A Highly Effective and Long-Lasting Inhibition of miRNAs with PNA-Based Antisense Oligonucleotides

Su Young Oh, YeongSoon Ju, and Heekyung Park*

MiRNAs are non-coding RNAs that play a role in the regulation of major processes. The inhibition of miRNAs using antisense oligonucleotides (ASOs) is a unique and effective technique for the characterization and subsequent therapeutic targeting of miRNA function. Recent advances in ASO chemistry have been used to increase both the resistance to nucleases and the target affinity and specificity of these ASOs.

Peptide nucleic acids (PNAs) are artificial oligonucleotides constructed on a peptide-like backbone. PNAs have a stronger affinity and greater specificity to DNA or RNA than natural nucleic acids and are resistant to nucleases, which is an essential characteristic for a miRNA inhibitor that will be exposed to serum and cellular nucleases.

For increasing cell penetration, PNAs were conjugated with cell penetrating peptides (CPPs) at N-terminal. Among the tested CPPs, Tat-modified peptide-conjugated PNAs have most effective function for miRNA inhibition. PNA-based ASO was more effective miRNA inhibitor than other DNA-based ASOs and did not show cytotoxicity at concentration up to 1,000 nM. The effects of PNA-based ASOs showed considerable stability at storage temperature. These results suggest that PNA-based ASOs are more effective ASOs of miRNA than DNA-based ASOs and PNA-based ASO technology, compared with other technologies used to inhibit miRNA activity can be an effective tool for investigating miRNA functions.

INTRODUCTION

MicroRNAs (miRNAs) are a newly discovered class of short non-coding RNAs of about 22 nt in length that are expressed in most species, including animals, plants and viruses. MiRNAs are implicated in post-transcriptional gene regulation, which plays a vital role in cellular processes such as developmental control, metabolism and apoptosis (Bushati and Cohe, 2007; Esquela-Kerscher and Slack, 2006; Kim, 2005; Xia et al., 2008). Additionally, many miRNAs have been shown to be involved in the regulation of cancer (Esquela-Kerscher and Slack, 2006).

Currently, various technologies and tools to investigate the mechanisms of miRNA function have been well documented (Wang et al., 2007). In particular, antisense oligonucleotides (ASOs) have been described that allow selective protection of miRNA binding to the 3′-UTR region of target mRNAs. Chemical modifications of ASOs are necessary not only to improve their affinity for target miRNAs but also to protect the ASOs against nuclease degradation. In addition, these modifications serve to promote tissue uptake of ASOs, which can be applied for in vivo delivery (Davis et al., 2006; Esau, 2008). Many studies using various types of ASOs, such as DNAs, RNAs, locked nucleic acid (LNA)-modified DNAs or 2′-O-methoxyethyl (2′-OMOE)-modified oligonucleotides, have been reported (Davis et al., 2006; Naguibneva et al., 2006; Orom et al., 2006).

Peptide nucleic acids (PNAs) are synthetic nucleic acid analogues in which the sugar-phosphate backbone of the nucleic acid has been replaced by an uncharged N-(2-aminoethyl)-glycine scaffold. The nucleic acid bases are attached to this scaffold via a methylene carbonyl linker. PNAs have several notable properties such as their exceptional biological and chemical stability compared with other nucleic acid analogues. Because the intra-molecular distances and the configuration of the bases are similar to those found in naturally occurring DNA molecules, hybridization occurs specifically between the PNA and its complementary RNA sequences. Compared with DNA-RNA duplexes, PNA-RNA duplexes exhibit increased thermal stability. Because of these favorable biochemical properties, a PNA-based ASO would be predicted to be an effective inhibitor of miRNA activity.

In the present study we tested the ability of a PNA-based ASO to inhibit miRNA activity. The inhibitory effect was evaluated by both the luciferase assay and real-time PCR. We demonstrated that PNA-based ASOs could be used to inhibit miRNA regulation of gene expression and function in mammalian cells more potent than other miRNA inhibitors.

MATERIALS AND METHODS

Construction of reporter plasmid

The plasmids used were the pGL3-control vectors (which encodes for the firefly luciferase) and the pRL-TK vectors which...
Table 1. Sequences of used cell penetrating peptides for delivery of PNA-based ASOs

<table>
<thead>
<tr>
<th>CPP name</th>
<th>Peptide sequence</th>
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<tbody>
<tr>
<td>R6 Pen</td>
<td>RQIKIWFQNRRMKWKK</td>
</tr>
<tr>
<td>Tat</td>
<td>GRKKRRQRRPPQQ</td>
</tr>
<tr>
<td>47Tat57</td>
<td>ggagggrkrrqrrr</td>
</tr>
<tr>
<td>Calionic</td>
<td>KKKK</td>
</tr>
<tr>
<td>C-myc tag</td>
<td>EQLKISEEDLNA</td>
</tr>
<tr>
<td>H region</td>
<td>AAVALLPAVALLLA</td>
</tr>
<tr>
<td>PTD-4</td>
<td>YARAAARQARA</td>
</tr>
<tr>
<td>Tranportan</td>
<td>AGYLGKINKALA-ALAKKIL</td>
</tr>
<tr>
<td>SSBP(I)</td>
<td>PKKRRKV</td>
</tr>
<tr>
<td>Tat-modified</td>
<td>RRRQRRKKRR</td>
</tr>
</tbody>
</table>

encodes for the renilla luciferase (Promega). DNA duplexes containing miRNA binding sequences (corresponding to each target site) with an EcoRI and a Pst overhang were ligated into the 3′ UTR of the firefly luciferase gene within the pGL3-control vectors. The presence of the ligated miRNA binding sites within the pGL3 vectors was confirmed by sequencing analysis.

Design and synthesis of a PNA-based ASOs

PNAs were synthesized as previously described (Lee et al., 2007). The PNA-based ASOs contained an O-linker at the N-terminus of the PNA to improve solubility and were subsequently conjugated to a modified peptide at the N-terminus of the PNA to mediate transfection into cells. In this study, we tested 10 types of cell penetrating peptides (CPPs) (Table 1) (Abes et al., 2007; Koning et al., 2003). Human miRNA sequences were obtained from the miRBase Sequence Database (http://miRNA.sanger.ac.uk, Release 10.1). A scrambled PNA-based ASO was used as a negative control (NC). The sequences of the PNA-based ASO used in study were as follows: PNA16: RRRQRRKKR-O-TATTTACGTGCTGCT; PNA21: RRRQRRKKR-O-TCACATCAGCTGA; PRRQRRKKR-O-PNA24; RRRQRRKKR-O-CGTTCTGGCTGAC; PNA31: RRRQRRKKR-O-ATTAACTGCGGACAA; LNA-modified ASO and 2′-O-methyl (2′-OMe)-modified ASO were purchased from Exiqon and Dharmacon respectively.

Cell culture and transfection

HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin. For experiments, cells were plated in either a 24- or 6-well plate and maintained in antibiotic-free media for 24 h. For lipid-based transfections, ASOs and target gene plasmids were mixed with Lipofectamine 2000 (Invitrogen) and added to cultured cells according to the manufacturer’s instructions. The cells were then harvested 48 h later.

Luciferase assay and Real time PCR

The cells were harvested after 48 h after transfection and assessed for luciferase activity using the Dual-Luciferase Reporter Assay protocol (Promega). For real-time PCR analysis of miRNAs, we used the extracted miRNA fraction from cells using the PureLink miRNA isolation Kit (Invitrogen). In brief, the RT-PCR was performed in 12 μl reaction volume containing 10 ng of miRNA and 3 μl of RT primer (Applied Biosystems). The levels of miRNAs were quantified using a TaqMan MicroRNA Assay Kit (Applied Biosystems) and a 7500 HT Fast Real-Time PCR System (Applied Biosystems). Analysis of bcl-2 expression was quantified with real time PCR using SYBR green (Takara) and normalized to the expression of β-actin. Primers for bcl-2 (Kang et al., 2005) and β-actin were synthesized from Bioneer and the sequences used for the PCR primers used are as follows: bcl-2 forward primer: 5′-GGTGGGTGAGAGGACCTCAGCA; bcl-2 reverse primer: 5′-TGACGCTTCACACACATGAC; β-actin forward primer: 5′-GCTCTGCTGGACCAAGGCTCT; β-actin reverse primer: 5′-CAACGATCTGGGTCATCTTCT.

Determination of cell viability

The MTT [3,4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide tetrazole] reduction assay was used to assess cell viability. Cells were transfected with target DNA and miRNA inhibitors and were incubated for 48 h. MTT was added to wells at final concentrations of 0.5 mg/ml and the plates were incubated at 37°C for 3 h. Following incubation, the growth medium was removed from the cultures and the blue formazan product was solubilized by adding 100 μl of dimethyl sulfoxide to each well. After agitating the plates for 15 min, the optical density of the solubilized formazan product in each well was measured using an automatic microplate reader with a 570 nm test wavelength and a 690 nm reference wavelength.

RESULTS

Selection of an optimal cell penetrating peptide for increasing the cell penetration properties of PNA-based ASOs

Unmodified PNAs have low efflux rates compared to other oligonucleotides (Wittung et al., 1995). However, it has been reported that the CPP conjugation increases the uptake of PNA-based ASOs into organs (Abes et al., 2007; Koning et al., 2003). To increase the cell penetrating properties of PNA-based ASOs for miRNAs, we conjugated a CPP to the N-terminus of a PNA-based ASO. We tested 10 types of well-known CPPs to determine an optimal CPP for use with our PNA-based ASOs (Table 1) (Abes et al., 2007; Koning et al., 2003). Among the CPPs tested, the Tat-modified and the R6 Pen peptide conjugated PNA-based ASOs were twice as effective at inhibiting miRNA function compared to naked, PNA-based ASOs (Fig. 1). We therefore used the Tat-modified peptide conjugated PNA-based ASOs to increase the cell penetration capabilities of our PNA-based ASOs.

Effect of PNA-based ASO on miRNA activity

Normalized luciferase ASO on miRNA activity

Normalized luciferase activity in the presence of a PNA-based ASO.
A 5-200 nM of PNA-based ASOs were treated to HeLa cells and cell lysates were analyzed by luciferase assay. (B) HeLa cells were treated with 200 nM PNA-based ASOs for miR-16. After 48 h, synthesized cDNA by RT-PCR was analyzed by real time PCR for bcl-2. (C) HeLa cell was transfected with 5-400 nM of NC. Cell viability was measured by the MTT assay. NC, negative control; C, no treatment.

ASO increased in a dose-dependent manner (Fig. 2A). Real-time PCR analysis demonstrated that expression of cellular miRNAs decreased in cells transfected with 200 nM of PNA-based ASOs (data not shown). These inhibition effects of miRNA by PNAs were also detected in K562, A549 and MCF7 cells (data not shown).

Many studies have shown that miR-16 negatively regulates Bcl-2 expression (Davis et al, 2006; Pellestor and Paulasova, 2004; Wang et al., 2007). To confirm that our PNA-based ASO was indeed able to inhibit the activity of miRNAs in living cells, we used real-time PCR to determine the bcl-2 mRNA level in cells that were exposed to a miR-16-specific PNA-based ASO (Fig. 2B). bcl-2 mRNA levels increased with increasing concentration of the PNA-based ASO that was administered. This result shows that a PNA-based ASO can influence the gene regulatory function of a miRNA.

While the effective concentration of miRNA inhibitors used in biological research varies, the concentration that is typically used is less than 100 nM. So, we measured the cytotoxicity of concentrations of PNA-based ASOs via the MTT assay. As shown in Fig. 2C, the viability of the cells remained unaffected when exposed to a PNA-based ASO at a concentration of 400 nM. These results show that over 200 nM of PNA-based ASO itself had no appreciable cytotoxic effect on mammalian cells.

Long lasting inhibition of miRNA by PNA-based ASOs
To confirm the maintenance of the PNA-based ASO function in living cells, we sustained cells for 9 d after transfection with PNA21. In treated cells with PNA21, miR-21 expression level decreased compared to control dose-dependently (Fig. 3A), however expression level of let7g is not regulated by PNA21 (data not shown). Treatment of PNA21 during 9 days is not effect on cell viability (Fig. 3B). These results suggest that PNA-based ASO was found to maintain its inhibitory effects until the 9 days.

Stability of PNA-based ASOs
We also tested stability of PNA-based ASOs to storage temperature (room temperature, 4°C and -20°C). PNA24 stored at room temperature, 4°C and -20°C showed no measureable decline in inhibitory activity until the 14 week (Fig. 4). Although optimal storage condition is -20°C, PNA24 that stored at room temperature also maintain the ability of miRNA inhibition. These observations suggest the PNA-based ASOs are stable for intracellular nuclease during long time and easy tool for use.

Comparison of PNA-based ASOs with other miRNA ASOs
Natural oligonucleotides are not suitable to use as antisense reagents because they are susceptible to nuclease digestion (Lebedeva and Stein, 2001; Rayburn and Zhang, 2008). To solve this problem, modified-ASOs such as LNA, 2′-OMe and 2′-OMOE have been used. These modifications increase the efficacy and stability of the antisense molecules (Rayburn and Zhang, 2008). However, there is some data to suggest that they impose a significant risk of hepatotoxicity (Larsen et al., 1999), while LNA-modified ASOs have the potential to improve potency.

Modified ASOs have been used in molecular biology studies including those that focus on miRNA function. In the present study, we compared the inhibitory effect of a PNA-based ASO with other modified-ASOs on miRNA activity. As shown in Fig. 5A, PNA-based ASOs were shown to be more effective than other miRNA inhibitors. When we transfected LNA-modified ASO into cultured cells, cell viabilities were decreased (Fig. 5B). These results illustrate the fact that LNA exhibit some cytotoxicity (Drygin et al., 2007; Larsen et al., 1999; Swayze et al., 2007) compared to PNA.

DISCUSSION
To gain a better understanding of miRNA function, ASOs targeting specific miRNAs have been widely used. The earliest report of miRNA inhibition using ASOs describes the targeting
of miRNAs by the microinjection of DNA oligonucleotides into Drosophila embryos (Boutla et al., 2003; Weiler et al., 2006). However, unmodified DNA oligonucleotides are not effective miRNA inhibitors because DNA is not resistant to nucleases digestion. Thus, these chemical modifications have made it possible to use ASOs as tools to study miRNA function.

PNA is not affected by nuclease such as RNase H and DNase. Additionally, non-ionic PNA has a higher affinity for target DNA or RNA sequences than naturally occurring oligonucleotides because of the lack of electrical repulsion. Another advantage of PNA is that it can be readily conjugated with amino acids or peptides to increase its rate of cellular uptake. Based on these properties, one would predict that PNA might make a very effective ASO to study miRNA activity (Braasch and Corey, 2002; Fabani and Gait, 2008; Larsen et al., 1999; Uhlmann et al., 1996). In the present study, we tested whether PNA-based ASOs could be used as miRNA inhibitors. To confirm miRNA inhibition by PNA-based ASOs, we used the reporter assay, which measures the efficiency of DNA transfection into cells by a transfection reagent such as Lipofectamine 2000. The previous report found that passive diffusion of unmodified PNAs across the lipid membrane was not an effective means of transport into cells (Wittung et al., 1995) and we also found that naked PNA-based ASOs had a weaker inhibitory effect on miRNA activity when compared to a Tat-modified peptide conjugated PNA-based ASO (Fig. 1). So, we used PNA-based ASOs conjugated with a Tat-modified peptide at the N-terminus of the PNA to increase cell penetration.

We tested over 135 types of PNA-based, miRNA-specific ASOs and found that all of the PNA-based ASOs could act as powerful miRNA inhibitors (data not shown). Inhibition of miRNA by PNA-based ASOs was confirmed by analysis of the expression of the miRNA-targeted mRNA or reporter protein and target miRNA. These effects of PNA-based ASOs were found to be dose-dependent (Fig. 2A). Up to a concentration of 400 nM, the PNA-based ASOs could inhibit miRNA function without having a cytotoxic effect on cultured cells (Fig. 2C).

Previous studies have suggested that increasing the nuclease resistance of ASOs could prolong their inhibitory activity. Chemical modification is the principal strategy used to improve the nuclease resistance of ASOs (Watts et al., 2008). ASOs with 2′-sugar modifications (including 2′-OMe, 2′-OMOE, 2′-fluoro (2′-F), and LNA), as well as a phosphorothioate backbone modification can be effective inhibitors of miRNAs in cell culture (Esau, 2008). All of 2′-modifications increases the affinity of the ASO to target RNA to some degree. However, the anti-miRNA activity of 2′-modified ASOs has not always correlated with affinity (Davis et al., 2006; Esau, 2008). The phosphorothioate backbone, while reducing the affinity of the ASO to target RNA to a certain extent, confers a significant increase in the resistance to nuclease degradation; this property may be necessary for long-term assays in cell culture (Esau, 2008). The 2′-modification increased the half-life of the ASO in serum from 3-5 min (for the unmodified ASO) to two to three days. This improved stability translated into higher efficacy in vivo (Watts et al, 2008). Previously, studies have shown that LNA-modified ASOs are more effective as miRNA inhibitors than 2′-OMe-modified ASOs (Esau, 2008; Swayze et al, 2007). However, there is some data that suggest that while LNA-modified ASOs may possess increased potency, they also present a significant risk of hepatotoxicity (Larsen et al., 1999).

In the present study, we compared the effect of our PNA-based ASO with other modified ASOs including 2′-OMe-modified and LNA-modified ASOs. The inhibitory effect of a PNA-based ASO on miRNA-24 activity was over two-fold greater than LNA-modified or 2′-OMe-modified ASOs (Fig. 5A). Transfection of LNA-modified ASO into cultured cell also decreased cell viabilities (Fig. 5B). These results confirm other reports that have shown the LNA-modified ASO to be cytotoxic (Drygin et al., 2007; Swayze et al., 2007). In fact, we compared not only PNA24 but also PNAlet7a, PNAlet7b, PNA10a, PNA16, PNA21, PNA20a and PNA31 with other modified-ASOs and observed that tested PNA-based ASOs have similar or better miRNA inhibition effects than others (data not shown).

We also tested the shelf life of PNA-based ASOs because the inhibitory activity of ASOs decreases as a function of storage temperature and time. This is important since storage re-
restrictions exist on ASOs that are shipped overseas. The PNA-based ASOs that were stored at room temperature and 4°C showed no measurable decline in inhibitory activity for up to 14 weeks. Other ASOs however, such as 2′-OME-modified and LNA-modified ASOs, showed decreased miRNA inhibitory activity, which correlated to the amount of time and the storage conditions that the ASOs were kept (data not shown). In addition, PNA-based ASO has long lasting effect on miRNA inhibition (Fig. 3). It is implicated that PNA-based ASOs could inhibit cellular miRNA in the presence of serum. One of the major challenges in ASO development has been the instability of conditions that the ASOs were kept (data not shown). In addition, PNA-based ASO has long lasting effect on miRNA inhibition, which correlated to the amount of time and the storage conditions that the ASOs were kept (data not shown). In addition, PNA-based ASO has long lasting effect on miRNA inhibition (Fig. 3). It is implicated that PNA-based ASOs could inhibit cellular miRNA in the presence of serum. One of the major challenges in ASO development has been the instability of ASOs in serum. Thus, these results demonstrate the improvements that PNA-based ASOs present over other ASOs.

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REFERENCES