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"Locked onto the Target": Increasing the Efficiency of Antagomirzymes Using Locked Nucleic Acid Modifications[†]

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ABSTRACT: This study highlights the effect of incorporation of locked nucleic acid (LNA) on improving the functional efficacy of DNAzymes against microRNAs (antagomirzymes). DNAzymes were designed against two different sites of miR-27a, which were encompassed both within the precursor and mature form of miRNA. The cleavage and functional activities of these DNAzymes have been compared to those of LNA-modified counterparts, containing LNA modification in each of the substrate binding arms. Preliminary examination based on in vitro cleavage demonstrated LNAzyme to be much more effective in the ensuing cleavage of target miRNA under both single- and multiple-turnover conditions. Evaluation of kinetic parameters indicated almost 5-fold higher cleavage efficiency, k_{obs}, for LNAzymes than for DNAzymes, leading to more efficient cleavage of the substrate. We attribute this enhancement in cleavage efficiency to the LNA-mediated improvement in the hybridization of the antagomirzyme·target complex. Functional validation of the relative activities was accomplished through the luciferase reporter assay and quantitative real-time polymerase chain reaction (qRT-PCR). Both the unmodified and LNA-modified antagomirzymes were very active in ensuing efficient miRNA knockdown; however, compared to the DNAzymes, the LNAzymes were almost 25% more active. A direct quantitative estimate of miRNA cleavage, conducted using qRT-PCR, further substantiated the data by indicating that LNAzyme effectively downregulated the levels of mature miRNA (up to 50%) versus the corresponding DNAzymes. Our data thus provide formative evidence of the successful employment of LNA-based antagomirzymes against miRNA.

MicroRNAs (miRNAs)[1] are short, noncoding, regulatory RNA molecules that post-trancriptionally regulate gene expression by base pairing to their target mRNAs and mediating mRNA cleavage or translational repression. Aberrant miRNA expression has been observed for a number of patho-physiological conditions. For cancer alone, both tumor suppressor and oncogenic miRNAs have been identified; however, whether this deregulated miRNA expression is a cause of cancer or simply an effect of the less-differentiated state of cancerous cells remains unclear (*1*). Given the significant role of miRNAs in orchestrating cellular processes, it becomes necessary to devise approaches that unveil the mysteries of miRNA function and regulation. This will not only augment our fundamental understanding of gene regulation mediated by miRNA but also provide a means of modulating their expression for therapeutic interventions.

The use of antisense oligonucleotides (antimiR/antagomiR) for this purpose has emerged as a perfect tool for characterization of miRNA expression patterns and functions. An antimiR binds to its target miRNA with Watson−Crick base pairing and, in turn, sequesters it in a duplex, making it unavailable for binding to its cellular targets (*2*). An antimiR, thus, does what an antisense oligonucleotide does to its target mRNA; however, while most of the antisense oligonucleotides lead to RNase H-mediated down-regulation of their targets, antimiRs are required to act in the cytoplasm (the site of localization of mature miRNA) and, therefore, work in an RNase H-independent mechanism. While the antimiR represents the most simplified and widely used form of miRNA inhibitors, other strategies for effective and long-term suppression of miRNA have also been developed. The "sponges" and "erasers" are a few of the newly developed strategies (*3*, *4*). Both these RNA molecules are expressed in the cell from transgenes and contain a tandem repeat of perfect or imperfect miRNA binding sites in their 3′-UTR, which eventually scavenge the endogenous miRNA away from its natural targets. An added advantage of these molecules over antimiRs is that these can be expressed either transiently from plasmids or stably form chromosome insertions to exhibit long-term effect. Another recent approach developed for antagonizing miRNA function relies on the use of classical model of 10−23 DNAzyme (*5*), hence, the name "antagomirzyme". These enzymes are catalytically active DNA molecules capable of cleaving even a highly structured, long, complementary RNA substrate in a site specific manner (*6*, *7*). Structurally, they consist of a centrally conserved ribozyme motif containing a catalytic core and two variable flanking regions (arms) on either end of the core. While the arms are complementary to the RNA substrate, the catalytic core serves to recognize and cleave a pyrimidine·purine junction at the cleavage site of the substrate. The inherent advantage of using such an enzyme-based antagonist is that it ensures complete cleavage of the substrate and in the process is regenerated to act on other

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[1]Abbreviations: LNA, locked nucleic acid; Lz, LNAzyme; Dz, DNAzyme; miRNA, microRNA; UTR, untranslated region; NS, non-specific; PHB, prohibitin; luc, luciferase; qRT-PCR, quantitative real-time polymerase chain reaction; PBS, phosphate-buffered saline; DMEM, Dulbeccoo's modified Eagle's medium.

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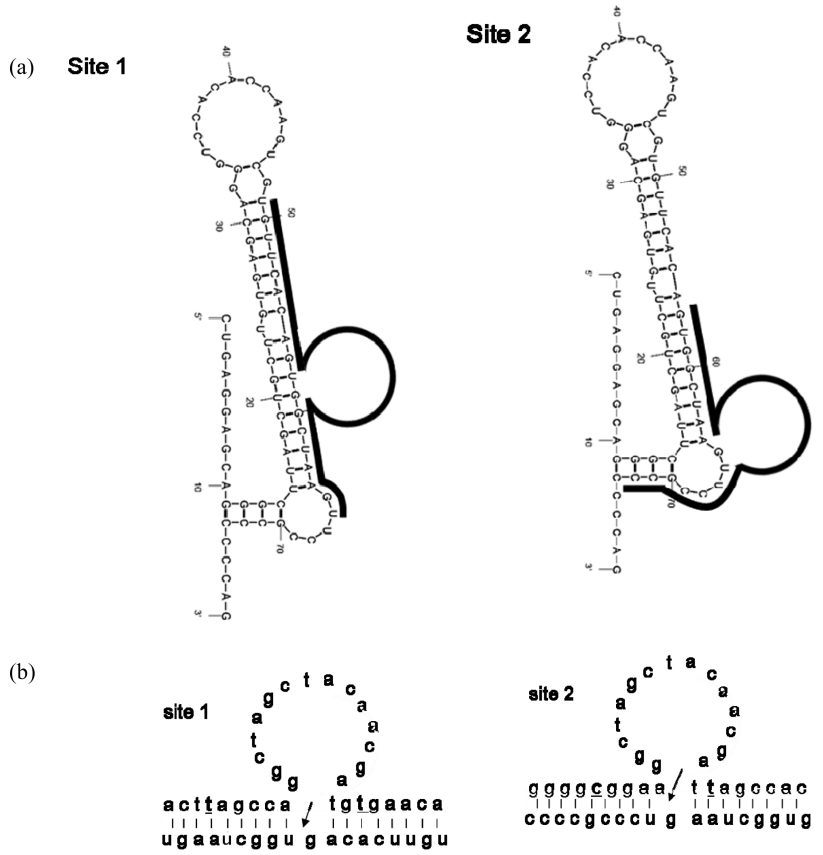
Scheme 1: Two Sites, Sites 1 and 2, within the Precursor Form of miR-27a, Targeted by the Antagomirzymes[a]



[a](a) The target region encompassed by the antagomizymes is depicted. (b) The precise cleavage site within each region is highlighted with an arrow. LNA modifications within the hybridizing arms are marked in bold and underlined.

molecules of the same miRNA. The working concentration of the enzyme is, thus, expected to be lower than that of the analogous antimiR, which itself is consumed in the process of target sequestration. Successful, implementation of DNAzymes have been documented even for the long and highly structured RNA substrates that are otherwise inaccessible to the conventionally used antisense agents (*8*, *9*). These encouraging results prompted us to extend this approach to miRNA downregulation, where for the first time we demonstrated the successful cleavage of both the precursor and mature forms of miRNAs, namely, miR-372 and miR-373, respectively, by 30-mer antagomirzymes (*10*).

While all these approaches are promising against miRNA, the detection and characterization of miRNAs over mRNA still present a technical challenge because of the small size of miRNA and the presence of nearly identical isoforms. Accurate quantification of miRNA, thus, calls for the development of robust and improved technologies allowing specific and sensitive detection of both mature and precursor miRNAs. The introduction of modified nucleotide analogues into the antimiRs greatly improves their efficacy by rendering characteristics like increased binding affinity, enhanced nuclease stability, excellent mismatch discrimination, improved cellular delivery, and low toxicity. One of these widely used engineered chemical modifications that has greatly influenced miRNA technology platform is the locked nucleic acid (LNA). These are the ribonucleotide analogues in which the 2′-oxygen is bridged to the 4′-position via a methylene linker to form a rigid, bicycle, locked 3′-endo conformation (*11*, *12*). This conformational restriction leads to unprecedented hybridization efficiency toward the complementary strand (*13*−*15*). Currently, clinical trials with LNA-based therapeutics are in progress against many aberrantly expressed oncogenic mRNAs or miRNAs (*16*). The excellent selectivity and sensitivity offered by LNA-based antimiRs are best suited for detection and characterization of miRNA function in vitro and in vivo (*17*).

This study presents another successful account of employing antagomirzymes against miRNAs, in particular miR-27a, which is implicated in a variety of cancers and chemotherapy resistance (*18*−*20*). More importantly, our study highlights the advantage of using LNA substitution to improve the functional efficiency of these enzymes under both in vitro and in vivo conditions. Antagomirzymes were designed against two different sites of miR-27a, and for each site, the functional efficiency of the unmodified form and the LNA-modified counterpart was compared. In vitro cleavage data demonstrated that substitution of one LNA in each of the hybridizing arms profoundly improved the ability of the enzyme to cleave the mature miRNA substrate. Further, enzyme kinetics data showed that relative to the unmodified DNAzyme, the higher rate constant of cleavage (K_{obs}) for LNAzyme results in more efficient cleavage of the substrate. This enhancement in cleavage efficiency primarily originates from LNA-mediated improvement in the hybridization of the antagomirzyme to the target. Functional validation of these data in cell culture-based assays was achieved using luciferase reporter analysis and real-time PCR. For both the assays, a stronger abrogation of miRNA function was observed for the modified antagomirzyme as compared to its unmodified counterpart. Our cell culture data thus correlated well with in vitro results indicating a promising application of LNA-based antagomirzymes against miRNA.

Table 1: Sequences of Antagomirzymes and Target miRNA Used in This Study[a]

description	nomenclature	sequence (5′−3′)
unmodified antagomirzyme against site 1	Dz 1	5′-acttagccaggctagctacaacgatgtgaaca-3′
LNA-modified antagomirzyme against site 1	Lz 1	5′-act**t**agccaggctagctacaacgatg**t**gaaca-3′
mutant antagomirzyme against site 1	mut 1	5′-acttagccaggctagctacaacatgtgaaca-3′
unmodified antagomirzyme against site 2	Dz 2	5′-ggggcggaaggctagctacaacgattagccac-3′
LNA-modified antagomirzyme against site 2	Lz 2	5′-gggg**c**ggaaggctagctacaacgat**t**agccac-3′
mutant antagomirzyme against site 2	mut 2	5′-ggggcggaaggctagctacaacattagccac-3′
mature miR-27a		5′-uucacaguggcuaaguuccgc-3′
nonspecific antagomirzyme	NS	5′-cgctcaaaggctagctacaacgagtcgcag-3′

[a]LNA modifications within the antagomirzymes are highlighted in boldface and are underlined.

MATERIALS AND METHODS

Oligonucleotides. HPLC-purified unmodified and LNA-modified antagomirzymes were purchased from Ocimum Biosolutions Pvt Ltd. The mature miR-27a RNA (5′-UUCACAGUGGCUAAGUUCCGC-3′) was purchased from Sigma Genosys. The solution concentration of each of the unmodified oligonucleotides was determined optically at 260 nm and 25 °C using the following molar extinction coefficients: 318500 for d(ACTTAGCCAGGCTAGCTACAACGATGTGAACA) and 315200 for d(GGGGCGGAAGGCTAGCTACAACGATTAGCCAC). These values were calculated by extrapolation of the tabulated values of the dimer and monomer nucleotides at 25 °C to high temperatures using protocols reported previously (*21*). For the modified oligonucleotides, the molar absorptivities were assumed to be identical to those of the DNA oligonucleotides.

Experimental Design. Two sets of antagomirzymes were designed for two different sites of microRNA, namely, miR-27a. The target sites were present in both the precursor and mature form of miRNA. Each set of antagomirzymes was comprised of a 32-mer unmodified antagomirzyme (Dz 1 and Dz 2) and its corresponding LNA-modified counterpart (Lz 1 and Lz 2), containing two LNA modifications, each within an internal thymidine residue of the hybridizing arm. Within this 32-mer antagomirzyme sequence, 15 nucleotides formed the catalytic core while 8 and 13 other nucleotides constituted the hybridizing arms. As depicted in Scheme 1, the antagomirzymes directed against site 1 encompassed a region from nucleotide 52 to 68, while antagomirzymes against site 2 accessed a region ranging from nucleotide 58 to 74. Additionally, control antagomirzymes (mutants 1 and 2) carrying a single deletion mutation at the G14 nucleotide from the 5′-end of the catalytic core were designed against each site. This guanosine is known to be highly conserved, and its substitution with any of the other naturally occurring nucleotides is known to completely abolish the enzyme activity (*22*). The sequences for all antagomirzymes and the mature miRNA target are listed in Table 1. Besides, an additional mock antagomirzyme, nonspecific to the miR-27a, was also used in cell culture-based studies to keep the total concentration of nucleic acid transfected in each well uniform.

Cloning and Vector Construction. miR-27a was overexpressed in the cell culture assay using the pSilencer 4.1 vector (Ambion, Austin, TX). Because the endogenous expression of this miRNA occurs in the form of miRNA cluster miR-23a∼27a∼24-2, we chose to clone the cluster instead of the individual miR-27a precursor form in the vector. The clone used was made as described previously (*23*). Further, to observe the antagomirzyme-mediated effects on miRNA levels, the 193 bp region of the 3′-UTR of the prohibitin gene (PHB), an established target of this miR-27a, was PCR amplified using forward primer 5′-CCGCTCGAGCACCCTGCCTGCACCTC-3′ and reverse primer 5′-ATAAGAATGCGGCCGCTGGATGTGAGAAGAATGAAAGA-3′ (*20*) and cloned within NotI and XhoI sites of the *Renilla* gene of the dual luciferase psi-check 2 vector (Promega). The relative change in the expression of *Renilla* luciferase activity was normalized with respect to the firefly luciferase control, present within the same vector. The resulting plasmids were sequenced to ensure accuracy.

Cell Culture and Differentiation. HEK 293T (human embryonic kidney) cells were maintained in Dulbecco's Minimal Essential Medium high glucose (DMEM) (GIBCO-BRL) supplemented with 10% fetal calf serum (Biological Industries), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma), and an antibiotic/antimycotic solution (100× stock) (Sigma) in a 37 °C humidified incubator with 5% CO_2.

In Vitro Cleavage Assay. The 21-nucleotide, mature miR-27a (5 μM) was radiolabeled using the KinaseMax Kit (Ambion) and purified using a Nuncaway purification column from Ambion. The substrate (300 nM) was incubated in a reaction buffer consisting of 50 mM Tris-HCl (pH 7.5). Both the labeled substrate and the antagomirzyme were heat denatured separately for 5 min at 85 °C, mixed, and cooled to 37 °C. The labeled substrate was incubated with four different concentrations of each of the antagomirzymes, at substrate:enzyme concentration ratios of 1:0.1, 1:1, 1:10, and 1:20 for 15 min. $MgCl_2$ (25 mM) was added to initiate cleavage reactions.

After 45 min, the cleavage reaction was stopped by addition of Gel Loading Buffer II (Ambion) followed by heat denaturation at 85 °C for 5 min. The intact substrate and the degraded product were separated on a 15% denaturing polyacrylamide gel.

In Vitro Cleavage Kinetics. Labeling and incubation of the substrate with antagomirzymes were performed at a substrate:enzyme ratio of 1:10 (single-turnover condition), as described above. Aliquots (20 μL) were taken from the reaction mix at regular intervals of 0, 5, 10, 20, 30, 45, 60, 75, 90, 120, and 180 min. The reaction was stopped by addition of Gel Loading Buffer II and heat denaturation at 85 °C for 5 min. The cleavage products were separated on a 15% denaturing polyacrylamide gel. The amount of cleavage was quantified using a Typhoon Trio phosphorimager and ImageQuant TL (GE Healthcare). The fraction of product formed at any time t (F_t) was calculated by dividing the amount of product by the amount of substrate and product. The data were then fit to a single-exponential decay function using the following equation in Origin (Microcal Software, Northampton, MA)

$$F_t = F_\infty(1 - e^{k_{obs}t}) \quad (1)$$

where F_∞ is the fraction of product at the end point of the reaction and k_{obs} is the rate constant of cleavage (*24*).

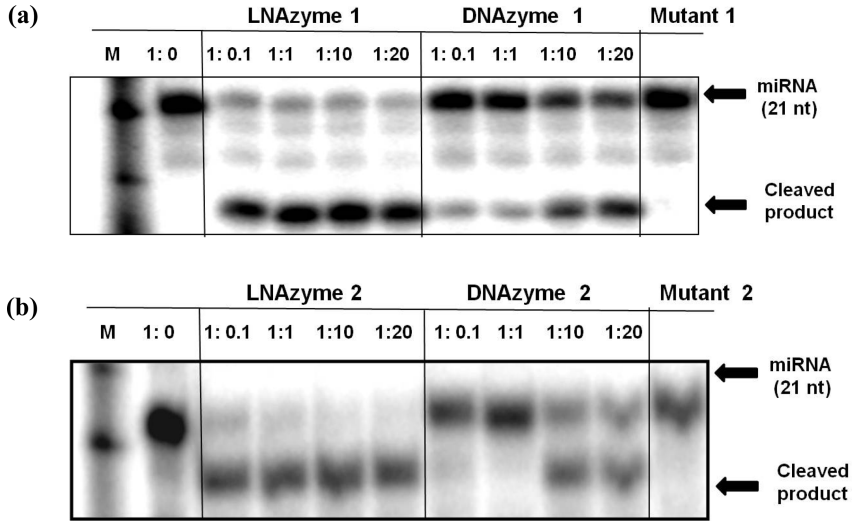


FIGURE 1: In vitro cleavage of mature miR-27a, by each set of antagomirzymes directed against (a) site 1 and (b) site 2, under multiple-turnover (substrate:enzyme ratio of 1:0.1) and single-turnover (substrate:enzyme ratios of 1:1, 1:10, and 1:20) conditions.

Luciferase Reporter Assay. Approximately 3×10^5 cells (HEK 293T) were seeded in each well of a 12-well plate, 24 h prior to transient transfection when 50−80% confluency was reached. Cells were washed once with Opti-MEM (GIBCO-BRL) and maintained in 1 mL of Opti-MEM. Transfection quality [Endo free plasmid maxi kit (Qiagen)] plasmids comprising the psi-check 2 vector alone or along with the pSilencer-miR-27a vector were cotransfected with 100 nM mock or miR-27a antagomirzymes, using Lipofectamine 2000 transfection reagent (Invitrogen); 48 h after transfection, cells were lysed in PLB buffer (Promega), and the total amount of protein was estimated using the BCA protein estimation kit (Sigma). The luciferase assay was performed using the dual-luciferase reporter assay kit (Promega), and luminescence was measured in a microplate luminometer (Berthold detection system). For data analysis, *Renilla* luciferase values were normalized using firefly luciferase luminescence values.

Real-Time Quantitative PCR. Total RNA was extracted using Trizol reagent, and reverse transcription was conducted with M-MuLV reverse transcriptase using stem−loop primers for miR-27a (5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCGGAAC-3′) and U6 control gene (5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGGAAC-3′). Amplification of cDNA was conducted using the miR-27a, forward primer 5′-TGCGGTTCACAGTGGCTAAG-3′, U6 forward primer 5′-TGCGGGTGCTCGCTTCGGCAGC-3′, universal reverse primer 5′-CCAGTGCAGGGTCCGAGGT-3′, and Syber Green PCR master mix (Applied Biosystems, Foster City, CA) (*20*). Results were normalized with respect to U6. The PCR conditions used were as follows: initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 30 s, and an additional cycle with extension at 72 °C for 10 min.

RESULTS

In Vitro Cleavage Assay. The functional performance of each of the antagomirzymes (DNAzymes and LNAzymes), designed against the two different sites of miR-27a, was tested using an in vitro enzyme cleavage assay. In the presence of divalent magnesium ions, all the antagomirzymes were highly effective in ensuring cleavage of the mature miRNA in a short span of incubation for 1 h. The mutant antagomirzymes containing single-nucleotide deletion mutations in the catalytic core, on the other hand, did not show any cleavage. The gel picture depicted in Figure 1 presents a comparison of relative antagomirzyme activities under multiple-turnover conditions (substrate excess) and single-turnover conditions (enzyme excess). Under single-turnover conditions (substrate:enzyme ratio of 1:10), better cleavage of the product was observed for the LNA-modified antagomirzymes compared to the unmodified enzymes. Noteworthy was the finding that while under multiple-turnover conditions (substrate excess) the unmodified enzymes failed to produce any considerable cleavage, the LNA-modified versions were very effective in ensuring significant cleavage of the substrate, suggesting higher potency of LNA-modified versus unmodified antagomirzymes. Furthermore, the control antagomirzymes carrying point mutations in the catalytic core did not exhibit any degradation of the substrate.

In Vitro Cleavage Kinetics. Cleavage kinetics of each of the antagomirzymes against mature miRNA substrate was followed under single-turnover conditions (substrate:enzyme ratio of 1:10). As depicted in Figure 2, complete saturation of cleavage activity could be seen over a period of 3 h. Densitometric analysis revealed better (faster) cleavage of the substrate for each of the miRNA sites considered for the LNA-modified antagomirzymes. Fitting of each of these curves using eq 1 allowed estimation of the cleavage rate parameter (k_{obs}) and the extent or fraction of substrate cleaved (F_∞) at infinity. As shown in Table 2, for site 1, a cleavage rate constant of 0.07 was observed for unmodified antagomirzyme, while in the presence of LNA, it was increased to a 4-fold higher value. Similarly, for site 2, a 5-fold higher cleavage rate constant was observed for LNAzyme with respect to DNAzyme.

Luciferase Reporter Assay. Functional validation of antagomirzyme's performance was achieved by cloning the target site (prohibitin) of miR-27a into the 3′-UTR of luciferase in a dual luciferase vector. Overexpression of miR-27a led to a decrease in luciferase activity of up to 25%. In the presence of antagomirzymes, however, significant reversion of reporter expression could be observed at an enzyme concentration of 100 nM. Figure 3 shows a significant increase in the level of luciferase expression, relating to abrogation of miRNA function, for LNA-modified

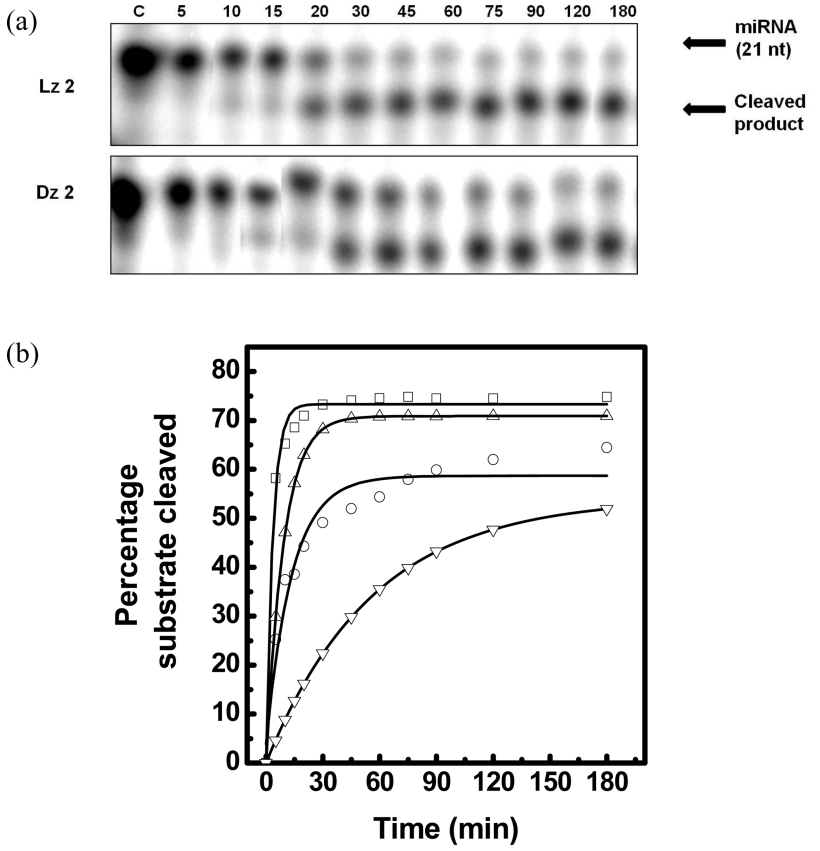


FIGURE 2: (a) Representative of the cleavage kinetics profile obtained for site 2 antagomirzymes (Dz 2 and Lz 2), upon incubation with the mature miRNA substrate at a substrate:enzyme concentration of 1:10. (b) Cleavage efficiencies obtained by plotting the percentage of substrate cleavage by different antagomirzymes [Lz 1 (□), Dz 1 (○), Lz 2 (△), and Dz 2 (▽)] over a time interval of 3 h. The resulting curve was fitted according to a single-exponential decay function, yielding kinetic parameter k_{obs} (min^{-1}).

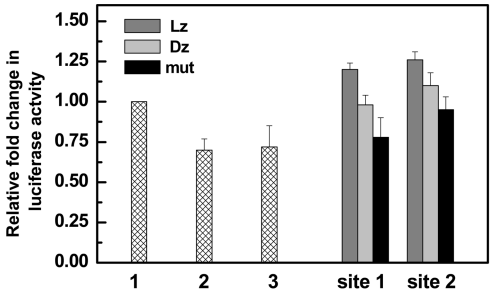
Table 2: Kinetic Parameters Obtained by in Vitro Cleavage of Mature miR-27a under Single-Turnover Conditions[a]

antagomirzyme	k_{obs} (min^{-1})	F_∞ (%)
Lz 1	0.28 ± 0.02	73.3 ± 0.8
Dz 1	0.07 ± 0.01	58.6 ± 1.8
Lz 2	0.10 ± 0.01	70.9 ± 1.4
Dz 2	0.01 ± 0.00	54.1 ± 3.7

[a]Experiments were conducted in 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl. k_{obs} refers to the rate constant, and F_∞ refers to the fraction of product formed at the end point of the reaction. For each reaction, the reported k_{obs} values are the average of three independent experiments.

antagomirzyme compared to the unmodified analogues. The nonspecific mock antagomirzyme (NS) did not produce any increment in luciferase expression, indicating the specificity of the enzyme·substrate reaction. The mutant antagomirzymes containing a single-nucleotide deletion mutation in the catalytic core though did produce an increase in the level of expression, however, not to the extent of the wild-type antagomirzymes.

Real-Time PCR Analysis. More direct evidence of the effect of antagomirzyme on miRNA function was found using quantitative real-time PCR. Antagomirzyme-mediated cleavage of the miRNA could well be visualized by monitoring the relative decrease in mature miRNA levels in the presence of LNA-modified and unmodified antagomirzymes. As seen in Figure 4, in the presence of LNAzymes, the relative level of expression of the mature form of miR-27a decreased to half of the original value. For Lz 1, a relative knockdown of 50% of the expression



	1	2	3	Site 1	Site 2
psicheck-PHB	+	+	+	+	+
psilencer-miR-27a	-	+	+	+	+
Antagomirzyme	-	-	+ (NS)	+ (Lz 1/Dz 1/ mut 1)	+ (Lz 2/Dz 2 /mut 2)

FIGURE 3: Dual-luciferase reporter assay for validating the in vivo function of DNA- or LNAzymes. The indicated vectors and antagomirzyme [LNAzyme (Lz), DNAzyme (Dz), mutant (mut), or non-specific (NS) control] designed against sites 1 and 2 were transfected into the HEK-293 cell line. The relative *Renilla* luciferase levels were monitored after 48 h and normalized to a firefly control, as shown. Error bars represent the standard deviation for three independent experiments.

was observed, which was almost 15% higher than that for Dz 1. Maximum downregulation could be observed for Lz 2, which

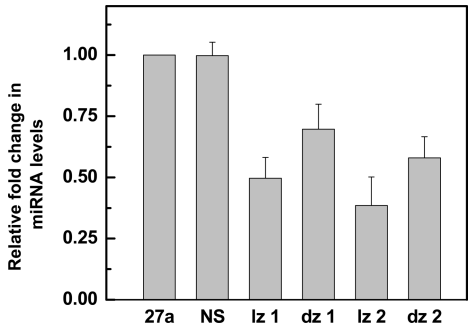


FIGURE 4: Quantitative real-time analysis for estimating the fold change in miR-27a levels in 48 h after cotransfection of antagomirzymes with the pSilencer-miR-27a vector. Error bars represent the standard deviation for three independent experiments.

gave > 50% of the downregulation, almost 20% higher than that for Dz 2.

DISCUSSION

Substitution with a modified nucleotide has long been pursued as a way to improve the fate and clinical outcome of miRNA-based therapeutics and diagnostics (*15*) . Evidence of the successful employment of LNA to improve the targeting efficiencies of the DNAzyme against conventional mRNA targets exists (*25*−*28*). We sought to employ LNA as means to improve the functional efficiency of previously described antagomirzymes against miRNA. When a DNAzyme is substituted, a choice of either modifying the hybridizing arms or placing the substitution in the catalytic core can be made (*29*−*32*). However, substituting the catalytic core might hamper the catalytic activity by destroying the three-dimensional structure of the enzyme. For the reason cited, we chose to modify the arms with one LNA modification each. In addition, we preferably modified the internal thymidines of the arms in accordance with earlier studies that showed that the effect of LNA substitution is most pronounced when the LNA substitution is made within the sugar ring of thymidine (*33*, *34*). An additional precaution while positioning LNA in a DNAzyme is to optimally limit the number of modifications, to avert the possibility of interaction between LNAzyme and the cleaved product. Such a scenario can greatly decrease the reaction rate and is usually encountered under multiple-turnover conditions. Earlier reports claim that LNAzymes with just one or two LNA monomers in each arm function considerably better than DNAzymes under multiple-turnover conditions (*25*, *35*). Considering this, we chose to modify only two residues in the overall architecture of the antagomirzyme.

A preliminary examination of the cleavage potency of all these antagomirzymes through an in vitro cleavage assay demonstrated that a mere 1 h incubation of the enzyme with the mature miRNA was sufficient to give effective cleavage of the substrate. As expected, LNAzymes were much more effective in cleaving the substrate than the unmodified DNAzymes under single-turnover conditions (excess enzyme). Noteworthy was the observation that unlike DNAzymes, the LNAzymes were functional even under multiple-turnover conditions, thereby suggesting that lower concentrations of the antagomirzyme would be needed in vivo to mediate the same extent of downregulation. Our in vitro cleavage kinetics data also support this finding, as for both sites we observed that the modified enzymes produce faster cleavage of the substrate, which is reflected in their higher cleavage rates. Kinetics for each of these antagomirzymes were followed until saturation and evaluated in terms of the fraction of substrate cleaved (F_t). It is worth mentioning that 100% cleavage of the substrate to product can never be achieved for such in vitro enzyme cleavage reactions, which can be ascribed to the fact that with the subsequent decrease in the substrate concentration, the relative binding affinity of the enzyme for the substrate decreases. A decrease in the number of hybridization events thus will appear as a plateau phase, as shown in Figure 2. A detailed analysis of these kinetic curves gave the rate constant of cleavage, i.e., k_{obs}, which is a direct estimate of the cleavage efficiency. In general, the kinetics of an enzyme·substrate reaction consists of two sequential steps, in the forward direction; step 1 involves formation of the enzyme·substrate bimolecular complex, and step 2 involves the dissociation of the cleaved products from this complex. The difference in the kinetics of single- and multiple-turnover reactions exists in the latter step, as the excess of enzyme under single-turnover conditions "overrides" the negative influence of step 2 on the reaction rate, thereby making it independent of product dissociation. Noteworthy is the fact that the presence of LNA affects the overall kinetics of the reaction by favoring hybridization between the enzyme and substrate in step 1; however, this same attribute could adversely affect the reaction rate by delaying dissociation of the cleaved product from the enzyme site in step 2. The fact that in the presence of LNA the cleavage activity of DNAzyme is enhanced for both single- and multiple-turnover conditions indicates that inclusion of LNA under both these conditions improves cleavage efficiency by stabilizing the enzyme·substrate complex (*36*).

Interestingly, the relative activity of DNAzymes versus LNAzymes is also known to be affected by the type of substrate (*24*). For short RNA substrates, as in this study (mature miRNA), the presence of LNA promotes complex formation by lowering the rate of substrate dissociation prior to the cleavage, while no change occurs in the substrate association step. For a long, structured target (like a precursor miRNA), the presence of LNA may also favorably affect the kinetics of enzyme·target "association". It is thus expected that the relative activities of DNAzymes and LNAzymes will differ in the cellular scenario, where the antagomirzymes will compete for both the mature and structured miRNA precursor target.

Functional validation of these enzyme's activities in the cellular scenario was conducted using the luciferase reporter assay. Here, the effect of overexpression of miR-27a in the presence and absence of antagomirzymes was calculated on its target gene prohibitin (PHB) whose 3′-UTR was cloned under *Renilla* luciferase. With LNA-substituted DNAzymes, a much stronger reversion of target expression could be observed. Interestingly, this increase in the level of expression of the luciferase prohibitin gene was even greater than that of controls. On the basis of these observations, we conclude that some endogenous miR-27a expression already existed in the cells, and therefore, cotransfection of antagomirzymes not only suppressed the activity of overexpressed miR-27a plasmid but also suppressed the expression of endogenous miR-27a, thereby further elevating the levels of the target luciferase gene versus the controls (bar 1 of Figure 4). Another noteworthy finding was the increase in the level of luciferase expression for mutant antagomirzymes containing a point mutation in their catalytic cores. This increase in the level of expression is expected, as the presence of the mutation in the catalytic core decimates the cleavage activity to zero (as seen in in vitro cleavage results), but at the same time, the complementary hybridizing arms of the enzyme enable it to inhibit miRNA

function by acting as a sequestering agent (antimiR). The mutant here, thus, mimics the effects of an antimiR. Our luciferase protein expression data thus establish that the antagomirzymes performed much better than an antimiR (here mutant antagomirzyme) designed for the same site.

We also performed real-time quantification of miRNA expression in the presence of antagomirzymes. It should be recalled that both the precursor and mature forms of the miRNA harbor the two target sites and, therefore, compete to interact with antagomirzyme when present inside the cell. We have quantified the changes in miR-27a expression for the mature form of miRNA; 48 h post-transfection, an almost 50% decrease in the level of target expression could be observed for LNA-modified antagomirzymes, which was clearly higher than the fold decrease in miRNA levels given by the unmodified enzyme. While in our case the analysis of the luciferase reporter assay and real-time data was performed after transfection for 48 h, it is the turnover (biogenesis) rates of the precursor and the mature forms of the miRNA in question that would eventually govern the extent and the time period over which this inhibitory effect will last. Besides this, the fine-tuning of the number of modifications within the antagomirzyme and careful selection of correct target sites on the miRNA and its precursors can greatly influence the potency of the antagomirzyme in the cellular milieu.

CONCLUSION

This study demonstrates the employment of LNA in improving the functional efficiency of conventionally used DNAzymes against miRNA. Incorporation of an LNA modification into each of the hybridizing arms of the DNAzyme increased the cleavage efficiency of the enzyme by favoring formation of enzyme·target complex. Because the higher stability of an oligonucleotide to its target directly correlates with its functional efficiency, we speculate that the presence of LNA improves the performance of antagomizyme by making it more selective and specific for the target, a prerequisite when developing an anti-miRNA therapeutic (*37*). Validation of this speculation in the cellular settings further substantiated the data that showed that the catalytic potential of antagomirzymes can well be harnessed against miRNA, and because of this, the incorporation of LNA can serve as a perfect means for improving the functional activity and specificity of this novel anti-miRNA tool. We would further like to emphasize that though the relative content and positioning of LNA within the antagomirzyme call for precise optimization, the choice of the target site within the precursor miRNA and the working concentration of antagomirzyme are equally critical.

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