). We had previously observed that the introduction of centrally located mismatches yields high selectivities and potencies.[37] We reasoned that the combination of mismatches and UNA substitutions might yield improved compounds.

Introducing a UNA nucleoside blocked inhibition of both alleles when the substitution was at positions 1−5 (Figure 3B,C and Figures 1 and 2 of the Supporting Information). This result is consistent with positions 1−8 acting as a critical seed sequence during RNAi. Increased flexibility can disrupt key seed interactions even if the potential for base pairing is maintained.

Other substitutions were more compatible with potent RNAi. When the substitution was at position 6, the IC_{50} for inhibition of the mutant allele was 14 nM and the potency decreased to <10 nM for substitutions at positions 7−13. Two duplexes (P9U9 and P9U13) had outstanding potencies (~2 nM) and >40-fold selectivities, demonstrating that the combination of UNA substitutions and mismatched bases has the potential to yield promising compounds. Moving the position of the UNA substitution by just one position in P9U14 reduced the potency dramatically, emphasizing that UNA substitutions must be positioned carefully. For several UNA-substituted duplexes (e.g., P9−U9 and P9−U11), we observed little or no inhibition of wild-type expression (Figure 4), making them excellent candidates for further investigation.

Effect of UNA Substitutions on the Cleavage of RNA Targets during RNAi. Argonaute 2 (AGO2) is the catalytic engine of RNAi.[45] When a duplex RNA is fully complementary to its RNA target, the anticipated outcome is that AGO2 will mediate recognition and cleavage of the target RNA. We had previously observed that a duplex RNA that is fully complementary to a CAG repeat will cause a modest decrease in RNA levels, while little or no reduction is observed when the duplex contains centrally located mismatches.

We tested whether UNA-modified duplexes reduce *HTT* mRNA levels and whether recognition of UNAs involves the key RNAi factor AGO2. We transfected UNA duplexes into fibroblast cells and used RNA immunoprecipitation (RIP) with the anti-AGO2 antibody to examine the recruitment of AGO2 to *HTT* mRNA. Effects on AGO2 recruitment were compared with those with a noncomplementary control duplex (CM). Both UNA-modified duplexes recruited AGO2 to *HTT* mRNA (Figure 5A), consistent with functioning through RNAi.

Next, we used qPCR to test how UNA-substituted duplexes would affect the level of *HTT* mRNA (Figure 5B). Duplex RNA BB is a positive control siRNA targeting an outside region of the CAG repeat in *HTT* mRNA, and as expected, it reduced *HTT* mRNA. We then tested four allele-selective potent UNA-modified duplexes and observed that all four duplexes caused little alteration of the *HTT* mRNA level. These findings are consistent with the conclusion that UNA substitutions disrupt AGO2-mediated cleavage of target mRNA in a fashion similar to that of the introduction of central mismatches.

We further examined the effect of UNA substitutions on RNA cleavage through the RNAi pathway using a radiolabeled substrate *HTT* RNA with CAG repeats (Figure 5C). UNA-modified RNAs were individually incubated with AGO2 for a predetermined time to allow loading and formation of an AGO2−RNA complex. The complex was then mixed with the radiolabeled RNA substrate. The duplex RNAs substituted with UNA induced cleavage less frequently than the fully complementary duplex, consistent with our qPCR data. Cleavage was almost undetectable when the duplex has both mismatch and UNA substitution at positions 9 and 12, respectively (P9−U12).

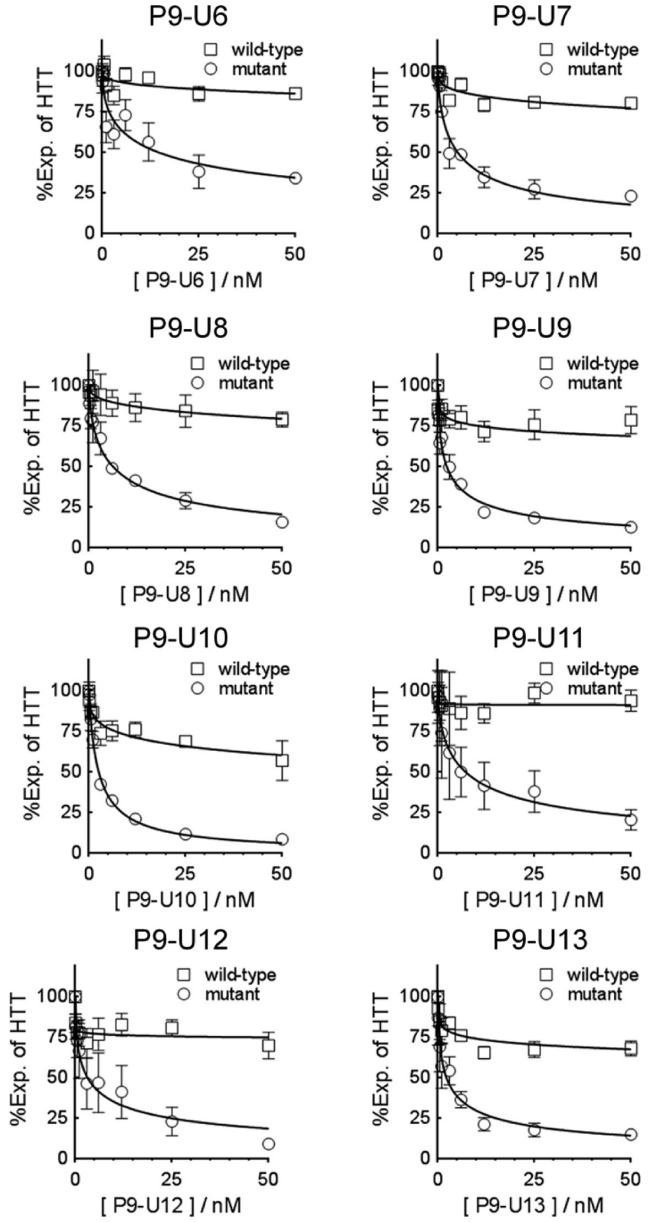

Figure 4. Dose−response data showing the effect of UNA substitutions on duplexes based on P9 siRNA containing a mismatched base at position 9 (P9) relative to the CAG repeat. Dose−response curves for P9−U6, P9−U7, P9−U8, P9−U9, P9−U10, P9−U11, P9−U12, and P9−U13 duplexes are shown. Dose curves are averaged data from three independent experiments and are based on Western analysis of HTT inhibition in GM04281 patient-derived fibroblast cells.

Taken together, data from RIP, qPCR, and *in vitro* cleavage assays show that the UNA substitution blocks cleavage of RNA substrates as effectively as introducing a mismatched base. These data suggest that allele-selective inhibition can be achieved without the need for cleavage of the target *HTT* mRNA and that these UNA-substituted duplexes function more like miRNAs than fully complementary siRNAs.

Effect of UNA Substitutions on the Inhibition of Expression of ATX-3 by Fully Complementary and Mismatch-Containing Duplexes. As noted above, several

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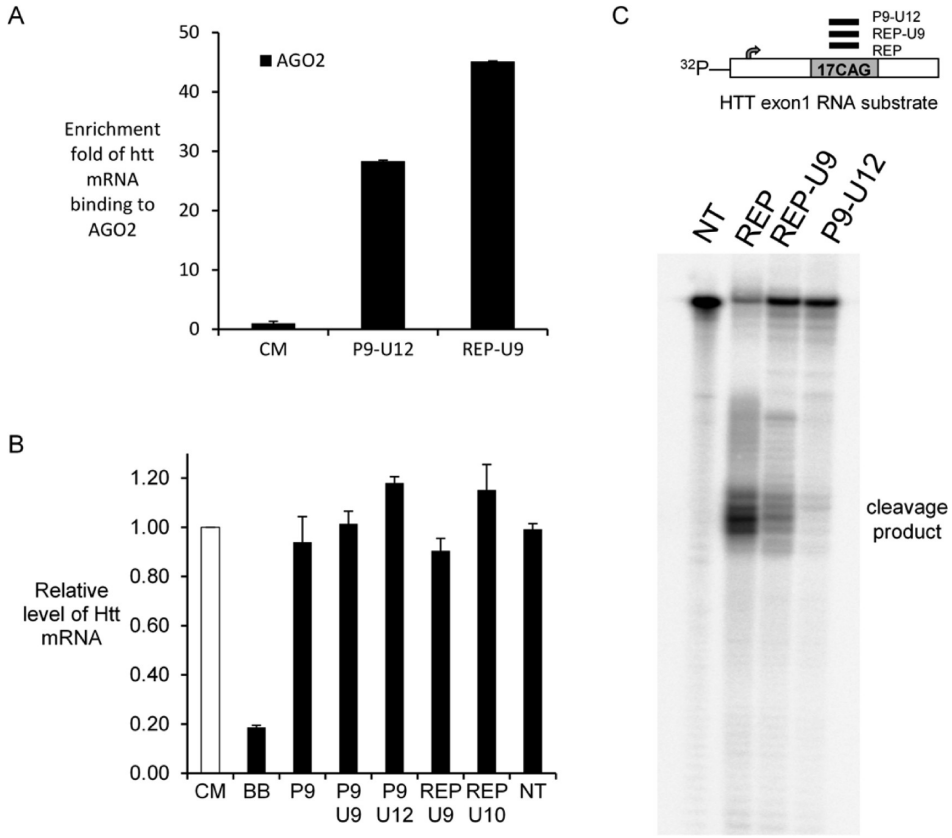
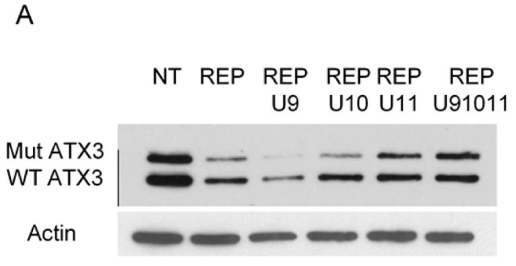


Figure 5. Effect of addition of UNAs on levels of *HTT* mRNA. (A) RNA immunoprecipitation (RIP) by use of an anti-AGO2 antibody to examine the association of the AGO2−UNA-modified siRNA complex with *HTT* mRNA. (B) Quantitative PCR (qPCR) showing the effect of UNA duplexes (25 nM) on levels of *HTT* RNA expression. (C) *In vitro* cleavage assay using RNA antisense strands and recombinant human AGO2 protein. NT means no treatment and no duplex added. BB is a duplex RNA that targets sequences of *HTT* mRNA outside the CAG repeat. CM is a control duplex lacking complementarity to *HTT* mRNA.



B

siRNA	mut IC_{50} (nM)	wt IC_{50} (nM)	Selectivity (fold)
P9	4.6	>50	>11
REP	12±4	24±9	2
REP-U9	3.9±0.7	29.6±7.8	7.5
REP-U10	6.0±1.6	>50	>8.3

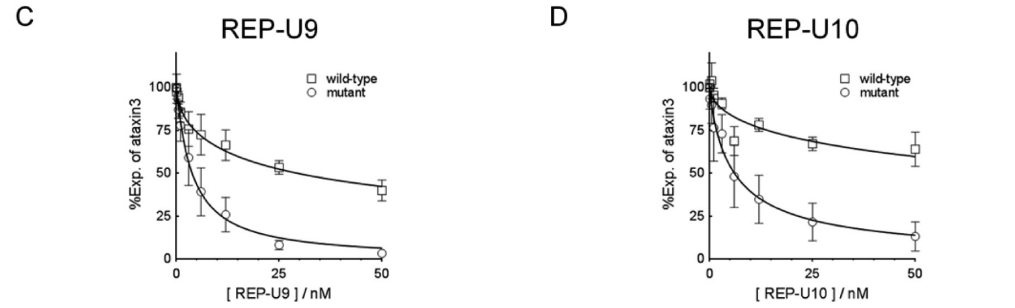


Figure 6. Effect of UNA substitutions on allele-selective inhibition of ATX-3 by RNA duplexes that are fully complementary to the CAG repeat. Western analyses of ATX-3 protein expression in GM06151 patient-derived fibroblast cells. (A) Effect of 25 nM UNA duplexes on inhibition of ATX-3 expression. (B) Summary of data. Dose−response curves showing the effect of adding (C) REPU9 and (D) REPU10 at increasing concentrations. NT means no treatment and no duplex added. Dose curves are averaged data from three independent experiments.

neurological diseases are caused by expanded CAG repeats, and a single anti-CAG compound may be able to treat several diseases. To understand the potential of UNA-substituted duplexes to inhibit expression of other disease genes, we examined their ability to allele-selectively inhibit ATX-3 expression in patient-derived fibroblast cells.

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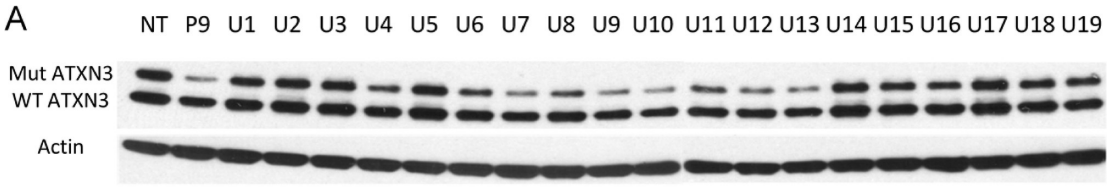

B

siRNA	mut IC_{50} (nM)	wt IC_{50} (nM)	Selectivity (fold)
P9-U12	5.5±1.5	35.2±10.1	6.4
P9-U13	13.0±2.8	42.5±7.9	3.2

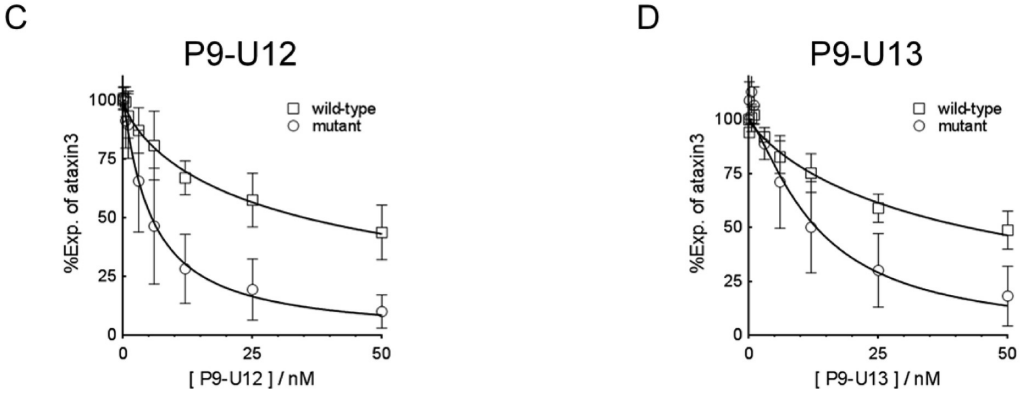

Figure 7. Effect of UNA substitutions on inhibition of ATX-3 expression. Duplexes were systematically substituted with UNA bases and contained a mismatched base at position 9 (P9) relative to the CAG repeat target. (A) Effect of 25 nM UNA duplexes on inhibition of ATX-3 expression, analyzed by Western analysis. (B) Summary of data. Dose–response curves for inhibition of ATX-3 expression by (C) P9–U12 and (D) P9–U13 duplexes. NT means no treatment and no duplex added. Dose–response curves are averaged data from three independent experiments.

Previously, we had observed that duplex RNAs that contained central mismatches could achieve allele-selective inhibition of ATX-3.[36] The potencies and selectivities of ATX-3 modulation, however, were lower than those for inhibition of HTT expression, and we identified fewer potent and selective duplexes. This result, that selectivity was different even though the CAG target sequence was found in both genes, suggested that the surrounding unique sequence of the *HTT* or *ATX-3* gene contributed to selectivity and that *ATX-3* was a more difficult target.

We tested UNA duplexes that were fully complementary to the CAG repeat and contained one or three UNA substitutions (Figure 2A). The three duplexes with one UNA substitution were allele-selective inhibitors (Figure 6). In contrast to inhibition of HTT expression, where inhibition by U9 or U11 had been more selective than inhibition by U10, for ATX-3 the highest selectivity was achieved by U10 with an IC_{50} value of 6.0 nM and >8.3-fold selectivity.

We also tested UNA duplexes that contained a single UNA substitution in combination with a mismatch at position 9 (Figure 3A). Several of these duplexes were allele-selective inhibitors of mutant HTT expression (Figure 7A), and P9–U12 and P9–U13 duplexes were chosen for further analysis (Figure 7B,C,D). The P9–U12 duplex possessed the best selectivity with an IC_{50} value of 5.5 nM and 6.4-fold selectivity.

DISCUSSION

Effects of the UNA Substitution on AGO2 Activity. RNA duplexes containing UNA modifications achieve allele-selective inhibition of HTT and ATX-3 expression. Both potency and selectivity are sensitive to shifting the position of UNA substitutions. Previously, we[34,35,37−39] and others[36] have shown that the introduction of a mismatch or abasic site that disrupts AGO2-mediated cleavage can lead to allele-selective anti-CAG duplexes. Allele-selective inhibition by UNA duplexes adds increased backbone flexibility as a new strategy for generating allele-selective duplexes.

Examination of the crystal structure data for human AGO2 in complex with RNA[46] suggests that stacking is disrupted and kinks appear between bases 6 and 7 and bases 9 and 10. Furthermore, the ribose–phosphodiester backbone curves around bases 13 and 14. Via introduction of UNA residues at positions 9–11, the flexibility of the surrounding RNA backbone is increased. Increased flexibility may lead to a slight displacement in strand position relative to catalytic residues, making the RNA strand a poorer substrate for cleavage by AGO2-mediated cleavage.

Trinucleotide Repeats and the Challenge of Allele Selectivity. Genes that contain CAG trinucleotide repeats are responsible for up to 19 different hereditary diseases. These diseases have severe consequences for patients, and there are currently no curative treatments available. Useful drugs are urgently needed. Nucleic acids, with their potential to silence expression of disease genes, offer significant advantages as a strategy for therapeutic development.[33]

One approach to silencing trinucleotide repeat genes involves non-allele-selective antisense oligonucleotides or duplex RNAs.[24,30,31] These compounds are active *in vivo* in mouse models of HD and may offer the most rapid route to human clinical trials. Reduced levels of the wild-type protein during non-allele-selective inhibition, however, may have adverse con-

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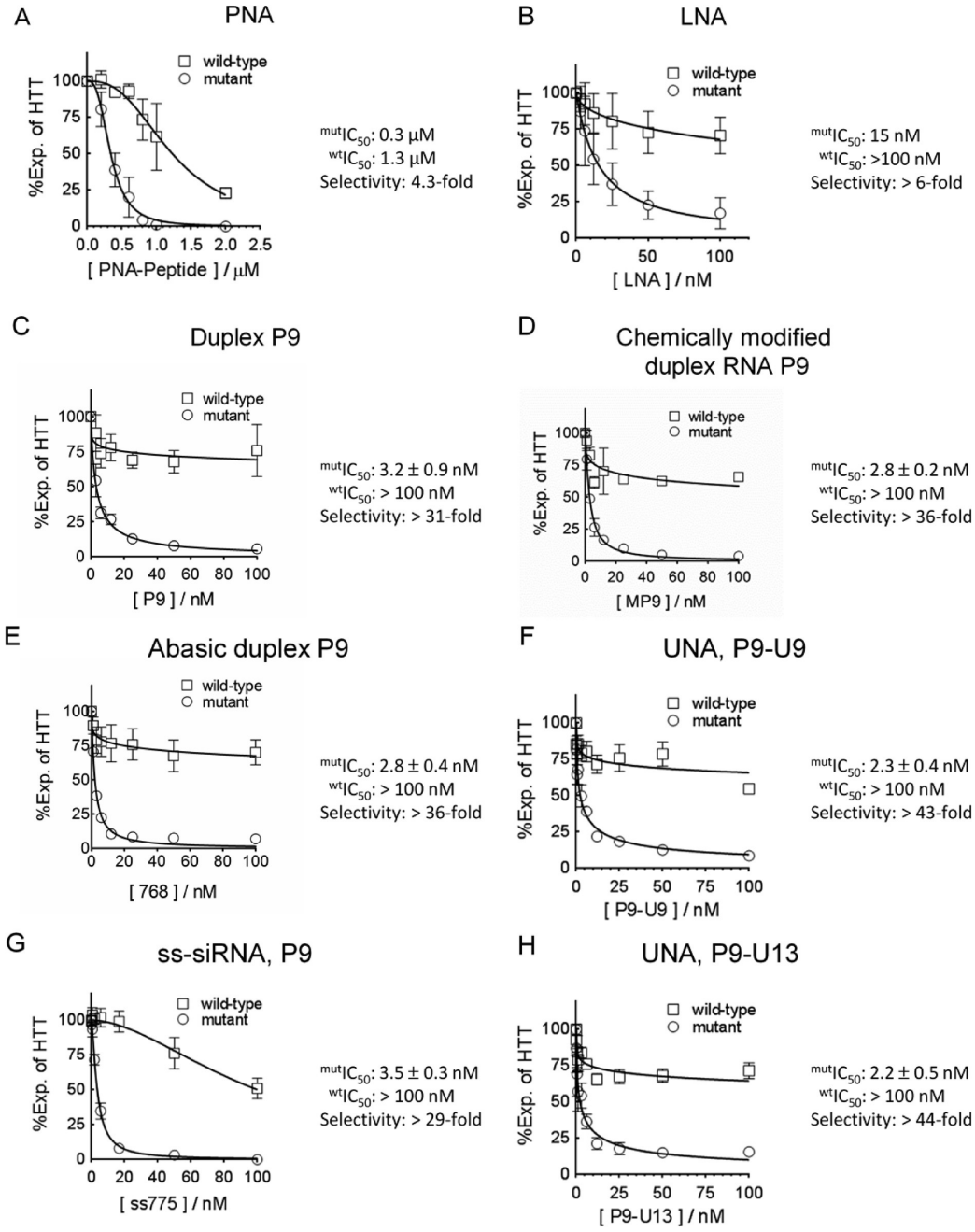

Figure 8. Representative does−response curves for inhibition of HTT expression by (A) PNA (peptide nucleic acid),[39] (B) LNA (locked nucleic acid),[40] (C) duplex siRNA with a central mismatch (P9),[37] (D) chemically modified duplex siRNA with a central mismatch,[41] (E) duplex siRNA with abasic substitution,[41] (F) the P9−U9 duplex, (G) single-stranded siRNA with a central mismatch,[42] and (H) the P9−U13 duplex. Dose−response curves are averaged data from three independent experiments. Note that the cellular delivery method for PNA differs from other oligomers and potencies cannot be compared directly.

sequences. Allele-selective strategies are a useful alternative in case trials in which non-allele-selective drugs encounter problems.

Allele-selective inhibition can be achieved by antisense oligonucleotides[23] or duplex RNAs[25] that take advantage of single-nucleotide polymorphisms to gain specificity for inhibiting expression of the mutant allele. While effective, the identity of SNPs varies between patients, and it would be necessary to develop several drugs for the treatment of most of the population for a disease like HD.[26] By contrast, the expanded CAG repeat exists in all patients. Targeting the CAG repeat might be useful for all HD patients, and because the expanded CAG repeat is common among several diseases, it is possible that one molecule might be able to treat several different hereditary pathologies.

We have shown that several different types of synthetic oligomer can achieve allele-selective inhibition of HTT or ATX-3 expression (Figure 8). These oligomers include peptide nucleic acid (PNA),[40] LNA or cEt-bridged nucleic acid,[40,44] mismatch-containing RNA duplexes with no chemical modifications,[37] mismatched duplexes with extensive chemical modifications,[40] mismatched duplexes with abasic substitutions,[41] and single-stranded silencing RNAs.[38,42] We find that potencies and selectivities for the best UNA match the best molecules from any other class of anti-CAG nucleic acid (Figure 8).

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In contrast to previous work showing that the introduction of acyclic substitutions could enhance RNase H activity,[18] we observe that UNA substitutions block cleavage activity by AGO2. This difference emphasizes that the effect of UNA substitution will depend on context, including the properties of the enzyme involved in silencing and the exact placement of the UNA modifications. It is also worth noting that there are other acyclic modifications, like the 2′,3′-secouridine modifications used in the previous study, and these might also provide a basis for allele-selective duplexes.

To be a drug, a nucleic acid inhibitor of mutant HTT expression will need to enter brain cells and be well-tolerated. This need to balance cellular uptake, potency, allele selectivity, and toxicity presents a challenge for drug development. An encouraging outcome from our studies is that diverse nucleic acid chemistries are compatible with effective inhibition of mutant alleles. These chemistries (UNA, abasic, ss-siRNA, and 2′-modified) can be combined with each other or with mismatched bases at varied positions to create a large number of duplexes that are potent and selective. This pool of candidates then becomes a reservoir for testing compounds in animals to identify agents with optimal *in vivo* properties.

■ ASSOCIATED CONTENT

Supporting Information

Representative gel images of the dose−response curves for inhibition of expression of HTT by UNA-modified P9 duplexes (Figures S1 and S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Y.A., J.H., and J.L. planned and executed experiments testing the inhibition of HTT. C.M. and Q.X. designed and synthesized UNAs. D.R.C. planned experiments and wrote the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

HTT, huntingtin; ATX-3, ataxin-3; UNA, unlocked nucleic acid; AGO2, argonaute 2; T_m, melting temperature; HD, Huntington's disease; MJD, Machado Joseph disease; RIP, RNA immunoprecipitation.

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