Implications of High-Affinity Hybridization by Locked Nucleic Acid Oligomers for Inhibition of Human Telomerase†

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ABSTRACT: Oligonucleotides that contain locked nucleic acid (LNA) bases have remarkably high affinity for complementary RNA and DNA sequences. This increased affinity may facilitate the recognition of nucleic acid targets inside cells and thus improve our ability to use synthetic oligonucleotides for controlling cellular processes. Here we test the hypothesis that LNAs offer advantages for inhibiting human telomerase, a ribonucleoprotein that is critical for tumor cell proliferation. We observe that LNAs complementary to the telomerase RNA template are potent and selective inhibitors of human telomerase. LNAs can be introduced into cultured tumor cells using cationic lipid, with diffuse uptake throughout the cell. Transfected LNAs effectively inhibited intracellular telomerase activity up to 40 h post-transfection. Shorter LNAs of eight bases in length are also effective inhibitors of human telomerase. The melting temperatures of these LNAs for complementary sequences are superior to those of analogous peptide nucleic acid oligomers, emphasizing the value of LNA bases for high-affinity recognition. These results demonstrate that high-affinity binding by LNAs can be exploited for superior recognition of an intracellular target.

Nucleic acid recognition is fundamental to applications ranging from gene expression arrays to antisense inhibition of gene expression (*1*). Even small improvements in the chemical properties of oligonucleotides and oligonucleotide mimics could lead to breakthrough developments in biotechnology and medicine. Recognizing this challenge, chemists have designed and synthesized a large variety of novel nucleic acids and nucleic acid mimics to enhance the affinity and specificity of hybridization (*2*).

Recently, the Wengel (*3*) and Imanishi (*4*) laboratories described the synthesis and hybridization of a novel nucleotide termed locked nucleic acid (LNA,[1] also known as bridged nucleic acid, BNA), with a subsequent report from Wang (*5*). LNA monomers contain a methylene bridge that connects the 2′-oxygen with the 4′-carbon of the ribose ring of RNA (Figure 1). This bridge results in a locked 3′-*endo* conformation, reducing the conformational flexibility of the ribose and increasing the degree of local organization of the phosphate backbone (*6*, *7*). This entropic constraint leads to improved binding to complementary RNA and DNA sequences, with a single LNA substitution increasing T_m values by as much as 10 °C (*3*, *4*; reviewed in refs *8* and *9*). LNAs also exhibit enhanced triplex formation (*10*, *11*), show evidence of antisense efficacy and low toxicity when injected into animals (*12*), and inhibit Tat-dependent transactivation

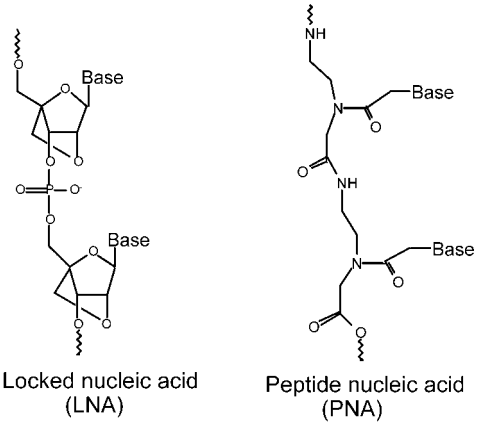

FIGURE 1: Structures of LNA and PNA.

(*13*). LNA bases can be interspersed with DNA bases, allowing binding affinity to be tailored for individual applications. The ability of LNA bases to confer dramatic increases in hybridization affinity suggests that they have great potential for optimizing nucleic acid recognition. Here we test this hypothesis by correlating high-affinity binding by LNAs with their ability to inhibit an important physiological RNA target, human telomerase.

Telomerase is a ribonucleoprotein and is responsible for maintaining telomere length by adding the repeated sequence TTAGGG to the ends of eukaryotic telomeres (*14*). Telomerase contains a reverse transcriptase domain (hTERT) that is responsible for catalyzing nucleotide addition (*15*, *16*), and an RNA domain (hTR) that provides a template for polymerization (*17*). Numerous studies have shown that telomerase activity is expressed in cancer cells but is absent from adjacent noncancerous cells (*18*). This correlation

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[1] Abbreviations: PNA, peptide nucleic acid; LNA, locked nucleic acid; TRAP, telomere repeat amplification protocol; PS, phosphorothioate.

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suggests that telomerase must be reactivated for sustained tumor proliferation and that telomerase inhibitors may provide a new approach to chemotherapy (*19*, *20*).

The RNA component of telomerase contains an 11-base sequence that binds telomeric DNA and guides the addition of six-base telomeric repeats (*17*). This template sequence is an excellent target for inhibition by oligonucleotides because its role in telomerase activity depends on it being accessible to hybridization by the telomere (*20*). Thus, unlike most other cellular RNA targets that are obstructed by RNA secondary structure, the template of hTR is relatively open to hybridization by synthetic oligonucleotides and oligonucleotide mimics because of the need for it to bind the telomere during normal function. Previously, we have shown that 13-base peptide nucleic acid (PNA) (*21*, *22*), 2′-*O*-methyl-RNA (*21*), and 2′-*O*-methoxyethyl-RNA (*23*) oligomers are potent inhibitors. PNAs and 2′-*O*-methyl-RNA introduced into cells cause telomere erosion, reduce cell proliferation, and lead to cell death (*21*, *22*). Gryaznov and colleagues have reported similar results with thiophosphoramidate DNA oligonucleotides (*24*). Inhibition was sharply dependent on the length of the oligomer; when eight-base oligomers were tested, the potency of inhibition decreased by 1000-fold relative to those of 13-base inhibitors (*25*).

We reasoned that the potential for high-affinity hybridization by LNAs and LNA−DNA chimera might allow them to be highly effective inhibitors of telomerase. Here we test this hypothesis and determine how high-affinity binding, the ratio of DNA and LNA bases, oligomer length, and phosphorothioate substitution affect the specificity and efficiency of inhibition. We find that LNAs are potent and selective telomerase inhibitors in cell extracts and inside cells, and that even relatively short LNAs are capable of inhibiting telomerase activity within cultured cells.

MATERIALS AND METHODS

Oligomer Synthesis. All oligomers containing LNA bases were obtained from Proligo LLC (Boulder, CO). LNAs and an LNA−DNA chimera were solubilized in certified DNase/RNase-free water (Invitrogen, Carlsbad, CA). Two MicroSpin G-25 Sephadex columns (Amersham Pharmacia Biotech, Piscataway, NJ) were prepared for each LNA or an LNA−DNA chimera to remove contaminants that might be toxic to cultured cells. Columns were equilibrated in DNase/RNase-free water. Solutions containing LNAs and an LNA−DNA chimera were heated at 65 °C for 15 min to dissociate aggregates and then applied to the desalting columns. PNAs were synthesized, purified by C-18 reverse phase HPLC, and analyzed by matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry on site as described previously (*26*).

Determination of Melting Temperatures. Samples for melting temperatures were prepared using an oligomer (100 μM stock concentration) and its respective complement (100 μM stock concentration) in 2.5× PBS [phosphate-buffered saline (pH 7.4) without calcium or magnesium chloride (Invitrogen)]. The sample was prepared using 5 μL of oligomers and 145 μL of 0.1 M Na_2HPO_4 buffer (pH 7.4) with a mineral oil (145 μL) overlay. Melting temperatures were determined using a Hewlett-Packard 8452A UV diode array spectrophotomer and an HP 89090A Peltier temperature control accessory or on a Cary 100Bio UV−visible spectrophotometer (Varian, Walnut Creek, CA) using Cary WinUV software. Measurements were performed in a 1.0 mL quartz cuvette [Spectrosil Far-UV Quartz type 26.100 stoppered cell with a 10 mm path length and a 15 mm *Z*-dimension (Uvonic Instruments, Plainview, NY)]. Data were collected using the TEMPCO software from 96 to 9 °C and from 9 to 96 °C in 3 °C increments with an equilibration time of 0.1 min at each temperature after an initial 5 min equilibration prior to starting the temperature ramping. Software for either instrument was set to ramp the block temperature at 2 °C/min. It was not necessary to anneal the samples prior to T_m determinations because the measurement runs started at a high temperature and then decreased in temperature. Data files were imported into Sigma Plot 6.0 for Windows (SPSS Science, Chicago, IL) for statistical and nonlinear curve fit analysis. Independent analyses were performed for the data corresponding to the denaturation and annealing profiles. T_m values were calculated on the basis of the van't Hoff equation (*27*). The reported values are averages of the T_m values derived from the annealing and denaturation profiles.

Analysis for Non-Watson−Crick Secondary Structure. Standard conditions devised by Hurley and co-workers (*28*) were used to determine if the eight-base LNAs, X and XI, were forming any type of secondary structure. Buffer conditions were as follows: 50 mM KH_2PO_4 (pH 7.0) with the addition of 100 mM KCl alone or with 5 mM $MgCl_2$. T_m values were determined using the instrumentation mentioned above.

Cell Culture. To ensure that experiments were performed using cells capable of forming tumors, DU145 cells (an immortal line derived from human prostate cancer, 5×10^6 cells) were injected into a Harlan nude athymic mouse, which was irradiated with 400 rads (Gamma irradiation) 24 h prior to injection. Tumors were harvested when they reached the size of approximately 400 mm^3. Tumors were minced and explanted back into tissue culture. Cells were passaged in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich Corp., St. Louis, MO) supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA), 100 units/mL penicillin, 0.1 mg/mL streptomycin (Sigma-Aldrich), 2.5 μg/mL Amphotericin B (Mediatech, Inc., Herndon, VA), and 50 μg/mL Tylosin (ICN, Costa Mesa, CA). These passaged cells will be termed DU145E cells.

Telomerase Assays. Telomerase activity from DU145E cells was determined with the telomere repeat amplification protocol (TRAP) using the TRAPeze telomerase detection kit (Serologicals, Norcross, GA) (*29*). Oligomers tested for inhibition were prepared over a concentration range of 100 μM to 1 nM in logarithmic steps by serial dilution. Oligomer at each concentration was incubated with 200 cell equivalents of DU145E cell lysate for 30 min at 37 °C. The TRAPeze reaction mixture was added to each sample and then the mixture incubated for 30 min at 37 °C, facilitating the extension of the radiolabeled primer by telomerase. The extended products were amplified with a two-step PCR cycle of 30 s at 94 °C followed by 30 s at 60 °C, repeated 27 times. In all experiments, activity measurements were within the linear range of the TRAP assay.

The following controls were included in every experiment. A sample containing buffer and amplification reagents to

which no cell lysate was added was used to ensure that false products were not being amplified by PCR. Cell lysate in the absence of oligomer inhibitor was tested to determine the maximum level of telomerase activity. An internal amplification standard was included to monitor the success of the PCR. As a final control, the oligonucleotides being tested as inhibitors were added at a concentration of 3.3 μM prior to the PCR amplification to confirm that the observed inhibition was due to binding of telomerase rather than interference with the template during PCR. No inhibition of the PCR step was observed for any of the oligomers that were tested.

Reaction products were resolved by nondenaturing PAGE (polyacrylamide gel electrophoresis) analysis, followed by PhosphorImager analysis (Molecular Dynamics, Inc., Amersham Pharmacia Biotech), which provided quantitative data reflecting the extent of telomerase inhibition. The internal standard served as a control for PCR amplification efficiency in each reaction and for quantitative analysis of the TRAP products. The lanes were divided into one region encompassing the telomerase products and another including the internal standard signal. The radioisotope density was integrated for each area, and the ratio of telomerase product to internal standard was determined. The extent of inhibition as a function of inhibitor concentration was plotted, and these graphs were used to derive IC_{50} values. All assays were performed in triplicate, and the reported values for inhibition are averages of these triplicate determinations.

Transfection of Cultured Cells with Anti-Telomerase Oligonucleotides. DU145E cells were plated at a density of 25 000 cells per well in a 24-well plate in DMEM supplemented as described. After cells were allowed to adhere overnight, they were transfected with 2 μL of LipofectAMINE (Invitrogen) and 500, 250, and 125 nM oligomer in 200 μL of OPTI-MEM (Invitrogen) per well, according to the manufacturer's directions. After incubation for 6 h at 37 °C in 5% CO_2, the transfection mixture was removed and growth medium (containing serum, penicillin, streptomycin, Amphotericin B, and Tylosin) was added. Cells were harvested 40 h post-transfection by washing the cells with 1× phosphate-buffered saline (PBS), followed by trypsinization with 100 μL of a trypsin solution (0.05% trypsin-EDTA, Invitrogen) per well. Inactivation of the trypsin was achieved through the addition of 400 μL of growth medium/well. Cells were counted using a Coulter Z Series Cell Counter (Beckman Coulter, Fullerton, CA). Lysates were prepared using 1× CHAPS lysis buffer (Serologicals), and samples were assayed for inhibition of telomerase.

Fluorescence Microscopy. An LNA−DNA chimera (Cy3-GTCTtccatttTACC) targeted to luciferase mRNA was designed to include a Cy3 fluorescent label at the 5′-terminus. The LNA was delivered into DU145E prostate cancer cells as described above. The LNA−lipid complex was dispensed in 200 μL per well. Controls of similar volume included lipid only, or LNA alone. Transfections were limited to 6 h followed by replacement of the transfection mixture with complete growth medium. Cells were washed four times in 1× PBS at room temperature with a 5 min room-temperature incubation between washes. After the last wash, cover slips were mounted onto slides using 5 μL of a mounting agent consisting of 45% VECTASHIELD Mounting Medium with DAPI (Vector Labs, Burlingame, CA), 45% PermaFluor Aqueous Mounting Medium (Shandon, Pittsburgh, PA), and 10% glycerol. Slides were analyzed using a Zeiss Axiovert 100M inverted fluorescence microscope equipped with a Cy3 filter, a digital imaging system, and Openlab imaging software (Improvision Inc., Lexington, MA).

RESULTS AND DISCUSSION

Design of LNAs and an LNA−DNA Chimera. To test the hypothesis that LNAs could offer advantages as telomerase inhibitors, we designed 13-base LNAs I−VIII, six-base LNA IX, and eight-base LNAs X−XIII (Table 1). We compared their melting temperatures (T_m) for recognition and evaluated their ability to inhibit human telomerase. These LNAs were fully complementary (LNAs I, III, V, VII, IX, X, and XII) to the RNA template of human telomerase or possessed one or two mismatched bases (LNAs II, IV, VI, VIII, XI, and XIII). The match and mismatch pairs were designed to test the effects of varying oligomer length, LNA substitution, and phosphorothioate modification.

LNAs I and II contained DNA bases to moderate their affinity and were expected to bind less well than LNAs III and IV that contained only LNA bases. LNAs V−VIII possess the same arrangement of DNA and LNA bases as I and II but contained phosphorothioate (PS) substitutions (*30*). We tested PS-substituted LNAs V−VIII because PS linkages are known to improve nuclease stability and pharmacokinetic properties of DNA oligonucleotides (*31*), suggesting that PS linkages will also be an important chemical modification for improving recognition by LNAs. LNAs IX−XIII were tested to examine binding by short LNA oligomers and to examine the hypothesis that high-affinity binding by LNAs could be exploited to design exceptionally short LNAs that can act as telomerase inhibitors.

T_m Values for LNA Hybridization. We determined melting temperatures (T_m) for the hybridization of LNAs to their DNA and RNA complements to confirm their potential for avid and selective recognition of complementary sequences (Tables 1 and 2 and Figure 2). Consistent with earlier observations (*3*−*5*), T_m values for hybridization of the LNAs used in our studies to their RNA or DNA complements were greatly enhanced. T_m values for LNA binding to RNA were increased from 29 to >49 °C relative to the values for analogous DNA−RNA hybrids. This increase is equivalent to 3−4 °C per LNA base (Table 1 and Figure 2A). For comparison, we also examined DNA−LNA hybridization and found T_m values were also increased (Table 1 and Figure 2B). Consistent with previous observations by Wengel (*8*), we found that RNA−LNA hybrids were usually more stable than DNA−LNA hybrids (differences of 2−10 °C, Table 1). The presence of mismatched bases in the RNA complement lowered T_m values by 49 °C (I vs II), >31 °C (III vs IV), 36 °C (V vs VI), and 35 °C (VII vs VIII) (Table 2).

All T_m measurements were performed under standard conditions [100 mM sodium phosphate (pH 7.6)]. It is likely that the T_m values would be somewhat higher if the experiments were carried out in the presence of 1.5 mM $MgCl_2$ as found in the telomerase assay buffer. Experiments were performed on exactly complementary sequences to eliminate any contribution from overhanging bases and ensure uniformity between measurements. One drawback of this approach is that short RNA complements may have less

Table 1: Melting Temperatures (T_m, °C) for LNA Oligomers I−XIII and Analogous DNA, RNA, and PNA Oligomers Hybridized to Fully Complementary RNA or DNA Oligomers[a]

LNA Sequence	T_m LNA:DNA	T_m LNA:RNA	T_m DNA:RNA	ΔT_m LNA:RNA-DNA:RNA	T_m PNA:RNA	ΔT_m LNA:RNA-PNA:RNA
(I) **CAG**tt**AGGGT**t**AG**	81	89	41	48	68	21
(II) **CAG**tt**AGAAT**t**AG**	65	75	35	40		
(III) **CAGTTAGGGTTAG**	>90	>90	41	>49	68	>22
(IV) **CAGTTAGAATTAG**	79	81	35	46		
(V) **CAG**tt**AGGGT**t**AG**	71	76	41	35	68	8
(VI) **CAG**tt**AGAAT**t**AG**	55	64	35	29		
(VII) **CA**G**tt**A**GGGT**t**AG**	74	83	41	42	68	15
(VIII) **CA**G**tt**A**GAAT**t**AG**	60	70	35	35		
(IX) **TAGGGT**	56	62	nd		47	15
(X) **TAGGGTTA**	73	76	nd		49	27
(XI) **TAGAGTTA**	73	75	nd			
(XII) **TA**G**GGTTA**	67	72	nd		49	23
(XIII) **TA**G**AGTTA**	51	57	nd		49	8

[a] All oligomers are shown from the 5′- to 3′-end. nd means not detectable. Boldface, uppercase bases are LNA. Lowercase bases are DNA. Underlined bases contain phosphorothioate linkages.

Table 2: Melting Temperatures (T_m) for the Hybridization to RNA of Corresponding Complementary or Mismatch-Containing LNAs and LNA−DNA Chimeras

LNA pair	Match[a]	T_m	Mismatch[a]	T_m	ΔT_m (match-mismatch)
(I/II)	**CAG**tt**AGGGT**t**AG** GUCAAUCCCAAUC	89	**CAG**tt**AGAAT**t**AG** GUCAAUC**CC**AAUC	40	49
(III/IV)	**CAGTTAGGGTTAG** GUCAAUCCCAAUC	>90	**CAGTTAGAATTAG** GUCAAUC**CC**AAUC	59	>31
(V/VI)	**CAG**tt**AGGGT**t**AG** GUCAAUCCCAAUC	76	**CAG**tt**AGAAT**t**AG** GUCAAUC**CC**AAUC	40	36
(VII/VIII)	**CA**G**tt**A**GGGT**t**AG** GUCAAUCCCAAUC	83	**CA**G**tt**A**GAAT**t**AG** GUCAAUC**CC**AAUC	48	35
(X/XI)	**TAGGGTTA** AUCCCAAU	76	**TAGAGTTA** AUC**C**CAAU	51	25
(XII/XIII)	**TA**G**GGTTA** AUCCCAAU	72	**TA**G**AGTTA** AUC**C**CAAU	43	29

[a] Boldface, uppercase bases are LNA. Lowercase bases are DNA. Underlined bases contain phosphorothioate linkages. Shaded bases reflect the mismatch position of the RNA complement.

structure than longer ones, leading to some variation of hybridization properties.

Introduction of phosphorothioate substitutions reduced the T_m values for hybridization relative to those for the analogous phosphodiester LNAs. However, the T_m values for hybridization to RNA by LNAs V−VIII were still greatly elevated relative to those of the analogous DNA oligomers, with increases in T_m of 29−42 °C (Table 1). Relatively short

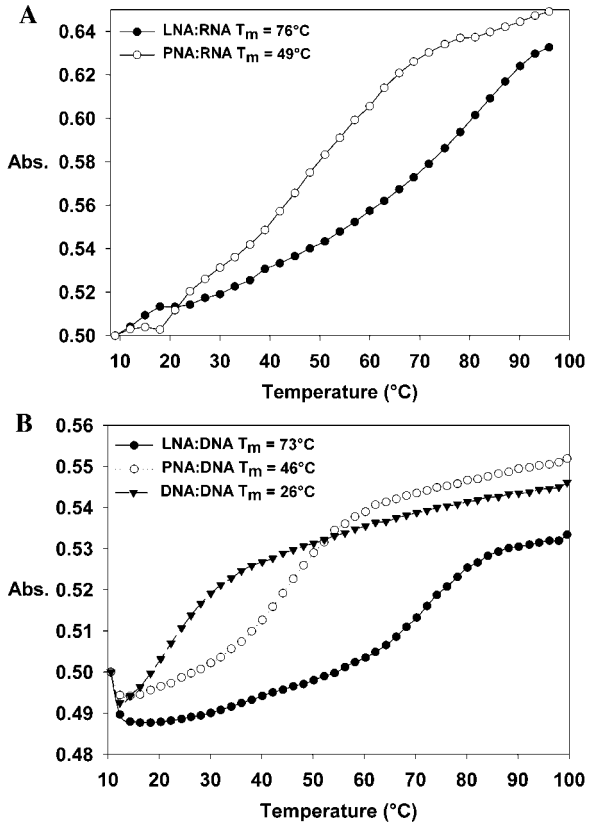

FIGURE 2: (A) Melting temperature profiles for analogous RNA−LNA and RNA−PNA duplexes. The thermal transition for the RNA−RNA or DNA−RNA hybrid could not be detected. (B) Melting temperature profiles for analogous PNA−DNA, LNA−DNA, and DNA−DNA complexes. LNA X and its PNA analogue were used to generate these data. Abs is the absorbance measured at 274 nM minus the background absorbance measured at 500 nM.

LNAs IX−XIII possessed melting temperatures of 62−76 °C, far above the T_m values of analogous DNA−RNA duplexes, and demonstrate the ability of LNA substitutions to confer exceptionally high affinity on short oligomers (Table 1 and Figure 2).

Comparison of T_m Values for Analogous PNA and LNA Oligomers. To put the hybridization stability of oligonucleotides containing LNA bases in perspective, T_m values for analogous PNA oligomers were determined. PNAs possess a neutral amide backbone, capable of extraordinarily high affinity for complementary sequences (*32*, *33*). Thus, PNAs serve as a good benchmark for the evaluation of LNAs and other new types of oligomers that appear to bind tightly to complementary sequences. Of the LNA sequences that were tested, including LNAs I, II, and V−VIII that were partially substituted with DNA bases, all possessed T_m values superior to those measured for the analogous PNAs for hybridization to either DNA or RNA (Table 1 and Figure 2). The observation that binding by LNAs can be more stable than that by analogous PNAs emphasizes the conclusion that LNAs offer important advantages for high-affinity recognition of nucleic acids.

IC_{50} Values for the Inhibition of Telomerase by 13-Base LNAs. To test whether high T_m values would correlate with the accurate recognition of a biologically important target, we examined inhibition of telomerase by LNAs I, III, V, and VII. Experiments were conducted using DU145E prostate cancer cell extract at a physiological temperature (37 °C).

Table 3: IC_{50} Values for LNA-Mediated Inhibition of Human Telomerase Activity from DU145 Cell Lysates

LNA Sequence[a]	Telomerase Inhibition IC_{50} (nM)
(I) CAGttAGGGTtAG	10
(II) CAGttAGAATtAG	>5000
(III) CAGTTAGGGTTAG	10
(IV) CAGTTAGAATTAG	1000
(V) CAGttAGGGTtAG	1
(VI) CAGttAGAATtAG	50
(VII) CAGttAGGGTtAG	1
(VIII) CAGttAGAATtAG	>5000
(IX) TAGGGT	1000
(X) TAGGGTTA	25
(XI) TAGAGTTA	>1000
(XII) TAGGGTTA	2
(XIII) TAGAGTTA	>5000

[a] Boldface, uppercase bases are LNA. Lowercase bases are DNA. Underlined bases contain phosphorothioate linkages.

LNA−DNA chimera I inhibited telomerase with an IC_{50} value of 10 nM, 500-fold more potently than mismatch-containing LNA II (Table 3 and Figure 3A). Fully substituted LNA oligomer III also inhibited telomerase with an IC_{50} value of 10 nM. The fully substituted LNA IV, which contains two mismatches, inhibited telomerase 100-fold less potently than the analogous fully complementary LNA III. The small amount of inhibition by LNA IV was surprising because it possesses a relatively high T_m value (59 °C) for binding to the telomerase RNA template sequence (Table 3). Presumably, binding to the RNA in the context of the ribonucleoprotein is a more complex process that demands greater stringency. The RNA target may be more structured or be partially blocked by protein. These results demonstrate that inhibition of telomerase by an LNA−DNA chimera and LNAs can be highly potent and highly selective.

Effect of Phosphorothioate Substitutions on Inhibition. PS substitutions are known to improve nuclease resistance and serum stability and enhance pharmacokinetic properties in vivo (*31*), which led us to test inhibition by PS-substituted LNAs V−VIII, XII, and XIII. LNA V inhibited telomerase with an IC_{50} value of 1 nM, while mismatch-containing LNA VI inhibited telomerase with an IC_{50} value of 50 nM (Table 1). The match versus mismatch discrimination was 50-fold, significantly less than the >500-fold discrimination afforded by analogous phosphodiester-substituted LNAs I and II.

The relatively strong inhibition by phosphorothioate-substituted LNA V suggests a mode of action involving both Watson−Crick base pairing with the hTR template and electrostatic interactions between the thioate linkage and the protein component (hTERT). The hypothesis that the phosphorothioate backbone promotes interactions with hTERT

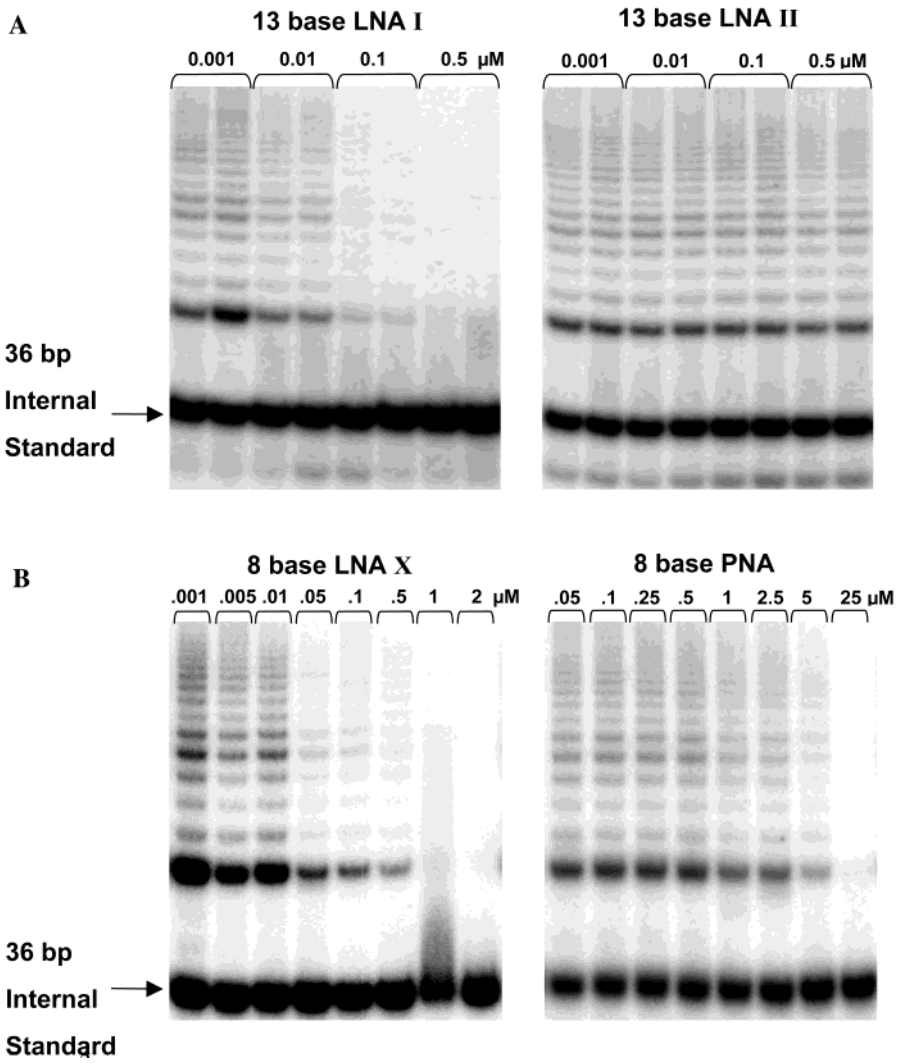

FIGURE 3: (A) Inhibition of telomerase by match and mismatch LNA oligomers. Inhibition of telomerase activity by LNA I which is fully complementary to the telomerase RNA template (left) and LNA II which contains two mismatches relative to the telomerase RNA template (right). (B) Inhibition of telomerase by analogous LNA and PNA oligomers. Telomerase inhibition by (left) eight-base LNA X and (right) an eight-base PNA composed of the same sequence as LNA X. Telomerase activity was monitored using the telomere repeat amplification protocol (TRAP) as described previously (*12*).

is consistent with previous reports that phosphorothioate substitutions can lead to non-sequence selective inhibition of human telomerase activity by PS-substituted DNA or 2′-*O*-alkyl RNA (*23*, *34*, *35*). It is also consistent with many reports that describe enhanced interactions between PS-DNA oligonucleotides and proteins (*36*).

Short LNAs as Telomerase Inhibitors. To explore whether the high binding affinity of LNAs could be exploited to reduce the oligomer length needed for potent inhibition, we evaluated the six-base LNA IX and the eight-base LNAs X and XI (Table 1). The match LNAs IX and X inhibited telomerase with IC_{50} values of 1000 and 25 nM, respectively. Inhibition was highly selective, with mismatch-containing LNA IX possessing an IC_{50} value of >1 μM. To put the potency of inhibition by these LNAs in perspective, we also examined inhibition by the analogous eight-base PNA. In contrast to effective inhibition by LNA X, the eight-base PNA (which also contained a C-terminal lysine) inhibited telomerase activity with an IC_{50} value of 5 μM, a 200-fold lower potency (Figure 3B). Superior inhibition by LNA X is consistent with its high T_m value relative to that of the analogous PNA (Table 1 and Figure 2) and suggests that the high T_m value can be directly translated into superior potency for telomerase inhibition.

We also obtained eight-base LNAs XII (fully complementary) and XIII (one mismatch relative to the telomerase template) that contained PS linkages at the 3′- and 5′-termini to enhance resistance to degradation by exonucleases. We observed that the short PS-substituted LNA was an effective inhibitor of telomerase, with an IC_{50} value of 2 nM, while the analogous mismatch-containing LNA inhibited the enzyme >2500-fold less effectively (IC_{50} value of >5000). These data further reinforce the conclusion that manipulation of LNA length and backbone substitution pattern can be used to tailor the properties of successful telomerase inhibitors.

Mechanism of Telomerase Inhibition. We observe that fully complementary LNAs or an LNA−DNA chimera effectively inhibits telomerase but that analogous mismatch-containing oligomers do not. These data are consistent with a competitive mechanism for inhibition whereby the LNAs hybridize to the hTR template by Watson−Crick base pairing and block the binding of the oligonucleotide used to prime strand elongation by telomerase. It is interesting to note that mismatch-containing LNAs, hybridized to complementary

RNA oligomers analogous to the template sequence of telomerase, exhibit T_m values (40−51 °C) (Table 2) that are higher than the temperature of the TRAP assay (37 °C). Were the potency of telomerase inhibition to exactly correspond to hybridization of LNAs to complementary RNA oligomers, one might have expected much higher levels of non-sequence selective inhibition. Instead, the fact that high-level mismatch discrimination is observed indicates that recognition of the telomerase template is a relatively complex process that acts to amplify the selectivity of oligomer binding.

The LNAs used in these studies contain three consecutive guanines, a motif that can promote formation of G-quadruplex structures within DNA and affect telomerase activity (*28*, *37*). To determine whether G-rich LNAs can form non-Watson−Crick structure and possibly affect telomerase inhibition, we performed T_m analysis on eight-base LNAs in the absence of complementary sequences. Assays were carried out in the presence of 150 μM potassium ion, a concentration significantly higher than the potassium concentration of 63 μM present in assay buffer for telomerase activity. We observed that the thermal transitions by LNA XI that contained an adenine in place of a central guanine were similar to that of LNA X that contained three guanines. We also observed that guanine-rich LNAs that are not complementary to the telomerase template do not block telomerase activity (results not shown). Our data are consistent with the conclusion that the LNAs examined here do not possess a substantial tendency toward quadruplex structure and that formation of G-quadruplex structure does not influence the mechanism of inhibition.

Inhibition of Telomerase within Cells by LNAs. To further test the properties of LNAs, we examined their ability to inhibit telomerase inside cells. LNAs were transfected into cells using the cationic lipid LipofectAMINE. To establish initial guidelines for cellular uptake, we used microscopy to follow the transfection of an LNA−DNA chimera labeled with the fluorophore Cy3 that we had been using for unrelated studies of antisense gene inhibition. No uptake was observed in the absence of LipofectAMINE (Figure 4A). When LipoFectAMINE was included, microscopy revealed rapid and diffuse uptake throughout the cell (Figure 4B,D). Punctate staining was also observed, consistent with subcellular compartmentalization, and DAPI reference staining indicated that this subcellular compartmentalization was outside of the nucleus (Figure 4C,D). Cellular uptake of the LNA−DNA chimera was similar to that reported by Gait and co-workers (*13*).

Cells were subsequently transfected with LNAs directed against the template region of human telomerase (I, III, V, VII, IX, X, or XII) or analogous mismatch-containing controls (II, IV, VI, VIII, XI, or XIII). Cells were washed carefully to remove oligomer that might be bound to the outside of cells and harvested 40 h after transfection. Lysates were prepared, and telomerase activity was measured using the TRAP assay. We observed that fully complementary LNAs I, III, V, and VII were able to inhibit >80% of telomerase activity. In contrast, treatment with mismatch-containing oligomers II, IV, VI, VIII, XI, and XIII resulted in a less than 25% decrease in activity (typical results for eight-base oligomers X and XI are shown in Figure 5).

Recognition of Targets by Short LNAs. Many chemical modifications are available for improving the properties of oligonucleotides, so when a new modification appears, it is important to define the advantages, if any, that it offers. Potent inhibition of telomerase by eight-base LNAs is an impressive demonstration of the potential for high-affinity recognition by LNAs. However, a potential problem with using small oligomers to recognize cellular RNA targets is that six- to eight-base sequences may occur many times

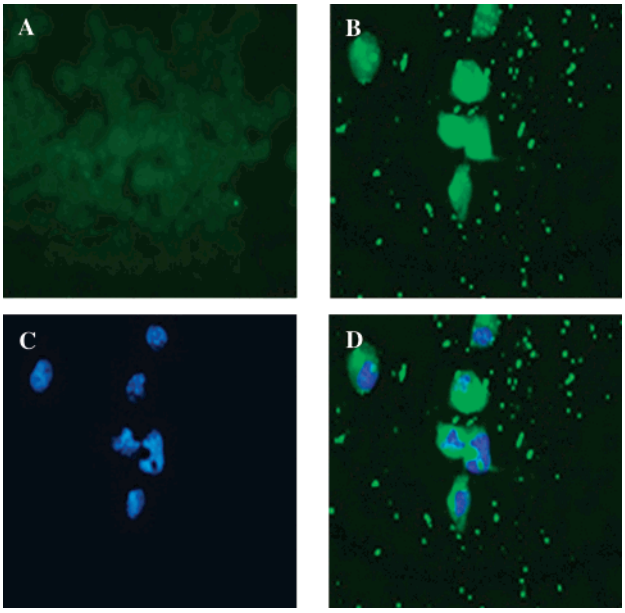

FIGURE 4: Fluorescence microscopy showing cationic lipid-mediated uptake of LNAs by DU145E cells. (A) DU145E cells incubated with a Cy3-labeled LNA−DNA chimera in the absence of lipid. (B) DU145E cells transfected with a Cy3-labeled LNA−DNA chimera complexed to LipofectAMINE (Cy3 filter). (C) DU145E cells reference stained with DAPI (DAPI filter). (D) Overlay of panels B and C. All images are at 630× magnification.

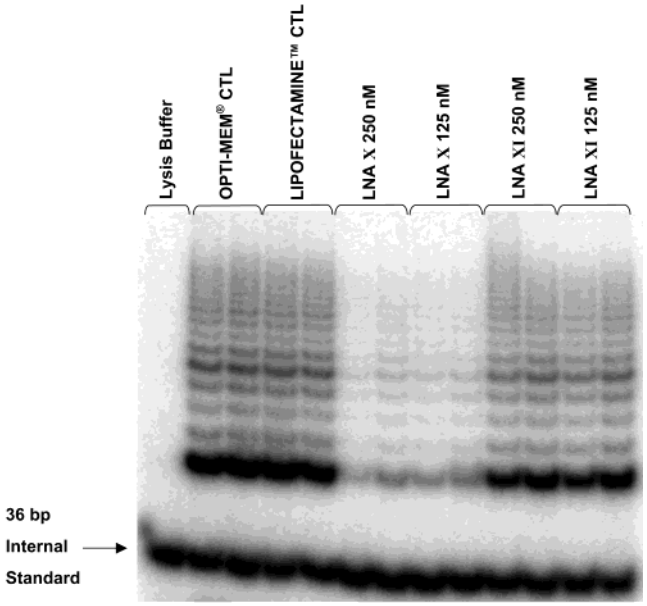

FIGURE 5: Telomerase activity assay showing inhibition of telomerase by LNAs that had been delivered into cells using cationic lipid. Inhibition by eight-base match (X) and eight-base mismatch (XI) oligomers are shown; similar results were obtained using the other match and mismatch LNAs described in Table 1. Cells were lysed, and telomerase activity was assayed 40 h after transfection. A 36-base internal amplification standard was used to ensure proper amplification during the PCR step and as a benchmark for quantitation.

within cellular mRNA and genomic DNA. Since a major advantage of LNA relative to other types of base substitutions appears to be the ability of short LNAs to bind with outstanding affinity, it is useful to consider the issue of whether adequate selectivity can be achieved inside cells.

Binding to genomic DNA may not prove to be a problem because DNA is double-stranded and largely protected from inadvertent hybridization by interactions with chromatin, rendering it relatively inaccessible to recognition by oligonucleotides. Even when DNA is bound, recognition of most DNA sequences is unlikely to yield DNA−LNA complexes capable of blocking transcription or replication. Binding to mRNA may not produce a high level of unintended consequences either, because all LNA oligomers do not efficiently activate RNase H (*12*), an enzyme that degrades RNA−DNA hybrids. Like other types of oligonucleotides that cannot readily activate RNaseH, it is likely that LNAs can affect translation by binding to the terminus of the 5′-untranslated region or by binding to splice sites and interference with RNA processing. Such sites are only a small subset of total RNA sequences, reducing, although not eliminating, the likelihood of recognition of important secondary sites. The most likely nonspecific effect would be observed through the unintentional binding of the 5′-terminal region of an RNA transcript but, unless lethal, would probably go undetected.

Supporting the suggestion that small oligomers can be useful biological agents, Matteuchi and co-workers have introduced seven-base propynyl-modified DNA oligomers into cells and have observed unaided cellular penetration, selective gene inhibition, and an absence of measurable nonselective effects (*38*). Our results concur with these findings. We observed no toxicity (measured in terms of cell number) or alteration of cell morphology upon transfection of the eight-base LNAs into cells followed by cell culture for up to 7 days post-transfection. These data suggest that the LNAs X−XIII do not bind to nontarget sequences involved in cellular survival. Clearly, these results are not conclusive, but they raise the possibility that short LNAs can possess adequate selectivity for targets inside cells. Gene array analyses of the effects of match, mismatch, and scrambled LNAs offer one strategy for addressing this issue.

The ability of short LNAs to stably bind complementary sequences suggests that it may be possible to use them to build hybrid molecules with optimized chemical properties. For example, one could start with a small LNA that binds a target sequence with modest stability, such as six-base LNA IX which possesses a T_m of 62 °C and inhibits telomerase with an IC_{50} of 1 μM. Such relatively weak recognition of the target of interest could be improved through the introduction of modifications at the 3′- and 5′-termini to take advantage of interactions with surrounding ribonucleoprotein surfaces. This approach is the one traditionally taken by medicinal chemists during the optimization of small molecule drugs. An advantage for this approach is that the binding of the hybrid LNA would be optimized for its specific target, further reducing the likelihood of unintended phenotypes due to binding nontarget RNA or DNA sequences. A second advantage is that the modifications used to create the hybrid LNA might serve to optimize pharmacokinetic properties and cellular uptake as well as improve the potency and specificity of target recognition.

SUMMARY

We have identified LNAs that not only bind to complementary sequences with exceptionally high affinities but also bind more strongly than analogous PNA oligomers. This result adds to the growing body of evidence that LNAs are promising tools for avid recognition of complementary sequences and offer advantages not afforded by the many other types of oligonucleotide and oligonucleotide mimics now available. LNAs complementary to the RNA template component of human telomerase act as potent and selective inhibitors, with IC_{50} values as low as 10 nM. A short eight-base LNA was a 200-fold better inhibitor than the analogous PNA and inhibits telomerase activity in the nanomolar range. While this short LNA is complementary to many sequences within the genome, there is no obvious toxicity to cells after transfection. Given their excellent binding affinities and ability to enter cells through simple transfection protocols, we anticipate that LNAs will prove to be an important addition to the chemical repertoire for nucleic acid recognition.

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