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Antisense and Antigene Inhibition of Gene Expression by Cell-Permeable Oligonucleotide−Oligospermine Conjugates

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Supporting Information

ABSTRACT: Oligonucleotides and their derivatives are a proven chemical strategy for modulating gene expression. However, their negative charge remains a challenge for delivery and target recognition inside cells. Here we show that oligonucleotide−oligospermine conjugates (Zip nucleic acids or ZNAs) can help overcome these shortcomings by serving as effective antisense and antigene agents. Conjugates containing DNA and locked nucleic acid (LNA) oligonucleotides are active, and oligospermine conjugation facilitates carrier-free cell uptake at nanomolar concentrations. Conjugates targeting the CAG triplet repeat within huntingtin (HTT) mRNA selectively inhibit expression of the mutant huntingtin protein. Conjugates targeting the promoter of the progesterone receptor (PR) function as antigene agents to block PR expression. These observations support further investigation of ZNA conjugates as gene silencing agents.

Inhibition of gene expression by oligonucleotides is an area of intense interest for both basic research and therapeutics. One of the primary challenges confronting efforts to develop improved oligonucleotides is their polyanionic nature. Negative charge complicates uptake through cell membranes, and there is a significant electrostatic penalty inherent to recognition of target nucleic acids, particularly when the target sequence is already base paired (e.g., structured RNA or duplex DNA).

One approach to overcoming these challenges is to conjugate a cationic group to either the 3′ or 5′ termini of oligonucleotides.[1] Such conjugation can alter the physical properties of the oligonucleotides while maintaining specific binding to complementary sequences. The ability of oligonucleotide−cation conjugates to improve hybridization in a cell-free system has been well documented.[2,3] However, it is more difficult to show improved recognition of DNA and RNA targets inside cells.

Oligonucleotide−oligospermine conjugates (Zip Nucleic Acids, ZNAs) are synthesized using phosphoramidate chemistry to build the cationic tail.[4] Direct attachment of cationic charge neutralizes the negatively charged backone and improves binding affinity and binding kinetics. Improved hybridization makes ZNAs promising probes for PCR.[5] The attached positive charge can facilitate cellular uptake and siRNAs bearing an oligospermine tail silence gene expression.[6]

We have synthesized oligonucleotides with 5′-oligospermine tails and tested them as antisense agents for inhibition of human huntingtin (HTT) and antigene agents for blocking transcription of the human progesterone receptor (PR). We have tested ZNAs against two types of cellular nucleic acid, mRNA (antisense approach) and chromosomal DNA (antigene approach).

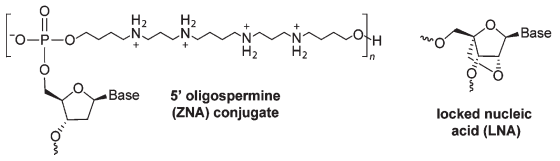

Figure 1. Chemical structure of a 5′ oligospermine (ZNA) conjugate and locked nucleic acid (LNA) nucleoside.

The oligonucleotide domains of these conjugates were either DNA or a mixture of locked nucleic acid (LNA) and DNA (Figure 1). LNA is a modified nucleotide containing a methylene linkage between the 2′ and 4′ positions of the ribose ring.[7] Introducing LNA bases can increase T_m values as much as 5−9 °C per substitution.[8]

All oligonucleotides were 19 bases long, contained oligospermine domains with 3-, 6-, or 9-spermine units, and possessed formal charges of −9, 0, or +9, respectively. These spermine conjugates were less soluble in water than unconjugated oligonucleotides, probably because reducing the overall charge increases aggregation. We overcame these solubility problems by dissolving ZNAs in concentrated phosphate buffered saline (2.5× PBS, pH 7.4, ∼350 mM salt).

We have previously reported inhibition of HTT expression by antisense oligomers targeted to the CAG repeat within HTT mRNA.[9,10] Mutant HTT causes Huntington's Disease (HD), an incurable neurodegenerative disorder.[11] A normal HTT gene possesses a repeat region containing no more than 36 CAG trinucleotides, while HD patients have an allele with greater than 37 repeats.[12] Agents that selectively inhibit expression of mutant HTT while leaving the wild-type allele unaffected represent a promising therapeutic strategy.[13] Our previous studies identified oligonucleotides that could take advantage of the relatively small difference in repeat length between the normal and mutant alleles to selectively reduce expression of mutant HTT. Advancing these lead compounds into the clinic, however, will benefit from discovery of more potent or cell permeable compounds.

We first introduced oligonucleotides into fibroblasts derived from HD patients by transfection with a cationic lipid (Figure 2A−D and Supporting Figure S1). A DNA oligonucleotide

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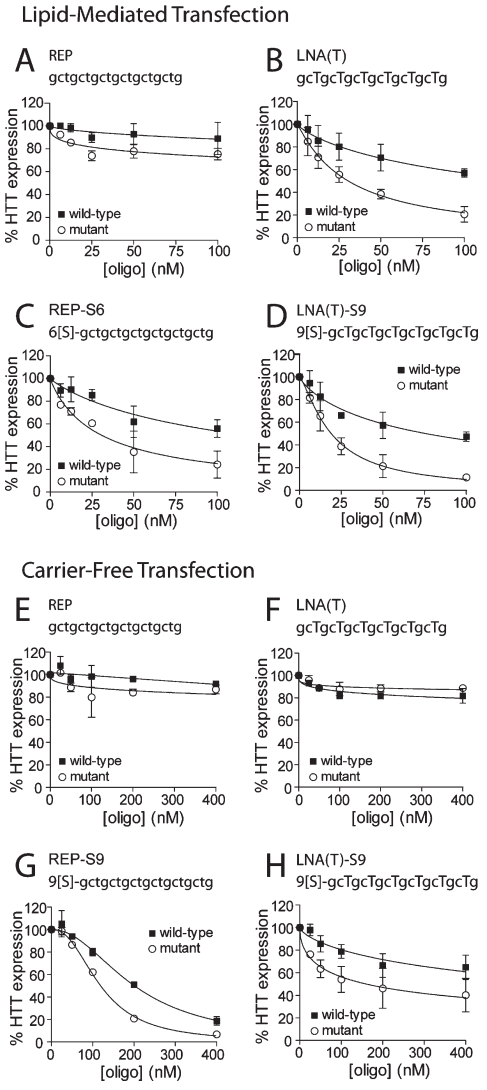

Figure 2. Allele-selective inhibition of mutant HTT expression with oligospermine-conjugated DNA and LNA. HTT protein expression in patient-derived fibroblasts in response to oligonucleotide or oligonucleotide-spermine conjugates transfected with lipid-based reagent (A–D) or without a carrier (E–H). Error bars are standard error of the mean (SEM). DNA nucleotides are lower case; LNA are upper case.

lacking the spermine domain (REP) was inactive, while an LNA [LNA(T)] selectively inhibited mutant HTT with an IC_{50} value of 30.5 nM (Table 1), similar to what we have observed previously.[9,10]

In contrast to the inactivity of the unmodified DNA, addition of spermines conferred allele-selective inhibition to the DNA conjugate. Conjugation of six spermines to DNA (REP-S6) yielded excellent inhibition (IC_{50} of 30.8 nM) as well as allele selectivity similar to that achieved with unconjugated LNA(T). Conjugation of spermines to LNA(T) improved inhibition of mutant HTT by lowering IC_{50} values to 16.4 and 19.1 nM for 3 or 9 spermines, respectively (Table 1).

Cellular uptake in the absence of lipid would make laboratory experiments more straightforward and increase the potential for potent effects in animals. To test conjugates without transfection reagents, we added conjugates directly to fibroblasts (Figure 2E-H and Supporting Figure S2). The unconjugated DNA and LNA

Table 1. Antisense ZNA Oligonucleotides

		IC_{50} (nM) (+) lipid		IC_{50} (nM) (−) lipid	
Name	Sequence (5′−3′)[a]	wt	mut	wt	mut
REP	gctgctgctgctgctgctg	>100	>100	>400	>400
REP-S3	[S3]-gctgctgctgctgctgctg	>100	>100	>400	>400
REP-S6	[S6]-gctgctgctgctgctgctg	>100	30.8	313	194
REP-S9	[S9]-gctgctgctgctgctgctg	>100	81.9	201	119
LNA(T)	gcTgcTgcTgcTgcTgcTg	>100	30.5	>400	>400
LNA(T)-S3	[S3]-gcTgcTgcTgcTgcTgcTg	61.2	16.4	>400	>400
LNA(T)-S6	[S6]-gcTgcTgcTgcTgcTgcTg	>100	30.2	>400	>400
LNA(T)-S9	[S9]-gcTgcTgcTgcTgcTgcTg	75.9	19.1	>400	160

[a] DNA nucleotides are lowercase; LNA nucleotides are uppercase. Number of spermines conjugated to the 5′-end is indicated in square brackets (e.g., [S3]).

oligonucleotides did not alter gene expression in the absence of lipid. In contrast, we observed inhibition when 6 or 9 spermines were attached. Nine spermine groups gave the best potencies (Table 1). Inhibition correlated with the number of spermines suggesting that attached spermine enhances cellular uptake and/or binding to the RNA target. Scrambled sequence ZNAs were not active demonstrating that inhibition was sequence-selective (Supporting Figure S3).

When DNA binds to mRNA a DNA−RNA hybrid is formed that can be cleaved by RNase H. The observation of HTT inhibition with DNA-oligospermine conjugates prompted us to investigate whether they supported RNase H activity (Figure 3A). We incubated oligonucleotides with RNase H and a 5′-radiolabeled RNA (REP69) containing the CAG repeat expansion and observed cleavage of the RNA substrate. Digestion products resolved on a denaturing polyacrylamide gel revealed robust induction of RNase H activity for DNA-spermine conjugates. LNA(T) contains evenly spaced LNA modifications which prevent efficient RNase H cleavage of hybridized target RNA,[10] and spermine conjugation did not affect this property.[14]

To determine if DNA-based ZNAs were directing RNase H cleavage of HTT mRNA inside cells, we performed qPCR on HTT mRNA after lipid-mediated transfection of oligonucleotide-spermine conjugates (Figure 3B). In contrast to results from cell-free assays, HTT mRNA levels were not substantially altered when anti-HTT oligospermine conjugates were introduced into cells. We conclude that spermine conjugation can support RNase H cleavage. However, mRNA cleavage does not appear to be the primary mechanism of HTT inhibition inside cells.

To generalize the effects of ZNAs inside cells we targeted a chromosomal sequence within a gene promoter. Adding cationic groups enhances recognition of duplex DNA in cell free assays and might also improve binding to chromosomal targets.[15] We have previously demonstrated that LNA or PNA oligomers complementary to the progesterone receptor (PR) promoter can inhibit transcription inside cells.[16] We tested ZNA analogues of both antigene DNA (agDNA) and LNA (agLNA) for their ability to inhibit PR expression (Table 2 and Figure 4). Two isoforms of PR, termed PR-A and PR-B, are expressed from the same gene and are visible as separate bands by Western blot.

The LNA−oligospermine conjugates inhibited PR expression, and the efficiency of inhibition decreased with increasing oligospermine tail length (Figure 4A and Table 2). We were surprised that attaching the ZNA tail caused a drop in efficacy,

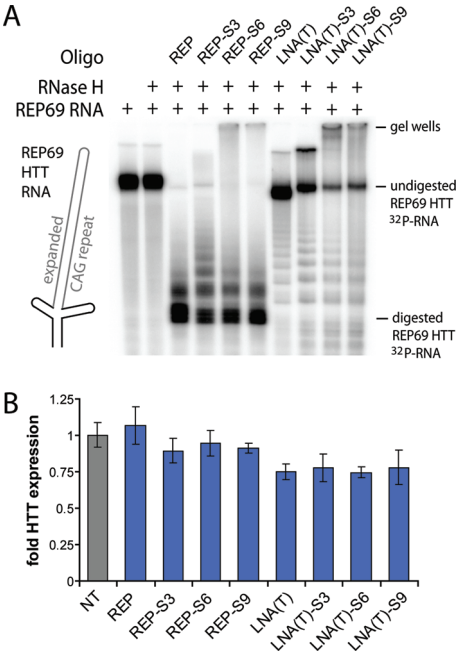

Figure 3. Potential for ZNAs to induce RNAse H activity. (A) RNase H digestion of radiolabeled substrate upon treatment with ZNAs. (B) Effect on target RNA levels of adding ZNAs (50 nM) to cells evaluated by real time quantitative PCR. Error bars are standard deviation. NT: not transfected.

especially since the positively charged tail was expected to aid in strand invasion and binding. The spermine tail may reduce uptake into the nucleus or interfere with other steps unique to targeting nuclear DNA.[17] DNA-based conjugates gave no inhibition.

To explore whether ZNAs could be cell-permeable antigene agents, we treated cells with ZNAs at 500 nM. There have been reports that unconjugated oligonucleotides can enter cells at high concentrations without lipid transfection.[18] We therefore also added 500 nM unconjugated LNA as a reference. Neither the unconjugated LNA nor the DNA conjugate showed significant gene inhibition (Figure 4B). In contrast, the agLNA−S9 conjugate showed about 40% inhibition, demonstrating that antigene inhibition can be achieved in the absence of lipid transfection reagents.

We observed substantial inhibition (>30%) upon treatment of cells with unconjugated agLNA complexed with spermine (Figure 4B). In contrast, for antisense inhibition of HTT, benchmark compound LNA(T) was inactive in the presence of unconjugated spermine (Table 1, Figure 2, Supporting Figure S3C). Differences between the antigene and antisense systems might explain why free spermine is partially active in the one case and not active in the other: we are using different cell lines (T47D cells vs fibroblasts), targeting different compartments (nucleus vs cytoplasm), and using different transfection protocols (reverse transfection, in which dissociated cells are added to the transfection solution, vs forward transfection, in which the transfection solution is added onto plated cells). While spermine has often been used as a component of transfection reagents, it has not been found to be a high-efficiency transfection reagent on its own.[19]

We carried out an MTS assay to evaluate cell viability after addition of antisense or antigene ZNAs (Supporting Figure S4). For the antisense series of ZNAs targeting HTT, no changes in cell growth were observed after treatment with 100 nM oligonucleotide in the presence of lipid. For lipid-free transfections at

Table 2. Antigene ZNA Oligonucleotides

Name	Sequence (5′−3′)[a]	% inhib 50 nM, (+) lipid[b]	% inhib 500 nM, (−) lipid[b]
agDNA-S3	[S3]-tgtctggccagtccacagc	n.i.	
agDNA-S6	[S6]-tgtctggccagtccacagc	n.i.	
agDNA-S9	[S9]-tgtctggccagtccacagc	n.i.	n.i.
agLNA	tGtctGGccAGtccAcAGc	86 ± 9	9 ± 4
agLNA-S3	[S3]-tGtctGGccAGtccAcAGc	70 ± 3	
agLNA-S6	[S6]-tGtctGGccAGtccAcAGc	65 ± 1	
agLNA-S9	[S9]-tGtctGGccAGtccAcAGc	45 ± 3	39 ± 8

[a] DNA nucleotides are lowercase; LNA nucleotides are uppercase. Number of spermines conjugated to the 5′-end is indicated in square brackets (e.g., [S3]). [b] n.i., no inhibition.

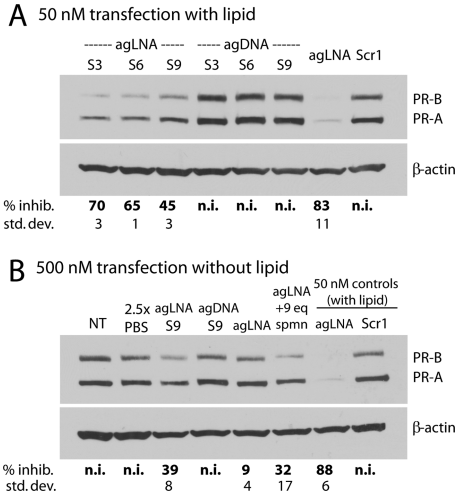

Figure 4. Transcriptional silencing by antigene ZNAs. PR protein expression treatment in (A) the presence or (B) the absence of cationic lipid. NT, nontransfected; n.i., no inhibition; spmn, spermine; Scr1, scrambled control of sequence cCacaGCtgTCcagTtGGc.

400 nM, conjugates LNA(T)−S6 and REP−S9 were the only compounds of this series to cause reduced cell proliferation.

For the antigene series, PR inhibition experiments are carried out using reverse transfection (lipid/ZNA added before plating cells, see methods). We measured effects on cell proliferation using both forward and reverse transfection protocols. For 50 nM oligonucleotide transfections in the presence of lipid, we observed up to a 70% loss in cell number, with effects varying depending on how the transfection was performed. We had previously observed that inhibition of PR expression reduces cell proliferation.[16a] Indeed, reduced cell proliferation correlated with the potency of inhibiting PR expression, and the unconjugated agLNA itself reduced cell numbers the most. Therefore, no spermine-related toxicity was observed. When 500 nM ZNA was added in the absence of lipid we observed reduced cell growth related to spermine tail length and independent of PR inhibition (both agLNA−S9 and agDNA−S9 reduced cell viability by about 40%). While high doses of oligospermine conjugates can slow cell growth, ZNAs can be used to control gene expression at concentrations that do not affect cell viability.

Previous results describing ZNA conjugates showed that the oligospermine tail increased binding affinity in a sequence-independent

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manner.[3] We characterized the effect of ZNA modification on melting temperature (T_m) using complementary sequences of exactly the same length (19 bases) or longer (39−41 bases). We observed a small but linear T_m increase upon oligospermine conjugation for all sequences (0.3 to 0.7 °C/spermine, Supporting Figure S5).

For the antisense oligomers, we carried out T_m experiments with both RNA and DNA target strands. While free spermine can bind to both B-form and A-form helical structures,[20,21] its interactions with the A-form helix are more energetically favorable.[20] Accordingly, oligospermine conjugation increased binding affinity toward RNA target strands more than DNA target strands (0.7 °C/spermine for RNA vs 0.3 °C/spermine for DNA; Figure S5D,E). This suggests that the stabilizing effect conferred by oligospermine conjugation is not simply due to charge neutralization, but contains a structural component.

The increased binding affinity is lower than previously reported.[3] Anti-CAG oligonucleotides form hairpins[10] and the oligospermine tail may bind the hairpin, reducing the net advantage of intermolecular binding. We determined T_m values for the anti-HTT oligonucleotides and observed that increasing oligospermine length increased T_m (Supporting Figure S5B).

In conclusion, we have shown that oligospermine-oligonucleotide conjugates (ZNA) are promising antisense and antigene agents. Their properties can be tuned by using sugar-modified backbones (e.g., LNA) and varying the oligospermine length. A nonfunctional DNA antisense oligonucleotide became an effective and selective inhibitor of mutant HTT protein after oligospermine conjugation. Spermine conjugation also aids in cellular uptake, giving IC_{50} values in the midnanomolar range in the absence of lipid. ZNA oligonucleotides warrant further development and optimization as gene silencing agents.

■ ASSOCIATED CONTENT

Supporting Information. Proliferation data, further data on HTT inhibition by ZNA oligos, all experimental methods, complete ref 12. This material is available free of charge via the Internet at http://pubs.acs.org.

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