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Single-Stranded RNAs Use RNAi to Potently and Allele-Selectively Inhibit Mutant Huntingtin Expression

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SUMMARY

Mutant huntingtin (HTT) protein causes Huntington disease (HD), an incurable neurological disorder. Silencing mutant HTT using nucleic acids would eliminate the root cause of HD. Developing nucleic acid drugs is challenging, and an ideal clinical approach to gene silencing would combine the simplicity of single-stranded antisense oligonucleotides with the efficiency of RNAi. Here, we describe RNAi by single-stranded siRNAs (ss-siRNAs). ss-siRNAs are potent (>100-fold more than unmodified RNA) and allele-selective (>30-fold) inhibitors of mutant HTT expression in cells derived from HD patients. Strategic placement of mismatched bases mimics micro-RNA recognition and optimizes discrimination between mutant and wild-type alleles. ss-siRNAs require Argonaute protein and function through the RNAi pathway. Intraventricular infusion of ss-siRNA produced selective silencing of the mutant *HTT* allele throughout the brain in a mouse HD model. These data demonstrate that chemically modified ss-siRNAs function through the RNAi pathway and provide allele-selective compounds for clinical development.

INTRODUCTION

Huntington disease (HD) is an incurable neurological disorder that afflicts at least 1:100,000 people worldwide (Walker, 2007; Finkbeiner, 2011). The disease is characterized by progressive neurodegeneration, and symptoms worsen steadily until death. HD is caused by a dominant heterozygous expansion of CAG trinucleotide repeats within the protein-encoding region of the huntingtin (*HTT*) gene. CAG is the codon for glutamine, and the average mutant *HTT* allele in patients contains ~45 consecutive CAG trinucleotides (MacDonald et al., 1993; Duyao et al., 1993; Kremer et al., 1994). Though the genetic origin of HD has been known for almost 20 years, curative drugs have not been identified. Effective agents that will benefit HD patients are urgently needed.

HTT protein is a difficult target for traditional small-molecule drugs because it forms interactions with many other proteins and because it is difficult to design small molecules that potently and selectively disrupt protein:protein interactions. Because the genetic origin of HD is localized to just one gene, inhibiting expression of HTT is a promising therapeutic option. Approaches to blocking HTT expression include use of single-stranded antisense oligonucleotides (ASOs) and duplex RNAs (dsRNAs) that target HTT mRNA (Sah and Aronin, 2011; Matsui and Corey, 2012). ASOs and dsRNAs that inhibit expression of HTT have been shown to alleviate symptoms and prolong survival in mouse HD models (Harper et al., 2005; DiFiglia et al., 2007; Boudreau et al., 2009; Drouet et al., 2009), with transient infusion yielding a sustained reversal of phenotype that persists longer than the HTT knockdown (Kordasiewicz et al., 2012). This success suggests that silencing HTT expression can be a productive strategy for developing drugs to treat HD.

HD is dominantly inherited, with patients expressing both mutant and wild-type *HTT* alleles. Simultaneously inhibiting both alleles may prove to be a successful clinical strategy, and studies in a mouse model have shown that reduction of both wild-type and mutant HTT has the same benefit as reduction of mutant HTT alone (Kordasiewicz et al., 2012). Multiple studies, however, suggest that reducing wild-type HTT levels may have deleterious effects (Nasir et al., 1995; Zeitlin et al., 1995; White et al., 1997; Godin et al., 2010; Omi et al., 2005; Huang et al., 2011). Allele-selective inhibitors that maximize reduction of mutant HTT and minimize loss of wild-type HTT would be ideal. One approach to achieving this goal exploits the existence of single-nucleotide polymorphisms (SNPs) that allow dsRNAs to distinguish the mutant and wild-type alleles (Miller et al., 2003; Schwarz et al., 2006; van Bilsen et al., 2008; Carroll et al., 2011). The identity of SNPs varies between patients, but some SNPs are common, and a few SNPs may be sufficient to cover a majority of HD patients in certain populations (Pfister et al., 2009; Lombardi et al., 2009; Carroll et al., 2011: Warby et al., 2009).

An alternative strategy for allele-selective inhibition exploits a universal difference between the mutant and wild-type

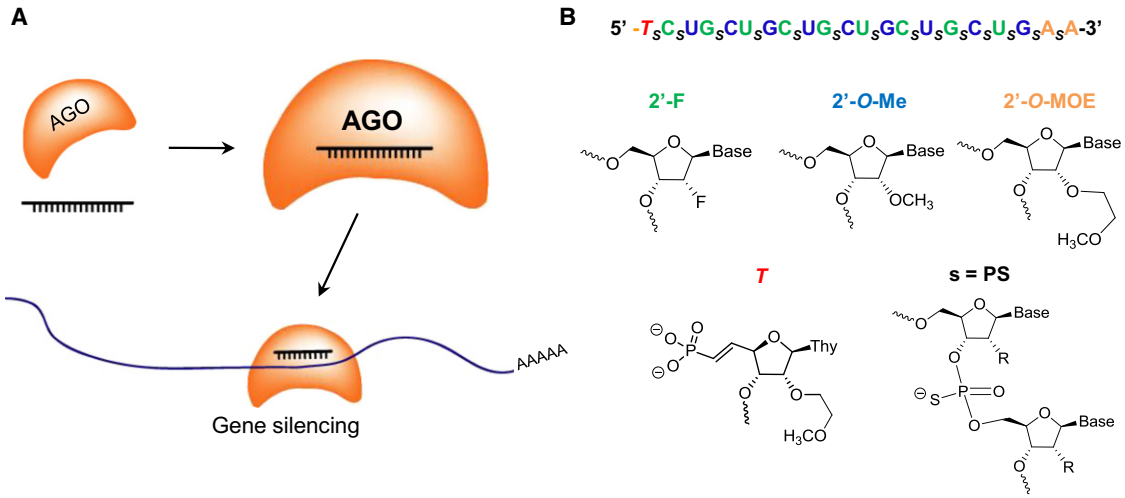

Figure 1. AGO-Mediated Gene Silencing and Chemically Modified ss-siRNAs

(A) Recognition of mRNA by ss-siRNA. ss-siRNA loads into AGO protein, and the complex recognizes a target sequence within an mRNA to silence gene expression.

(B) Sequence and chemical modifications of a typical ss-siRNA. The 5′-thymidine base is modified with an *(E)*-vinylphosphonate.

alleles: the mutant alleles have more trinucleotide repeats. The longer poly-CAG tract in mutant *HTT* mRNA offers more binding sites for complementary oligomers. In addition, trinucleotide repeats can form hairpin self-structures (Michlewski and Krzyzosiak, 2004; de Mezer et al., 2011; Krzyzosiak et al., 2012), and the expanded mutant repeats will likely form structures that differ from wild-type. These mutant structures may be more susceptible to recognition and selective binding by oligonucleotides and allow preferential inhibition of the mutant allele. We initially used single-stranded ASOs to test the hypothesis that oligomers complementary to CAG repeats could be allele-selective inhibitors (Hu et al., 2009; Gagnon et al., 2010; Hu et al., 2011; Gagnon et al., 2011). We identified several allele-selective ASOs but did not achieve selectivities of greater than 4- to 8-fold.

The mechanism of RNAi differs from that of ASOs (Watts and Corey, 2012), and we reasoned that changing our silencing strategy to RNAi might improve selectivities. Our initial tests with fully complementary siRNAs generated potent inhibition but little selectivity (Hu et al., 2009). Fully complementary duplexes function through an siRNA pathway that involves cleavage of target mRNAs, whereas mismatch-containing duplexes can act through a micro-RNA (miRNA)-like pathway that suppresses translation (Filipowicz et al., 2008). To test whether duplexes that resemble miRNAs might afford greater selectivity, we altered the mechanism of gene silencing by generating duplexes that mimicked miRNAs by introducing mismatches into the central region of the dsRNA. The mismatches were at positions predicted to disrupt cleavage of the target by Argonaute 2 (AGO2) (Wang et al., 2008), an essential protein for substrate recognition and degradation during RNAi (Liu et al., 2004). Using this strategy, we identified mismatch-containing RNAs that were potent and selective inhibitors (Hu et al., 2010). Krzyzosiak and colleagues also reported allele selectivity using RNA duplexes with mismatches at positions 13 and 16 (Fiszer et al., 2011).

Compounds that combine the favorable biodistribution and simpler synthesis of single-stranded oligonucleotides with the potency of duplex RNAs would offer an ideal strategy for silencing gene expression. Single-stranded RNAs (ssRNAs) have been reported to enter the RNA-induced silencing complex (RISC) under specific conditions and inhibit gene expression (Martinez et al., 2002; Schwarz et al., 2002; Holen et al., 2003). Unlike duplex RNA, which is stable in serum, the half-life of ssRNA in serum is measured in seconds to minutes (Braasch et al., 2003). Most ssRNAs would be likely degraded by nucleases before entering cells and inhibiting gene expression. Chemical modifications can stabilize RNA, and one study has reported that chemically modified boranophosphate RNA single strands could be active inside of cells (Hall et al., 2006). There was no experimental follow-up and little exploration of mechanism for unmodified or modified single-stranded RNAs, leaving it unclear whether the approach could have practical application. Another report of gene silencing by chemically modified ssRNAs has appeared (Haringsma et al., 2012), but their robustness and mechanism of action remain unclear.

Recently, the action of ssRNAs that function through the RNAi pathway has been revisited (Lima et al., 2012). Systematic chemical modifications and iterative design improvements led to stabilized single-strand small interfering RNAs (ss-siRNAs) (Figure 1A) that efficiently enter the RNA pathway and silence gene expression. Here, we describe potent and allele-selective inhibition of mutant HTT expression in HD-patient-derived cells and HD model mice with ss-siRNAs targeting CAG repeats.

RESULTS

ss-siRNAs Are Potent and Allele-Selective Inhibitors of Mutant HTT Expression

The ss-siRNAs used in these studies contain a mixture of 2′-fluoro (2′-F), 2′-O-methyl (2′-O-Me), and 2′-methoxyethyl (2′-MOE)

ribose modifications (Figure 1B). The ss-siRNAs possess both phosphodiester and phosphorothioate internucleotide linkages. The 5′ terminus was capped with either a phosphate or a *(E)*-vinylphosphonate. We initially tested ss-siRNAs 537787 (fully complementary to the CAG repeat) and ss-siRNAs 537775 and 537786 (containing mismatched bases at positions 9 [P9] or 10 [P10], respectively).

These ss-siRNAs were introduced into HD-patient-derived fibroblast cell line GM04281 (69 CAG repeats/mutant allele, 17 CAG repeats/wild-type allele) by standard transfection methods. From our previous studies, oligomers with centrally located mismatches relative to their mRNA targets are predicted to inhibit expression of HTT protein but have little effect on *HTT* mRNA (Hu et al., 2010), leading us to focus on measuring protein levels. HTT is a large protein—347 kDa in molecular weight—and the expanded repeat leads to only a few kDa increase. The small molecular weight difference makes discrimination between alleles challenging, but mutant and wild-type HTT proteins can be efficiently resolved using temperature-controlled SDS-PAGE (Hu et al., 2009).

ss-siRNAs 537787, 537775, and 537786 inhibited HTT expression with varying potencies and selectivities (Figures 2 and 3). Fully complementary ss-siRNA 537787 possessed an IC_{50} value of 8 nM and a selectivity of >13-fold (Figure 2A). This selectivity is better than the selectivity of the analogous unmodified duplex RNA (2-fold) (Hu et al., 2009). ss-siRNA 537775 (single mismatch at position 9) possessed an IC_{50} value of 3.5 nM and a selectivity of >29-fold (Figure 2B). ss-siRNA 537775 was the best inhibitor, with potency and selectivity values similar to the most selective dsRNAs identified (Hu et al., 2010). ss-siRNA 537786 (single mismatch to P10, one base shift relative to 537775) possessed an IC_{50} value of 22.3 nM and a selectivity of >4-fold (Figure 3), making it less effective than the analogous mismatch-containing dsRNA (Hu et al., 2010).

These results suggest that chemical modifications and the precise positioning of mismatched bases affect allele-selective recognition and silencing. In one case, selectivity was better relative to the analogous dsRNA; in another, selectivity was similar; and in the third example, selectivity and potency were worse. Though ss-siRNAs appear to function similarly to dsRNAs, the exact outcome of recognition depends on sequence.

We examined inhibition of HTT by ss-siRNA 537775 over time. ss-siRNA was added only once at the beginning of the experiment. During this period, cells doubled three to four times, diluting out the ss-siRNA. We observed >80% inhibition of mutant HTT expression for up to 8 days, with expression gradually returning to the original levels after 2 weeks (Figure 2C).

To provide a comparison with a silencing strategy that is non-allele selective and does not involve RNAi, we tested a gapmer ASO complementary to a region outside the CAG tract. The "gapmer" ASO contains a central DNA "gap" to recruit RNase H that is flanked by chemically modified bases to improve binding (Watts and Corey, 2012). The gapmer inhibited HTT expression with an IC_{50} value of 7.4 nM and a selectivity of 1.7-fold (Figures 2D and 3). These data suggest that ss-siRNAs targeted to the CAG repeat can achieve potencies that are similar to those achieved using ASOs, a gene silencing approach broadly used in clinical testing (Watts and Corey, 2012), while having the added benefit of being allele-selective.

As a further comparison, we examined silencing by anti-CAG ssRNAs that lacked chemical modifications. These unmodified ssRNAs were not active when tested at concentrations of up to 400 nM (Figure 2E). These results contrast with reports that unmodified ssRNAs possessed silencing activity in mammalian cells (Martinez et al., 2002; Schwarz et al., 2002) possibly due to differences in the cell lines, transfection techniques, the mRNA target, or the sequence of the silencing RNA.

Optimizing ss-siRNA Design

ss-siRNAs 537787, 537775, and 537786 contain 5′ (*E*)-vinylphosphonate moieties designed to improve stability and potency in vivo. This modification is not needed for testing in cell culture (Lima et al., 2012), and substitution with a phosphate moiety facilitates the synthesis of the large number of compounds needed to identify improved inhibitors and investigate mechanism. To determine whether potent and allele-selective inhibition could be achieved with 5′-phosphate ss-siRNAs, we tested compounds 553819, 553822, and 553821 (containing no mismatch, a P9 mismatch, and a P10 mismatch, respectively). These ss-siRNAs possessed potencies and selectivities similar to their phosphonate analogs (Figures 3 and 4A and Figure S1 available online).

We chose the phosphate design for large-scale tests and synthesized compounds that varied in the number and placement of mismatched bases (Figure 3). Several compounds possessed good potencies and selectivities, with the best combination of potency and selectivity achieved by ss-siRNA 557426. ss-siRNA 557426 contained three centrally located mismatched bases and combined an IC_{50} value of 3.3 nM with >30-fold selectivity (Figures 3 and 4B).

We had previously observed that a mismatched base at position P6 within a dsRNA abolished dsRNA-mediated inhibition of HTT. P6 is within the seed sequence (bases 2–8), a region that is critical for efficient RNAi (Lim et al., 2005). ss-siRNA 556888, an ss-siRNA that contained a mismatched base at P6, was the only single-mismatch compound to not inhibit HTT (Figures 3 and 4C). This result suggests that ss-siRNAs and duplex RNAs share critical recognition elements and supports the hypothesis that ss-siRNAs act through the RNAi pathway.

Most HD patients have mutant *HTT* alleles containing 40–50 repeats (Duyao et al., 1993; MacDonald et al., 1993). To determine whether ss-siRNAs might also be an effective strategy for allele-selective inhibition in this patient cohort, we tested inhibition in GM04719 patient-derived fibroblast cells (44 mutant repeats/15 wild-type repeats) (Figure 4D). We found that phosphate ss-siRNA 553822 inhibited expression of mutant HTT with an IC_{50} value of 0.9 nM and an allele selectivity >100-fold, demonstrating the potential to achieve allele-selectivity in cell lines within the median range of CAG repeat copy number. We note that inhibition of wild-type HTT levels off at ~45%, suggesting a population of wild-type mRNA that may contain some species that are refractory to silencing by anti-CAG ss-siRNAs.

Another challenge for agents that target trinucleotide repeats is the existence of other genes that contain trinucleotide repeats (Kozlowski et al., 2010). We observed no inhibition of

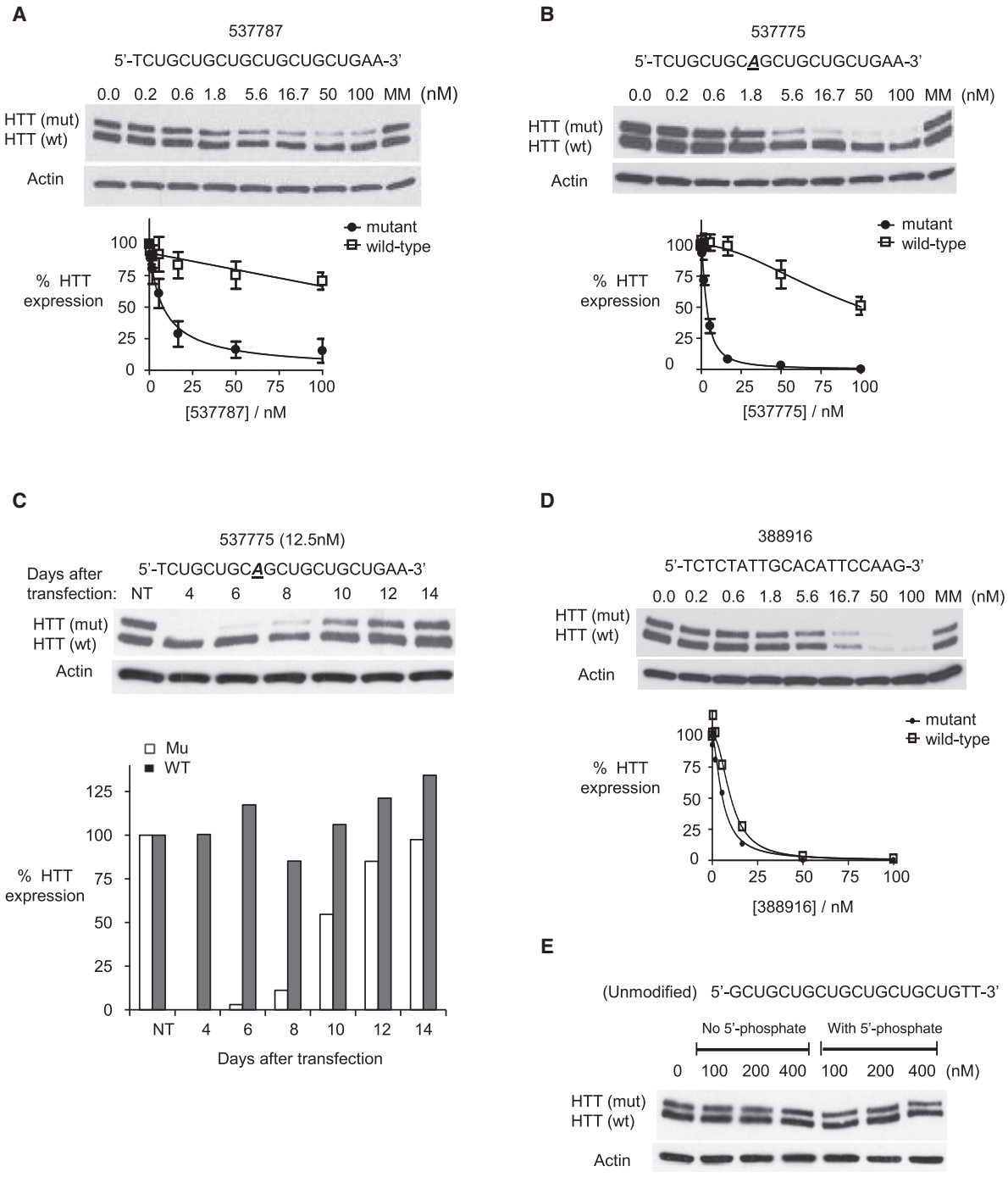

Figure 2. ss-siRNAs Inhibit HTT Expression

(A) Western analysis of inhibition of HTT expression by ss-siRNA 537787 (no mismatches).

(B) Western analysis of inhibition of HTT expression by ss-siRNA 537775 (one mismatch at P9).

(C) Western analysis of inhibition of HTT expression by ss-siRNA 537775 over 14 days with quantitation.

(D) Western analysis of inhibition of HTT expression by a methoxyethyl antisense oligonucleotide that targets a nonrepeat region of *HTT* mRNA. The graphs in (A), (B), and (D) show amounts of both wild-type and mutant HTT protein.

(E) Western analysis of inhibition of HTT expression by a fully complementary single-stranded RNA lacking any chemical modifications with and without a 5′-terminal phosphate.

MM, an RNA duplex containing multiple mismatches. Western analysis is representative data of at least duplicate experiments. Error bars are standard error of the mean (SEM) for dose-response studies from three or more independent experiments.

No.	Abbr. Name	Sequence	Position of Mismatch	Tm, ˚C	mut IC_{50} (nM)	wt IC_{50} (nM)	Selectivity (fold)
ssRNAs with 5'-*(E)*-vinyl phosphonate chemistry:							
537787	REP	**5' -*T*$_s$C$_s$UG$_s$CU$_s$GC$_s$UG$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	None	90-91	8.0 ± 1.7	> 100	>13
537775	P9	**5' -*T*$_s$C$_s$UG$_s$CU$_s$GC$_s$*A*G$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	9	84-85	3.5 ± 0.3	> 100	>29
537786	P10	**5' -*T*$_s$C$_s$UG$_s$CU$_s$GC$_s$U*A*$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	10	84-85	22.3 ± 2.6	> 100	>4
ssRNAs with 5'-phosphate chemistry:							
553819	REP	**5'-P -T$_s$C$_s$UG$_s$CU$_s$GC$_s$UG$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	None	> 95	5.7 ± 1.4	71.8 ± 15.6	13
556886	P4	**5'- P-T$_s$C$_s$U*A*$_s$CU$_s$GC$_s$UG$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	4	85	8.8	61.1	7
556887	P5	**5' - P-T$_s$C$_s$UG$_s$*A*U$_s$GC$_s$UG$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	5	82-89	17.2 ± 2.6	>100	>6
556888	P6	**5' - P-T$_s$C$_s$UG$_s$C*A*$_s$GC$_s$UG$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	6	81-85	N.I.	N.I.	N/A
556889	P7	**5' - P-T$_s$C$_s$UG$_s$CU$_s$*A*C$_s$UG$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	7	83-85	11.2 ± 1.7	51.1 ± 8.5	5
556890	P8	**5' - P-T$_s$C$_s$UG$_s$CU$_s$G*A*$_s$UG$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	8	83-85	4.1 ± 0.7	29.5 ± 4.3	7
553822	P9	**5' - P-T$_s$C$_s$UG$_s$CU$_s$GC$_s$*A*G$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	9	85	4.9 ± 0.8	90.4 ± 9.7	18
553821	P10	**5' - P-T$_s$C$_s$UG$_s$CU$_s$GC$_s$U*A*$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	10	82-86	17.8 ± 3.5	>100	>6
557407	P10R	**5' - P-T$_s$C$_s$UG$_s$CU$_s$GC$_s$U*U*$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	10	77-80	15.3 ± 2.4	>100	>7
556891	P11	**5' - P-T$_s$C$_s$UG$_s$CU$_s$GC$_s$UG$_s$*A*U$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	11	78-81	12.8 ± 1.7	>100	>8
556892	P12	**5' - P-T$_s$C$_s$UG$_s$CU$_s$GC$_s$UG$_s$C*A*$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	12	81-84	3.4 ± 0.6	72.0 ± 12.3	21
557406	P13	**5' - P-T$_s$C$_s$UG$_s$CU$_s$GC$_s$UG$_s$CU$_s$*A*C$_s$UG$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	13	78-82	4.2 ± 0.6	>100	>24
557408	P16	**5' - P-T$_s$C$_s$UG$_s$CU$_s$GC$_s$UG$_s$CU$_s$GC$_s$U$_s$*A*$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	16	81-84	8.1	27.3	3
557409	P910	**5' - P-T$_s$C$_s$UG$_s$CU$_s$GC$_s$*AA*$_s$CU$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	9, 10	78-81	6.3 ± 0.5	>100	>16
557426	PM3	**5' - P-T$_s$C$_s$UG$_s$CU$_s$GC$_s$*AA*$_s$*A*U$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	9, 10, 11	74	3.3 ± 0.5	>100	>30
557427	PM4	**5' - P-T$_s$C$_s$UG$_s$CU$_s$G*A*$_s$*AA*$_s$*A*U$_s$GC$_s$U$_s$G$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	8, 9, 10, 11	65-66	11.8 ± 1.9	>100	>8
557428	RM3	**5' - P-T$_s$C$_s$U*A*$_s$CU$_s$GC$_s$U*A*$_s$CU$_s$GC$_s$U$_s$*A*$_s$C$_s$U$_s$G$_s$A$_s$A-3'**	4, 10, 16	65	22.3	>100	>4
557429	RM4	**5' - P-T$_s$C$_s$*A*G$_s$CU$_s$G*U*$_s$UG$_s$CU$_s$*A*C$_s$U$_s$G$_s$*U*$_s$U$_s$G$_s$A$_s$A-3'**	3, 8, 13, 17	65-66	N.I.	N.I.	N/A
557430	REPU	**5' - P-T$_s$G$_s$CU$_s$GC$_s$UG$_s$CU$_s$GC$_s$UG$_s$C$_s$U$_s$G$_s$C$_s$U$_s$A$_s$A-3'**	None	>95	19.4 ± 4.7	>100	>5
Control oligomers:							
388916	Gapmer	**5'-TCTCTA$_d$T$_d$T$_d$G$_d$C$_d$A$_d$C$_d$A$_d$T$_d$T$_d$CCAAG-3'**	N/A	N/A	7.4 ± 0.7	12.6 ± 1.3	1.7
387898	Gapmer	**5'-CTCGAC$_d$T$_d$A$_d$A$_d$A$_d$G$_d$C$_d$A$_d$G$_d$G$_d$ATTTC-3'**	N/A	N/A	5.8	7.1	1.2
522247	Neg.Ctrl	**5' - T$_s$U$_s$AU$_s$CU$_s$AU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3'**	N/A	N/A	N.I.	N.I.	N/A
N/A	LNAT	**5'-GCTGCTGCTGCTGCTG-3'**	None	97	40 ± 7	>100	>2.5
N/A	CM	**5'-GCUAUACCAGCGUCGUCAUAA-3' 3'-TTCGA UAUGGUCGCAGCAGUA-5'**	N/A	N/A	N.I.	N.I.	N/A

Color schemes represent different chemical modifications as follows:

U: 2'-fluoro; A: 2'-*O*-methyl; A: 2'-methoxyethyl (2'-MOE); T:locked nucleic acid (LNA); *T:* phosphonate; s: phosphorothioate; d: deoxyribose.
All other sugars are riboses unless specifiied otherwise.

Figure 3. ss-siRNAs and Other Oligonucleotides Used in These Studies

N.I., no inhibition; N/A, not available. Except for CM (a dsRNA species), all T_m's are measured using ss-siRNAs duplexed with equimolar amounts of unmodified ssRNA 5′-CAGCAGCAGCAGCAGCAGCAGC-3′. Gapmer oligonucleotides have backbones containing only phosphorothioate linkages. All data were obtained using HD-patient-derived fibroblast cell line GM04281. Selectivity is calculated by dividing the IC_{50} for inhibition of wild-type HTT expression by that for mutant HTT. Error ranges represent SEM of IC_{50} values from biological replicates.

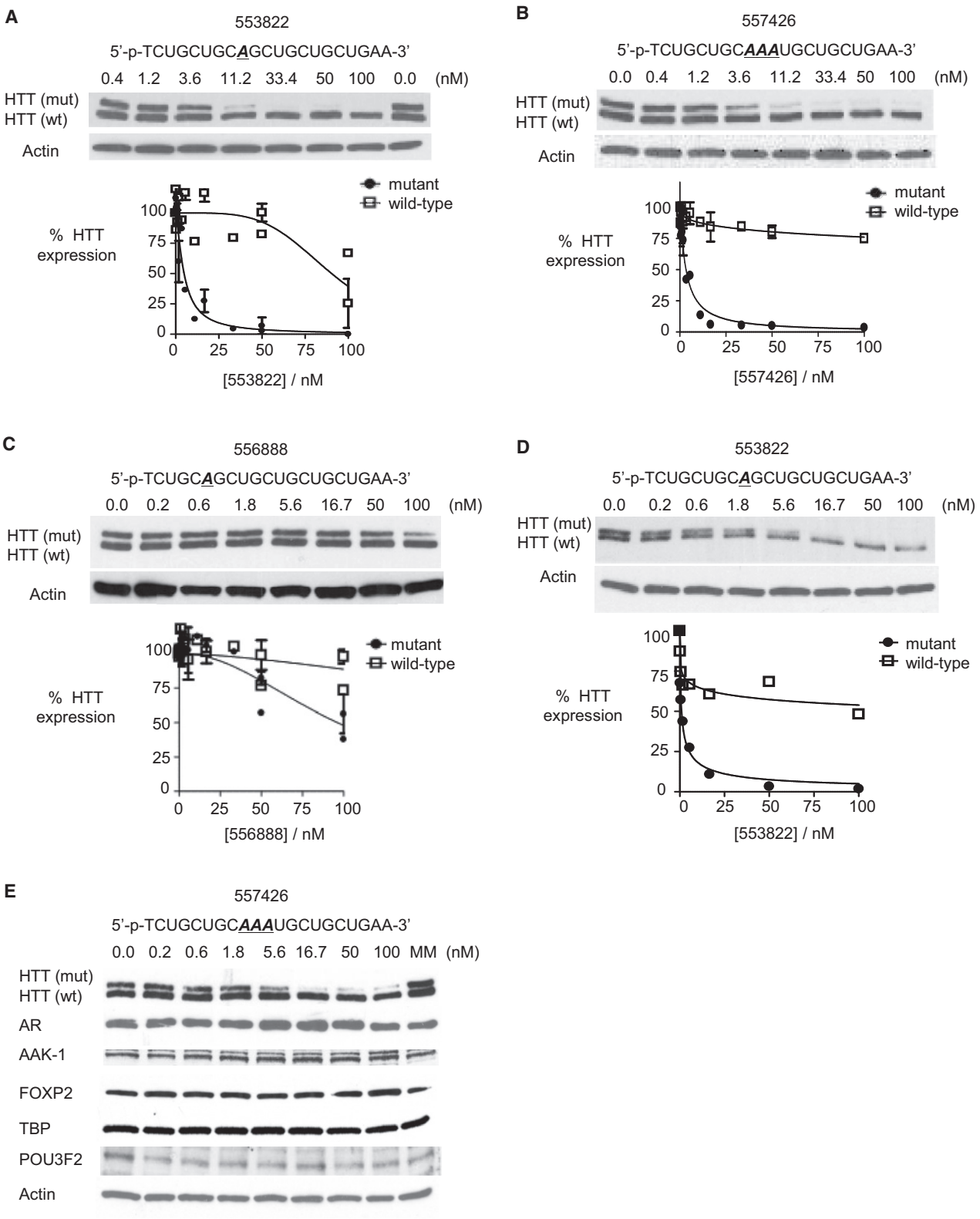

Figure 4. Characterization of Inhibition by Modified ss-siRNAs

(A) Western analysis of inhibition of HTT expression by ss-siRNA 553822 (mismatched base at P9) containing a 5′-phosphate.

(B) Western analysis of inhibition of HTT expression by ss-siRNA 557426 containing three central mismatches.

TATA-box-binding protein (TBP, 19 CAG repeats), androgen receptor (AR, ~20 CAG repeats), AAK-1 (6 CAG repeats), POU3F2 (6 CAG repeats), or FOXP2 (40 glutamines encoded by a mix of CAG and CAA trinucleotides) (Figure 4E) at concentrations well above those needed to achieve selective inhibition of mutant HTT.

Involvement of AGO2 Protein

AGO2 is a key protein involved in RNAi (Liu et al., 2004; Meister et al., 2004). There are four *AGO* genes in human cells. *AGO2* is the best characterized and the only variant with endonucleolytic activity. AGO1, AGO3, and AGO4 are also expressed, but their functions are less well defined. To determine which AGO variant is involved in gene silencing by ss-siRNAs, we used siRNAs targeting mRNAs encoding AGO1-4 to reduce expression (Figure S2). We observed that reducing AGO2 levels reversed silencing by 537775, consistent with involvement of AGO2 (Figure 5A). By contrast, silencing AGO1, AGO3, or AGO4 had little effect on allele-selective inhibition of mutant HTT.

To further investigate involvement of AGO, we used RNA immunoprecipitation (RIP) to examine the ability of ss-siRNA 537775 (P9 mismatch, (E)-vinylphosphonate 5′ terminus) to promote association of AGO2 with *HTT* mRNA. We transfected ss-siRNA 537775 into cells, harvested extracts, immunoprecipitated AGO-bound material using an anti-AGO2 antibody, and assayed the abundance of *HTT* mRNA.

We observed that *HTT* mRNA could be recovered upon transfection of ss-siRNA 537775 and RIP with anti-AGO2 antibody, but not when we treated with a noncomplementary ss-siRNA (Figure 5B). A locked nucleic acid (LNA) ASO that targets the CAG repeat and inhibits mutant HTT expression with an allele selectivity >6-fold (Hu et al., 2009; Gagnon et al., 2010) did not recruit AGO2 to *HTT* mRNA. The difference between the ss-siRNA and the LNA ASO underlines a fundamental difference in the mechanisms of action: ss-siRNAs rely on AGO2, whereas ASOs do not. Taken together, results from gene silencing and RNA immunoprecipitation support the conclusion that AGO2 is required for the action of ss-siRNA and that silencing proceeds through the endogenous RNAi pathway.

AGO is typically thought to mediate recognition of mRNA inside of cells by dsRNA consisting of a guide strand hybridized to a passenger strand. To determine the functional necessity of passenger strand, we created a heteroduplex by annealing ss-siRNA 537775 to an unmodified RNA passenger strand. This heteroduplex inhibited HTT expression with an IC_{50} of 5.4 nM and a selectivity of >15-fold (Figure 5C), similar to the ss-siRNA alone (IC_{50}: 3.7 nM; selectivity: >29-fold). This result demonstrates that introduction of chemically modified bases into the guide strand does not interfere with strand loading and that ss-siRNA can function through RNAi pathways that were once thought to require a passenger strand. This finding is significant because it shows that ss-siRNA is the only active species during gene silencing and the passenger strand is not necessary. During standard dsRNA-mediated RNAi using unmodified RNA duplex, the main role of the passenger strand is likely to protect the guide strand from digestion by nucleases, ensuring that it survives long enough to reach its target mRNA.

Inhibitory ss-siRNAs Do Not Reduce *HTT* mRNA Levels

siRNAs that are fully complementary to their target mRNAs are usually thought to cause AGO2-mediated mRNA cleavage and reduction of mRNA levels. The introduction of centrally located mismatches is predicted to interfere with strand cleavage without affecting binding (Wang et al., 2008). To test this hypothesis, we measured RNA levels by quantitative PCR (q-PCR) (Figure 5D). A duplex siRNA that targets a sequence outside of the CAG repeat reduces *HTT* mRNA levels by >80%. By contrast, 5′-(E)-vinylphosphonate ss-siRNA 537775 and 5′-phosphate ss-siRNA 553822 that contain mismatches at position P9 do not reduce RNA levels. This result is consistent with a mechanism that involves blocking protein translation rather than degradation of mRNA.

We also examined the potential for cleavage using an in vitro assay combining purified AGO2 or RNase H, different ss-siRNAs, and an in-vitro-transcribed *HTT* mRNA transcript containing 17 CAG repeats. ss-siRNAs 537775, 556887, 553822, and 553819, all potent and selective inhibitors inside of cells, did not lead to transcript cleavage (Figure 5E). By contrast, a control duplex RNA targeting a non-CAG sequence yielded cleavage products of the expected size. A control DNA oligonucleotide yielded cleavage upon addition of RNase H. These data demonstrate that ss-siRNAs targeting the CAG repeat do not cause RNA cleavage through the RNAi or RNase H pathways.

Inhibition by ss-siRNAs Is Cooperative

The repetitive region within a mutant *HTT* mRNA with 69 repeats is predicted to bind up to 9–10 twenty-base-long oligomers. A wild-type mRNA with 17 repeats, by contrast, can bind no more than 2. It is possible that binding of multiple oligomers at adjacent sites can lead to cooperative inhibition and contribute to allele-selective recognition of expanded mutant repeat regions. To determine whether inhibition is cooperative, we examined inhibition of mutant HTT by ss-siRNA 553822 over a wide range of concentrations (Figure 5F). After fitting the data to the Hill equation, we calculated Hill coefficients (n^h) of 2.2 and 1.2 for inhibition of mutant and wild-type HTT expression, respectively. These data are consistent with cooperative inhibition and suggest that association of the ss-siRNA with the expanded mutant repeat is likely to involve multiple binding events.

An ss-siRNA Is an Allele-Selective Inhibitor in HD Model Mice

To test ss-siRNAs in animals, we used $Hdh^{Q150/Q7}$ heterozygous knockin HD model mice (Lin et al., 2001). The $Hdh^{Q150/Q7}$ heterozygous mice carry one mouse huntingtin allele with 150 CAG

(C) Western analysis of inhibition of HTT expression by ss-siRNA 556888 containing a mismatch at P6.
(D) Western analysis of inhibition of HTT expression by ss-siRNA 553822 (mismatched base at P9) in 44-CAG-repeat GM04719 cells.
(E) Effect of ss-siRNA 537775 on other genes containing trinucleotide repeats.
MM, an RNA containing multiple mismatches. Western analysis for (A)–(C) is representative data from three or more experiments, and error bars are SEM.

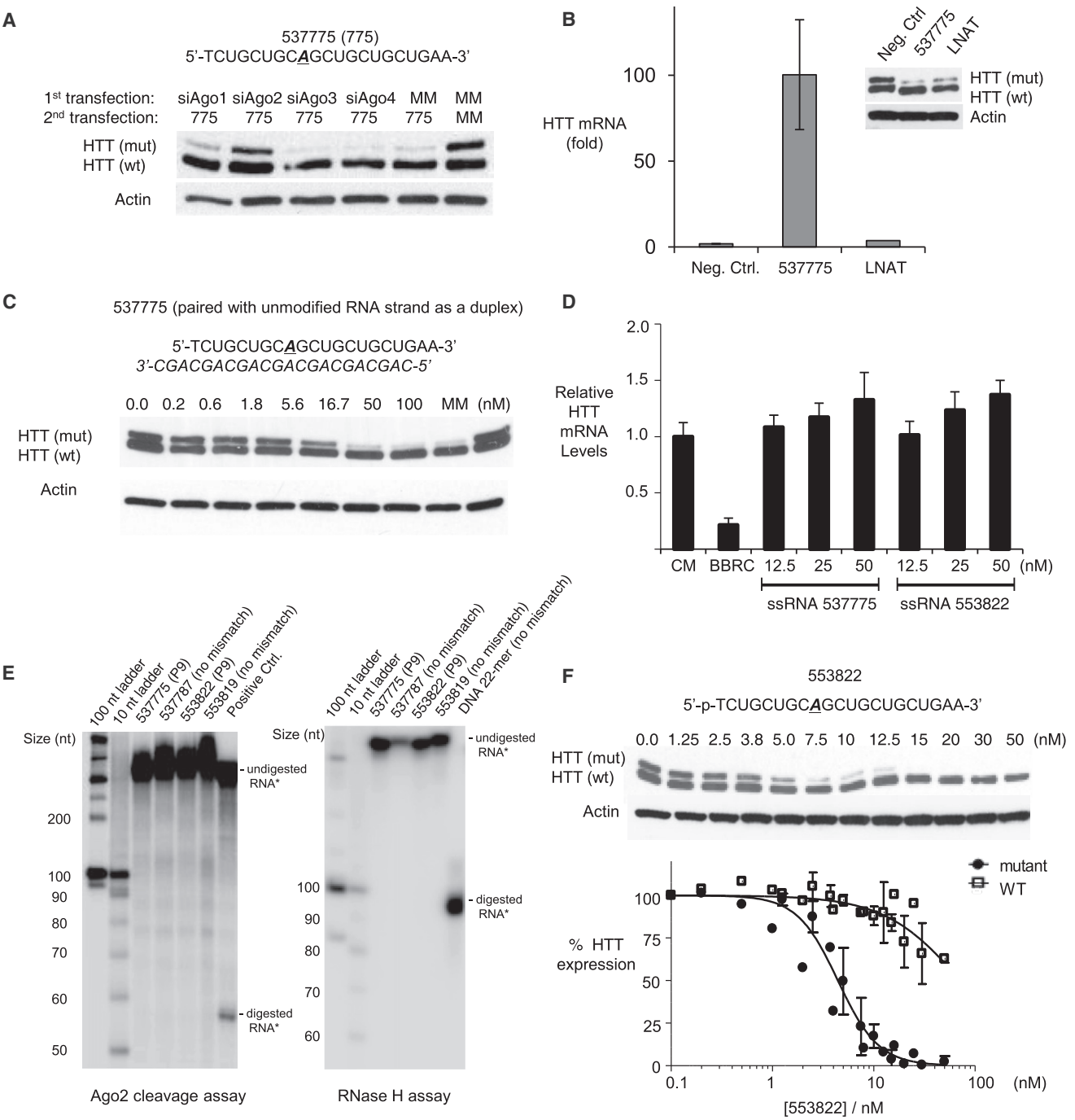

Figure 5. Mechanism of Allele-Selective Inhibition of HTT by ss-siRNA

(A) Western analysis of the effect of siRNA-mediated reduction of AGO1-4 expression on allele-selective inhibition by ss-siRNA 537775.

(B) RNA immunoprecipitation (RIP) using anti-AGO2 antibody after transfection of ss-siRNA 537775, a control ss-siRNA 522247 not targeting HTT, or an allele-selective single-stranded ASO (LNAT) (Hu et al., 2009) at 25 nM. y axis measures fold enrichment of *HTT* mRNA of anti-AGO2 versus IgG pull-down.

(C) Western analysis of inhibition of HTT expression by ss-siRNA 537775 in complex with a complementary unmodified RNA.

(D) Effect of ss-siRNAs 537775 or 553822 on levels of *HTT* mRNA evaluated by q-PCR.

(E) In vitro assays using recombinant RNase H and Ago2 proteins do not show efficient substrate cleavage by ss-siRNA.

(F) Primary data and Hill plot used for determining cooperativity of HTT inhibition by ss-siRNA 553822. x axis shows ss-siRNA concentration in logarithmic scale. Hill's coefficient (n^h) is 2.2 ± 0.3 for mutant HTT and 1.2 ± 0.2 for wild-type HTT.

Error bars from western quantitation and RIP are SEM from three or more independent experiments, and error bars on *HTT* mRNA levels are standard deviations (SD) from replicate data.

See also Figure S2.

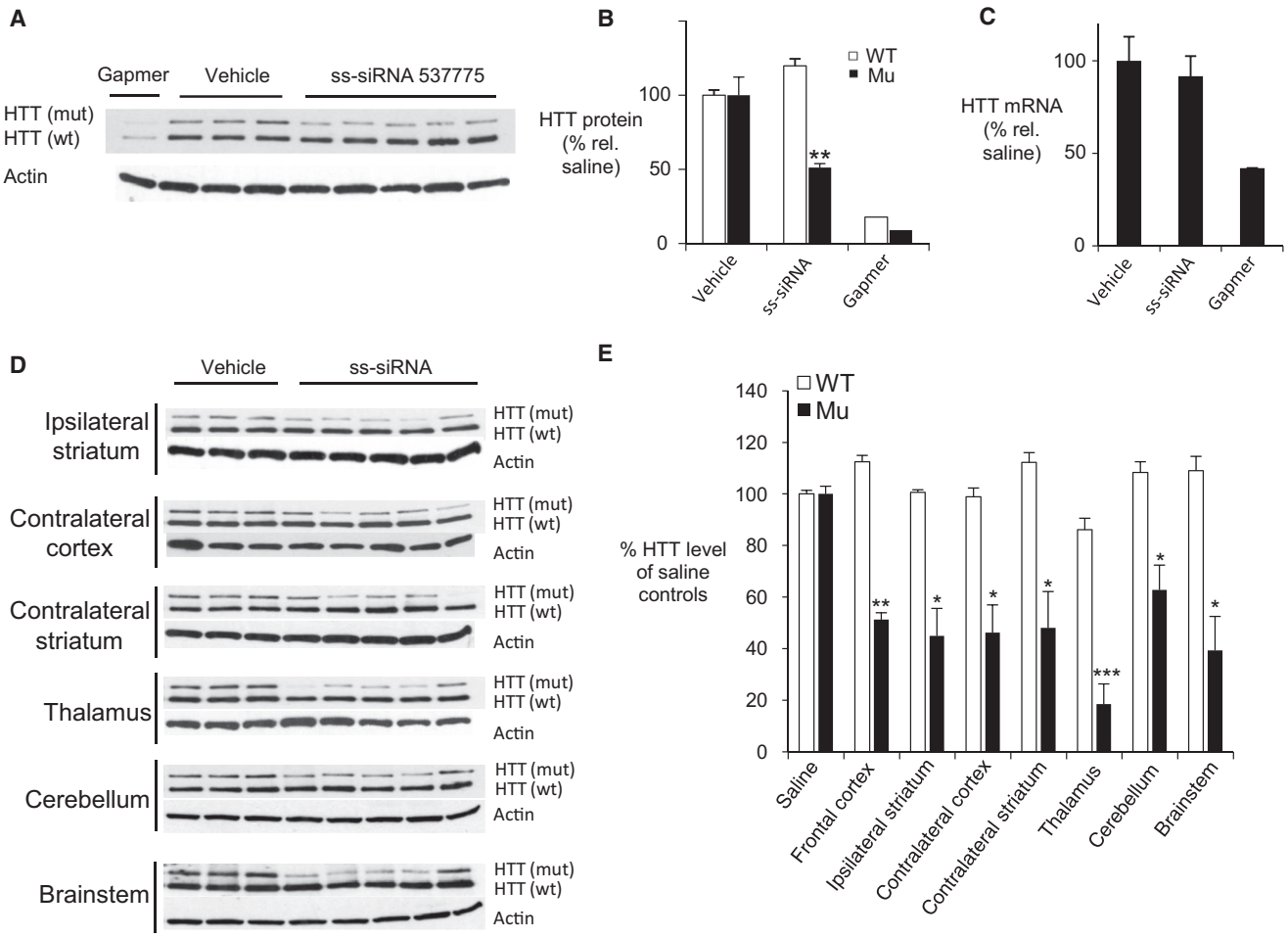

Figure 6. Allele-Selective Inhibition of HTT by ss-siRNA in HdhQ150 HD Mouse Model

(A) Western analysis of HTT expression on allele-selective inhibition by ss-siRNA 537775 (n = 5) in *Hdh*$^{Q150/Q7}$ mouse frontal cortex.

(B) Quantitation of wild-type and mutant HTT protein levels shown in (A).

(C) q-PCR analysis of *HTT* mRNA levels in mouse frontal cortex after treatment with ss-siRNA, vehicle, or control gapmer ASO.

(D) Western analysis HTT expression after allele-selective inhibition by ss-siRNA 537773 (n = 5) in different brain regions.

(E) Quantitation of western analysis from (D). Results from each treatment group/brain section were averaged.

Error bars represent SEM after averaging quantitation results from multiple gel images. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

See also Figure S3.

repeats knocked into exon 1 (Q150) and a second allele with a wild-type mouse huntingtin gene (Q7). The two *HTT* alleles in the *Hdh*$^{Q150/Q7}$ animals differ only in the length of the CAG repeat, making them ideal for determining whether an ss-siRNA can discriminate between the expanded and wild-type huntingtin transcripts in vivo.

To best mimic the human treatment paradigm, 5′-(E)-vinylphosphonate ss-siRNA 537775 was introduced into the cerebral spinal fluid of the right lateral ventricle to achieve distribution throughout the CNS, including brain regions implicated in HD pathology. ss-siRNA 537775 was continuously infused into the right lateral ventricle for 28 days (300 μg/day). Due to the long in vivo half-life of the huntingtin protein and the need to monitor protein levels rather than RNA, animals were treated for 4 weeks to ensure that reduced huntingtin synthesis could be detected.

We analyzed brain tissue for HTT expression by western analysis and q-PCR. As a positive control, we used a nonallele-selective gapmer ASO complementary to a region outside the CAG repeat (administered at 75 μg/day for 14 days). We observed allele-selective inhibition of HTT protein expression in the frontal cortex of all five mice in the experimental cohort relative to animals treated with saline (Figures 6A and 6B). q-PCR showed no decrease in *HTT* mRNA levels in animals treated with ss-siRNA (Figure 6C), consistent with results in cultured cells showing that inhibition does not result from cleavage of mRNA.

We then assayed inhibition in other brain regions, including contralateral cortex, thalamus, ipsilateral striatum, contralateral striatum, cerebellum, and brainstem, all of which displayed a reduction in levels of the mutant HTT protein when treated with ss-siRNA 537775 (Figures 6D and 6E). Consistent with our

results in cultured cells, injection of ss-siRNA 537775 did not reduce expression of other proteins containing CAG trinucleotide repeats (Figure S3). These experiments demonstrate that ss-siRNAs can distribute broadly throughout the central nervous system and inhibit mutant HTT expression.

DISCUSSION

Therapeutics that slow or reverse progression of HD are a major unmet medical need. Trinucleotide expansions cause numerous other hereditary diseases (Orr and Zoghbi, 2007), and anti-CAG agents that treat HD might also advance treatments for these conditions. ss-siRNAs combine strengths of dsRNAs and ASOs, and our objective for this study was to determine whether they would provide an alternate starting point for HD drug development. Substantial challenges confront the application of gene silencing strategies to neurological disorders (Sah and Aronin, 2011; Davidson and McCray, 2011), and optimizing the chemical properties of inhibitory molecules for maximal biological effect is a central goal. Our results demonstrate that ss-siRNAs can mimic miRNAs to allele-selectively suppress translation and inhibit mutant HTT expression with potencies and allele selectivities that are at least equal to those possessed by duplex RNAs and ASOs.

More broadly, gene silencing strategies that use synthetic nucleic acids have the potential to provide a new class of clinical agents for treating diseases that are currently incurable or for which current therapies are inadequate (Watts and Corey, 2012). ss-siRNAs provide a starting point for drug development and an additional option for overcoming roadblocks to successful clinical application. For basic research, ss-siRNAs provide a fresh perspective on the mechanism of RNAi.

ss-siRNAs Function through RNAi

miRNAs function through the RNAi pathway, and almost all miRNAs contain mismatched bases relative to their targets. We provide several lines of evidence that mismatch-containing anti-CAG ss-siRNAs also act through RNAi: (1) the maximum selectivity of inhibition by ss-siRNAs (>30-fold) (Figure 3) is much closer to that produced by duplex RNA (>30-fold) (Hu et al., 2010) than to that yielded by the non-RNAi-mediated ASOs (>4- to 8-fold) (Hu et al., 2009; Gagnon et al., 2010); (2) reduction of AGO2, a key RNAi factor, leads to less-efficient silencing of mutant HTT (Figure 5A); (3) addition of ss-siRNA, but not an allele-selective ASO, leads to robust recruitment of AGO2 to *HTT* mRNA (Figure 5B); (4) adding an unmodified RNA guide strand to the ss-siRNA does not affect its activity (Figure 5C); and (5) as observed for dsRNA (Hu et al., 2010), introduction of a mismatch at position 6 within the putative seed sequence for recognition by ss-siRNA largely abolishes inhibition of HTT (Figure 4C).

Action through the RNAi pathway is accompanied by potent inhibition of HTT expression. Several compounds possess IC_{50} values less than 10 nM, and the best ss-siRNAs have potencies that are almost identical to those observed for duplex RNAs (Hu et al., 2010). Easy identification of multiple potent and selective compounds that function through RNAi also has implications for therapeutic development. It is likely that many other compounds, with different mismatch positions or patterns of chemical modifications, will also be active. This large design space is compatible with allele-selective inhibition and provides many options for optimizing drug-like characteristics of ss-siRNAs and their subsequent therapeutic development. If one compound has a toxic effect related to its sequence or chemical composition, numerous other compounds can be developed instead.

Though all data indicate that ss-siRNAs function through the RNAi pathway, knowing how a dsRNA functions will not always fully predict the properties of an analogous ss-siRNA. For example, fully complementary ss-siRNAs were allele-selective inhibitors of HTT expression (Figure 2A), whereas the analogous fully complementary dsRNA was not selective (Hu et al., 2009). In another example, we had previously observed that duplex RNAs with mismatches at positions 9 or 10 are equally potent and selective inhibitors (Hu et al., 2010). ss-siRNAs with mismatches at positions 9 or 10, by contrast, are quite different in potency (Figure 3).

The origin of functional differences between dsRNAs and ss-siRNAs likely lies in the chemical differences between modified and unmodified RNA. ss-siRNAs have 2′-F and 2′-O-methyl sugar modifications, as well as phosphorothioate internucleotide linkages. The 2′ modifications tend to increase affinity, whereas phosphorothioate linkages tend to decrease affinity. The countervailing and sometimes unpredictable effects of these modifications are apparent from our data. Compared to analogous unmodified RNAs, some RNAs have lower melting temperature (T_m) values for association with complementary sequences, whereas others have higher values. In addition, the extensive chemical modification may affect mRNA recognition, AGO loading, and subsequent inhibition of gene expression. Understanding the intrinsic properties and potential of ss-siRNA will be an important goal for future research. Many chemically modified bases exist that can be substituted within ss-siRNAs, and it is likely that chemical optimization of recognition, potency, and selectivity will prove a productive area of investigation.

Mechanism of Allele-Selective Inhibition

We have shown that our anti-CAG ss-siRNAs recruit and require AGO2, suggesting that the first steps of allele-selective inhibition by ss-siRNAs involve recognition of AGO2 and subsequent association with *HTT* mRNA. Mutant and wild-type *HTT* mRNAs both contain CAG repeats, but the lengths of the repeat regions differ. In our most extreme case, selectivity is achieved even though the difference in the number of repeats in GM04719 cells (44 mutant repeats versus 15 wild-type repeats) is only 29. One explanation is that the mutant allele provides more binding sites for the ss-siRNA. For example, a wild-type allele with 20 repeats would have space for no more than 3 ss-siRNAs, whereas a mutant allele with 40 repeats would have space for as many as 6 ss-siRNAs. Our observation of cooperative effects on inhibition of HTT expression supports the conclusion that multiple ss-siRNAs bind to the expanded repeat target and leads to a preference for the mutant over the wild-type transcript. Indeed, the potential for multiple binding of small RNAs to adjacent sites to lead to cooperative gene silencing has been noted previously using expression constructs containing 3′-untranslated regions

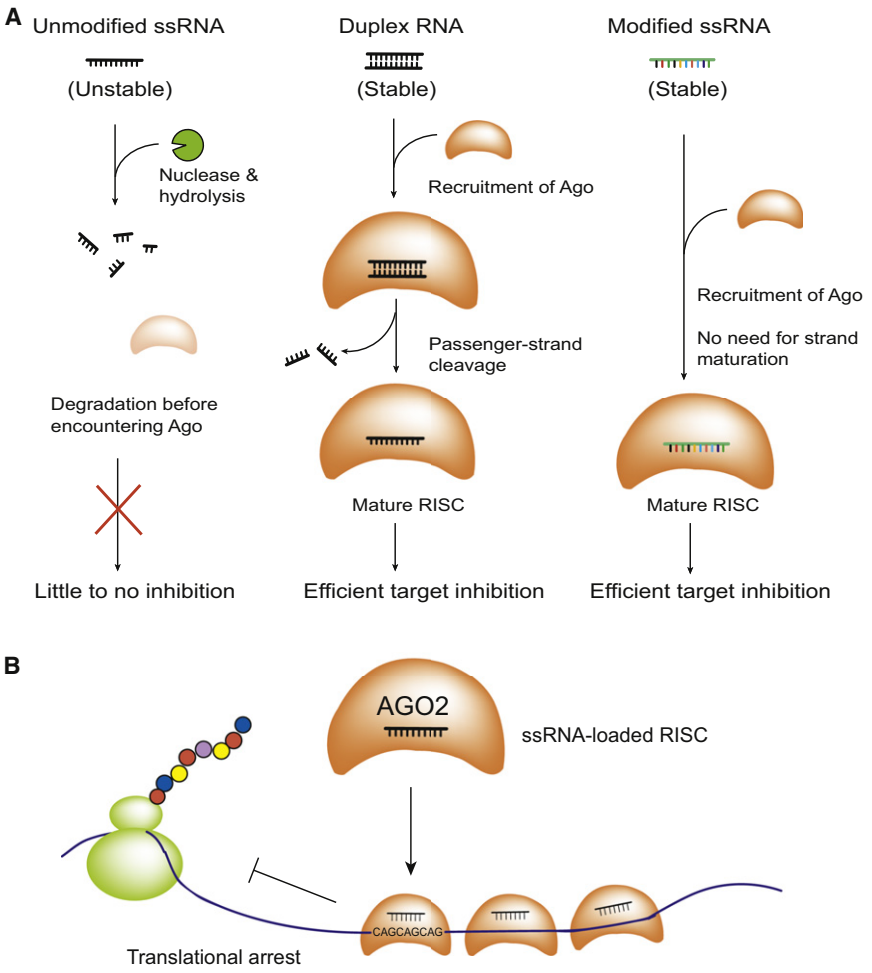

Figure 7. Action of Chemically Modified ss-siRNAs and Allele-Selective Inhibition of HTT

(A) Chemical modifications allow ss-siRNA to be stable and function through the RNAi pathway inside cells.

(B) Binding of multiple anti-CAG ss-siRNA:AGO2 complexes to expanded trinucleotide repeats contributes to allele-selective inhibition.

with varying numbers of target sequences (Broderick et al., 2011).

Once bound, inhibition leads to potent reduction of mutant HTT protein expression but no change in mRNA levels. Inhibition by ss-siRNAs is much more efficient than by analogous unmodified ssRNAs (Figure 2E) and is as efficient as analogous duplex RNAs (Hu et al., 2010). The guide strand, therefore, appears to be the only strand necessary for efficient RNAi (Figure 7A). For experiments with conventional duplex RNAs, the passenger strand serves as a delivery agent protecting the critical guide strand.

For mismatch-containing ss-siRNAs, the observed reduction in protein, but not mRNA, is consistent with our initial design assumptions that mismatches would disrupt AGO-mediated cleavage of mRNA. After multiple ss-siRNAs bind in complex with AGO to the repeat, they likely act as a roadblock to ribosome progress and prevent protein translation (Figure 7B). This "steric blocking mechanism" is similar to that used by ASOs that lack the ability to recruit RNase H and cannot cause cleavage of mRNA except that, in this case, the ss-siRNA is delivered by the endogenous RNAi machinery that has greater potential to facilitate efficient gene silencing. Multiple binding sites within expanded repeats permit cooperative binding and discrimination relative to shorter wild-type repeats.

miRNA versus siRNA Mechanisms

Crooke and coworkers also have reported inhibition of gene expression by ss-siRNAs in cell culture and animals (Lima et al., 2012). Both manuscripts demonstrate that the passenger strand is not necessary for gene silencing, and dispensing with it has the potential to improve the in vivo pharmacology of compounds that function through the RNAi pathway. However, the mechanisms of action differ. Crooke and colleagues use ss-siRNAs that are fully complementary to their target sequences and would be predicted to function through a siRNA-like pathway. Consistent with this expectation, they observe cleavage of target sequence in vitro, 5′-RACE products consistent with AGO2-mediated cleavage of the predicted target site, and reduction of mRNA in cell culture and in vivo.

By contrast, we use mismatch-containing RNAs that resemble miRNAs and have the potential to act through an miRNA-like pathway. Our target is the expanded CAG repeat within mutant *HTT* mRNA, which offers multiple sites for binding. Consistent with action through a mechanism that resembles that used by miRNAs, we do not observe cleavage of substrate in vitro, nor do we observe reduced mRNA levels in cell culture or animals. The observation of ss-siRNA silencing through both the siRNA and miRNA pathways suggests a broad compatibility with RNAi machinery and cellular RNA targets.

Implications for Therapeutic Gene Silencing

Advances in nucleic acid chemistry, a better understanding of nucleic acid pharmacology, and a more mature appreciation of the basic science underlying diseases have led to substantial recent clinical progress for nucleic acid therapeutics (Watts and Corey, 2012). It is now possible to cite several examples of nucleic acid drugs that have potent effects on target gene expression in humans. For example, Mipomersen, a drug designed to treat familial hypercholesterolemia, has been shown to benefit patients in multiple phase III trials and is now awaiting FDA review. Even brain disorders are becoming more amenable

to nucleic acid silencing. ASOs have been shown to inhibit superoxidase dismutase in the spinal cord of primates, and a phase I trial designed to test treatment of patients with familial amyotrophic lateral sclerosis (ALS) is ongoing.

Our results introduce ss-siRNAs as a strategy for treating neurodegenerative disease that provides an alternative to ASOs or dsRNAs. Chemically, ss-siRNAs are similar to ASOs because they both possess a single chemically modified antisense strand. Mechanistically, they resemble duplex RNAs that function through RNAi. ss-siRNAs combine strengths of the two existing approaches, possess unique advantages, and provide a distinctive new strategy for silencing gene expression.

Here, we demonstrate that the first generations of anti-CAG ss-siRNAs achieved potencies and selectivities for inhibiting HTT that are similar to those achieved by well-established gene silencing technologies. By further optimizing the type of chemical modification, placement of mismatched bases, or other design features, it is likely that subsequent generations of inhibitory ss-siRNAs will possess even more favorable properties. The availability of a gene silencing strategy that combines the strengths of siRNAs and ASOs will provide an important option for transforming the potential benefits from nucleic-acid-based silencing into practical benefits for patients.

EXPERIMENTAL PROCEDURES

Cell Culture

ss-siRNAs were synthesized by Isis Pharmaceuticals Inc. (Carlsbad, CA, USA) and reconstituted in nuclease-free water. Patient-derived fibroblast cell lines GM04719 (44 CAG repeat) and GM04281 (69 CAG repeat) were obtained from the Coriell Institute (Camden, NJ, USA) and transfected as described (Hu et al., 2010). Cells were plated in 6-well plates at 60,000 cells/well in supplemented MEM media 2 days prior to transfection. 6-well plates were used to provide the number of cells necessary for western analysis. Cells were transfected using lipid RNAiMAX (Invitrogen) per manufacturer's instructions. Cells were harvested 4 days after transfection for protein analysis and 3 days after transfection for RNA analysis.

Analysis of HTT Protein Expression

SDS-PAGE (separating gel: 5% acrylamide-bisacrylamide [50:1], 450 mM Tris-acetate [pH 8.8]; stacking gel 4% acrylamide-bisacrylamide [50:1], 150 mM Tris-acetate [pH 6.8]) was used to separate wild-type and mutant HTT proteins as described (Hu et al., 2010).

Analysis of *HTT* mRNA Expression

q-PCR was performed as described. Experiments were performed in replicates and error reported as standard deviation (SD). The q-PCR cycles are as follows: 50°C for 2 min; 95°C for 5 min; (95°C for 15 s; 60°C for 1 min) × 40 cycles.

Analysis of AGO2 Binding by RNA Immunoprecipitation

HD-patient-derived GM04281 (69 CAG repeat) fibroblasts were grown in 150 ccm^2 dishes and transfected with chemically modified ss-siRNA 24–48 hr postseeding. Two media changes were done 24 and 72 hr after transfection, and cells (~4×10^7, or six dishes per treatment) were harvested by trypsinization 96 hr after transfection in growth media. A small quantity of cells are saved and harvested for protein to check knockdown efficiency by western blot. RNA immunoprecipitation was performed as described using 4 μg anti-AGO2 (AB) antibody or 4 μg57113, Abcam) normal mouse IgG (12-371, Millipore, for IP) antibody in 0.75 ml of IP buffer at 4°C on rotator for 3–4 hr. Results were normalized by the two following parameters: (1) ratios of *HTT* mRNA to GAPDH mRNA (a housekeeping control) to eliminate small variations of total RNA across all samples; (2) binding of *HTT* mRNA to anti-AGO2 antibodies over that of IgG to measure fold enrichment of *HTT* mRNA in AGO2 IP relative to the nonspecific IgG background binding.

IC_{50}, Selectivity, and Cooperativity Calculations

Protein bands were quantified from autoradiographs using ImageJ software. The percentage of inhibition was calculated as a relative value to a no-treatment control sample. The program GraphPad Prism 4 was used to draw the fitting curves for dose-response experiments. The Hill equation was used for fitting in the following form: $Y = 100[(1 - X^n/(K^n + X^n)]$, wherein Y is percentage of inhibition, X is the ss-siRNA concentration, K is the IC_{50} value, and n is the Hill coefficient. At least three experiment data sets were used for curve fitting. The error is standard error of the mean (SEM), calculated from combining the data of each individual dose curve. Selectivity was calculated by taking the ratio of the IC_{50} for inhibition of the mutant HTT protein over that of the wild-type protein. Cooperativity was measured by obtaining the Hill coefficient that best fits the plotted curve, which corresponds to the value n in the equation.

In Vitro Ago2 Activity Assay

The reaction mixture contained 3 μl of recombinant hAgo2 protein, 3 μl of ss-siRNA (250 nM stock), 0.5 μl of tRNA (10 mg/ml), 0.5 μl of NTP mix (25 mM), 0.25 μl of Superase-IN (Ambion, 10,000 U), and 1 μl of 10× reaction buffer (0.5 M Tris [pH 7.4], 20 mM $MgCl_2$, 5 mM DTT, 2.5 mM ATP, 1 M KCl, 0.5 M NaCl). The mixture was allowed 1.5 hr at room temperature for preloading of ss-siRNAs, after which 50,000 cpm of radio-labeled 17-CAG RNA substrate was added to each reaction. AGO2-cleavage reactions were allowed to proceed for 2–2.5 hr at 37°C and then quenched with $LiClO_4$ in acetone. After centrifugation and acetone wash, the reconstituted RNA was run on 10%–14% sequencing gel and visualized on phosho-imager after overnight exposure in the dark.

Dosing and Surgical Procedure

HdhQ150 (CHL2) animals (Lin et al., 2001) were obtained from Jackson laboratories and maintained on the congenic C57BL/6 background. To continuously deliver compounds, osmotic pumps delivering 0.25 μl/hr (Model 2004) were used to deliver 300 μg/day of ss-siRNA or phosphate-buffered saline (PBS) (Sigma Aldrich) for 28 days, and pumps designed to deliver 0.5 μl/hr (Model 2002) were used to deliver 75 μg/day of the positive control MOE ASO for 14 days. Pumps (Durect Corporation) were filled with ss-siRNA or MOE diluted in sterile PBS and then incubated at 37°C for 24 or 48 (Model 2004) hours prior to implantation. Mice were anesthetized with 2.5% isofluorane, and a midline incision was made at the base of the skull. Using stereotaxic guides, a cannula was implanted into the right lateral ventricle and secured with Loctite adhesive. A catheter attached to an Alzet osmotic mini pump was attached to the cannula, and the pump was placed subcutaneously in the midscapular area. The incision was closed with 5.0 nylon sutures. Animals were sacrificed 4 weeks after initiating treatment. Brains were sectioned into 1–2 mm coronal sections and frozen on dry ice and stored at −80°C. Brain regions were harvested for RNA and biochemical analysis using 2 mm punches.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.08.002.

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