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Sequence Selective Recognition of Double-Stranded RNA at Physiologically Relevant Conditions Using PNA-Peptide Conjugates

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Abstract

Conjugation of short peptide nucleic acids (PNA) with tetralysine peptides strongly enhanced triple helical binding to RNA at physiologically relevant conditions. The PNA hexamers and heptamers carrying cationic nucleobase and tetralysine modifications displayed high binding affinity for complementary double-stranded RNA without compromising sequence selectivity. The PNA-peptide conjugates had unique preference for binding double-stranded RNA, while having little, if any, affinity for double-stranded DNA. The cationic PNAs were efficiently taken up by HEK293 cells, while little uptake was observed for unmodified PNA.

Peptide nucleic acids (PNA) have become important research tools for molecular recognition of double helical DNA.[1] Although PNA-DNA triple helices have been studied in detail,[2] analogous PNA-RNA triplexes were virtually unknown prior to our recent work.[3–5] We discovered that PNA formed stable and sequence selective Hoogsteen triple helices with RNA at mildly acidic (pH 5.5) conditions.[3] We also found that isolated pyrimidine interruptions in the purine rich strand of an RNA duplex could be recognized using nucleobase-modified PNA at pH 6.25.[4] Most recently, we found that 2-aminopyridine (**M**, Figure 1) nucleobase modification enabled stable triple helix formation at physiologically relevant pH, salt concentration and temperature.[5] Herein we report that conjugation of short cationic peptide (Lys_4) to **M**-modified PNA significantly improved RNA binding affinity without compromising sequence selectivity. The doubly modified PNAs had unique selectivity for double stranded RNA (dsRNA) compared to double stranded DNA (dsDNA). Furthermore, PNAs carrying **M** and Lys modifications were efficiently taken up by HEK-293 cells, while the unmodified PNA showed little uptake.

The groups of Corey[6–8] and Gait[9, 10] demonstrated that short oligolysine peptides greatly enhanced the delivery of conjugated PNA in cultured cells. Nielsen and co-workers[2] showed that pseudoisocytidine (**J**) nucleobase modification and conjugation of PNA with four lysines were both required for full stability of PNA-DNA triple helix at physiologically relevant conditions. Our interest in testing short oligolysine peptides was further stimulated

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Supporting Information Available Details of PNA synthesis, ITC experiments and data, UV and CD melting curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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by a study of Strömberg and co-workers,[11] who showed that addition of cationic peptides to UV melting buffer enhanced the thermal stability of 2'-*O*-MeRNA/RNA duplexes, but had no effect on the stability of DNA/DNA duplexes. We hypothesized that the stability enhancement was due to the cationic peptides binding selectively in the deep and narrow major groove of RNA, but not in the wider major groove of DNA. Since the triple helical binding of PNA also occurs in the major groove of RNA, we further hypothesized that conjugation of short oligolysine to PNA may significantly increase stability of PNA-RNA triple helices. If these hypotheses were true the resulting conjugates should be selective ligands for dsRNA, while binding less strongly to dsDNA. This would be an important benefit for potential in vivo applications in addition to the cell permeability. Herein we present results that support the above hypotheses.

In our previous studies we found that unmodified PNA dodecamers did not bind to double stranded RNA at physiologically relevant pH.[3–5] Attachment of four additional D-lysines to a PNA hexamer led to detectable binding of **PNA1** to dsRNA (Figure 2 and Table 1) at pH 7.4 and 37 °C in 10 mM sodium phosphate buffer containing 1 mM $MgCl_2$, 50 mM NaCl and 0.1 mM EDTA. However, **PNA1** had no sequence selectivity binding to all four hairpins featuring the variable base pair with approximately equal affinity (Table 1). At a higher salt concentration of the physiologically relevant buffer (2 mM $MgCl_2$, 90 mM KCl, 10 mM NaCl, 50 mM potassium phosphate), pH 7.4 and 37 °C we could not observe any binding of **PNA1** to the matched hairpin **HRP2** (Supplementary Figure S5). These results were consistent with binding being driven by the cationic peptide with relatively little PNA contribution due to unfavorable cytosine protonation.

Substitution of cytosine with 2-aminopyridine (**M**) led to sharp increase in binding affinity at physiologically relevant conditions (Table 1); **PNA2** bound to the matched **HRP2** with an association constant (K_a) of 1.7×10^8 M^{-1}. Despite the highly charged nature, **PNA2** had very good sequence selectivity. The high affinity was an additive effect of both **M** and Lys modifications, as **PNA3** lacking the four additional lysines at the amino end had only modest binding affinity for **HRP2**. This result was confirmed using another PNA sequence, **PNA4**, which had four lysine modifications at the carboxyl end and targeted a different region in **HRP2** (Table 1).

The RNA targets **HRP1–HRP4** were chosen from our previous studies, so that we could compare the lysine modified PNA with other modified PNAs tested in our earlier work.[3–5] The sequences of **PNA2** and **PNA4** were chosen so as to leave sufficient space in the major groove of the relatively short **HRP2** for binding of the tetralysine residue. The lower affinity of **PNA4** compared to **PNA2** suggested that future studies will be needed to explore sequence dependency and amino vs. carboxyl end lysine modification.

UV and CD thermal melting results were consistent with ITC data. UV melting traces showed relatively weak transitions for triplex dissociation (Supplementary Figure S15). For the matched triplexes **PNA2-HRP2** and **PNA4–HRP2**, the triplex dissociation overlapped with hairpin melting giving one transition at around 90 °C (the t_m for **HRP2** only is 91 °C). Consistent with a relatively higher affinity of PNAs for the mismatched **HRP1**, the UV melting curves showed weak transitions at 46 (**PNA2–HRP1**) and 36 (**PNA4–HRP1**) °C, while other mismatched triplexes had $t_m < 35$ °C. The triplex melting was also observed in temperature dependent CD spectra (Supplementary Figures S16 and S17) that showed characteristic transitions at 230–240 nm and 300–320 nm and melting temperatures similar to those observed in UV experiments. In contrast to UV melting curves, in CD melting plots we observed transitions that could be assigned to triplex dissociation of the matched **PNA2-HRP2** and **PNA4–HRP2** at around 70 °C. The CD melting data were consistent with our previous results on PNAs having only **M** modifications.[5] Taken together, the results of ITC,

UV and CD experiments confirmed our hypothesis that the cationic **M** and Lys modifications would provide mutual and additive stabilization of PNA-RNA triple helices.

Most remarkably, we could not detect any binding of either **PNA2** or **PNA4** to their matched DNA hairpin **HRP5** (Figure 2). Low affinity of short PNA for dsDNA was consistent with Nielsen's report that strong PNA-DNA triple helices were formed by PNA 15-mers while no binding could be detected for a PNA 10-mer.[2] Nielsen and co-workers used pseudoisocytosine (**J**) and lysine modified PNA similar to our PNA compounds reported here. Thus, the unusually high affinity of PNA hexamer **PNA2** and heptamer **PNA4** for the matched RNA target **HRP2** was the surprising result that provided strong support to our hypothesis that the cationic peptides would bind selectively in the major groove of dsRNA and confer unique RNA selectivity (as compared to the same DNA sequences) on our PNAs. The high sequence selectivity (mismatch discrimination) and RNA preference of **PNA2** and **PNA4** becomes especially remarkable if one considers that these PNAs may carry up to eight positive charges when binding to RNA. Such selectivity is in sharp contrast to other cationic RNA binding ligands, e.g., aminoglycosides, which are notoriously promiscuous RNA binders.

While we do not have data providing insight into the unique RNA selectivity of our PNAs, it is conceivable that the deep and narrow major groove of RNA presents a better steric fit for the PNA and peptide ligands than the wider major groove of DNA. In support of this notion, Dervan and co-workers[12] recently reported that the DNA minor groove-binding polyamides do not bind in the shallow minor groove of RNA. Taken together our and Dervan's results emphasize that the distinct conformations of dsRNA and dsDNA make the molecular recognition of each of these biopolymers a unique task. Peptide based ligands have been previously used for molecular recognition of pri-miRNAs by helix-threading peptides[13, 14] and TAR RNA of HIV by Tat-derived[15] and, recently, de-novo designed branched peptides.[16] While good binding affinity and selectivity was observed, the structural details of peptide binding to dsRNA have not been elucidated.

Poor cellular uptake of unmodified PNA has been a major bottleneck for practical applications.[1, 17] Based on the aforementioned results by Corey[6–8] and Gait,[9, 10] we envisioned that the cationic nucleobase and lysine modifications, besides enhancing the triple helix stability, might also improve the cellular uptake of PNAs. To obtain a preliminary insight into cellular uptake of our cationic PNAs we prepared a fluorescein labeled Fl-Lys-$(eg1)_2$-D-Lys-MTETMMMM-$(D\text{-Lys})_3$ (**PNA5**, Figure 3A), a variant of this sequence without **M**-modifications (**PNA6**) and an unmodified **PNA7**. In these PNAs, eg1 is 2-(2-aminoethoxy)ethoxy acetic acid spacer and **E** is 3-oxo-2,3-dihydropyridazine nucleobase (for full structures see Supplementary Figure S18). The **E**-modification, designed to recognize T-A and U-A interruptions in polypurine tracts,[18] was prepared following our previously reported procedures.[4] The specific PNA sequence chosen targets a purine rich tract (modeled in **HRP6**, Figure 3A) of pri-miRNA-155, an oncogenic microRNA overexpressed in various B-cell cancers.[19] We were interested in cellular uptake of **PNA5** as the next step toward exploring potential anticancer effects of pri-miRNA-155 binding by triple helix forming PNA.

Live HEK293 cells were incubated with 4 μM PNAs in Dulbecco's Modified Eagle's Medium at 37 °C for 24 h. The cells were washed with PBS buffer and immediately imaged without fixation (Figure 3B and 3C). In the first experiment, confocal fluorescence microscopy showed that the **M**- and Lys-modified **PNA5** efficiently penetrated HEK293 cells as judged by strong fluorescence in the upper panel of Figure 3B. In contrast, we could see little, if any, uptake of unmodified **PNA7** as judged by low fluorescence of HEK293 cells in the lower panel of Figure 3B. In the second experiment, comparison of PNAs

carrying different modifications (Figure 3C) showed that both **M**- and Lys-modifications contributed significantly to the efficient uptake of **PNA5**. While **PNA6**, bearing only additional Lys-modifications showed some uptake, the fluorescence levels where significantly lower than that for the doubly modified **PNA5**. As in the first experiment, the unmodified **PNA7** was not taken up by the HEK293 cells.

Finally, we studied the binding of **PNA5** to **HRP6** modeling the purine rich region (highlighted in blue in Figure 3A) of pri-miRNA-155. Analysis of the ITC trace (Supplementary Figure S19) showed that, as expected, **PNA5** recognized **HRP6** with high affinity ($K_a = 3.4 \times 10^7\ M^{-1}$) and close to 1:1 stoichiometry under physiologically relevant conditions. Consistent with our previous results on a similar recognition of pri-miRNA-215 hairpin,[5] the non-canonical structures in the pri-miRNA hairpin, the UoG wobble pair and A-bulge did not prevent formation of a stable PNA-RNA complex.

Biologically relevant double helical RNAs typically do not contain long polypurine stretches. However, it is common to find eight and more contiguous purines interrupted by one or two pyrimidines in ribosomal RNAs[20] and miRNAs.[21] While such short sequences are not unique in human genome, the fact that the triple helix forming PNAs require RNA to be in a double-stranded conformation increases the uniqueness of the recognition site. For example, BLAST search of a comprehensive non-coding RNA database[22] reveled that in humans 5′-GAUAGGGG, the pri-miRNA-155 recognition site in **HRP6**, is also found in several Piwi-interacting RNAs and non-coding RNA regions (total of 11 hits). However, in these RNAs the eight nucleotides are not part of double-stranded helices and, thus, are not expected to be viable recognition sites for **PNA5**. Among human pri-miRNAs, 5′-GAUAGGGG is unique to pri-miRNA-155; however pri-miRNA-3152 and pri-miRNA-6505 share a seven nucleotide sequence, 5′-AUAGGGG.[21] If necessary, the specificity of PNA for pri-miRNA-155 can be further enhanced by extending the triplex recognition site to 5′-GUGAUAGGGG.

In summary, PNAs carrying multiple cationic **M** and lysine modifications displayed high affinity and favorable sequence selectivity in triple helical binding to dsRNA. Attachment of short lysine peptides to PNA strongly enhanced binding of PNA to dsRNA while no binding could be observed to matched dsDNA. This is important for potential future applications in cells to eliminate non-specific binding of PNA to nuclear dsDNA, which may cause undesired off-target effects. From this perspective the high RNA selectivity of our cationic PNAs is very encouraging. The results supported our initial hypotheses that cationic peptides: 1) prefer binding into the deep and narrow major grove of RNA over the major groove of DNA and 2) will provide additional stabilization of PNA-RNA triple helices. Finally, we observed encouraging cellular uptake of the **M**- and Lys-modified PNA. Both modifications were important for efficient uptake of our PNAs. Our results suggest that PNAs carrying cationic **M**- and Lys-modifications may be promising compounds for modulating the function of biologically relevant double stranded RNA species in live cells.

METHODS

For experimental details, see Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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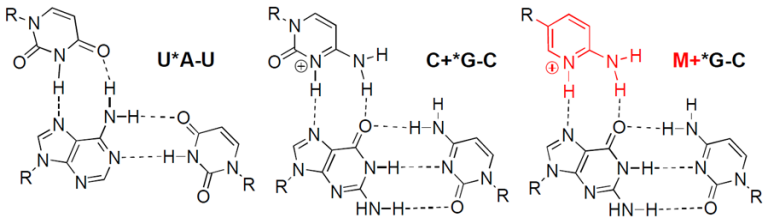

Figure 1.
Hoogsteen triplets used in RNA recognition.

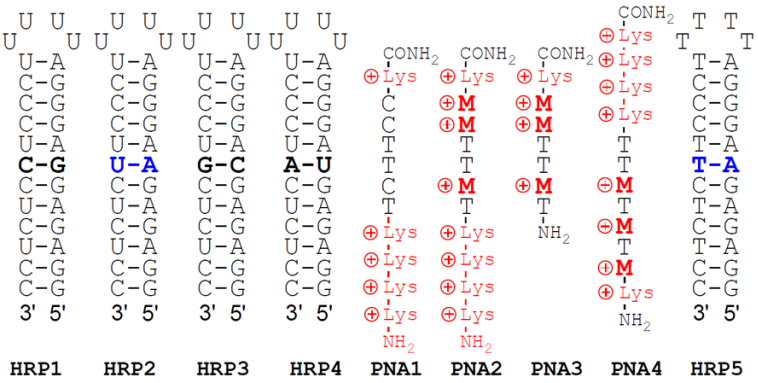

Figure 2.
Sequences of PNA, and RNA and DNA hairpins.

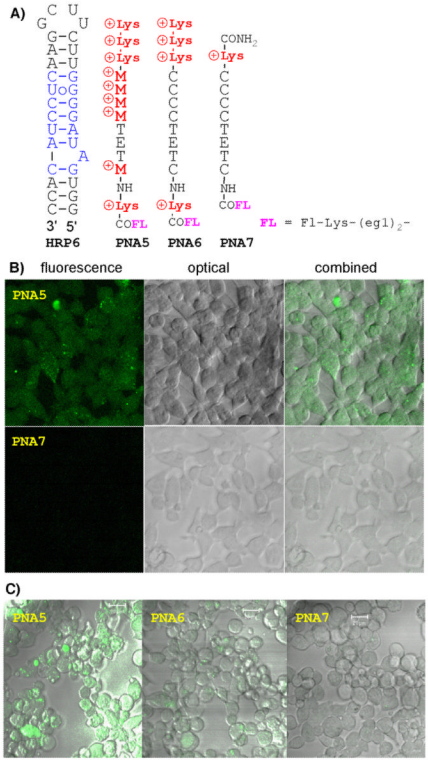

Figure 3.

A) Structures of **HRP6** modelling the PNA binding site of pri-miRNA-155 and fluorescein labeled PNAs; B) Uptake of **PNA5** (upper panel) and **PNA7** (lower panel) in live HEK293 cells monitored using confocal laser microscopy. The columns (left to right) represent fluorescence, optical and combined images taken with a 40 × objective; C) The combined fluorescence and optical images comparing uptake of **M**- and Lys-modified **PNA5**, Lys-modified **PNA6**, and unmodified **PNA7** in live HEK293 cells.

Table 1

Binding of M- and Lys-modified PNAto RNA targets[a]

PNA	HRP1 (C-G)	HRP2 (U-A)	HRP3 (G-C)	HRP4 (A-U)
PNA1 [b]	0.34	**0.19**	0.31	0.03
PNA2	0.52	**16.5**	0.01	NB[c]
PNA3 [d]	NB[c]	**0.4**	NB[c]	NB[c]
PNA4	0.23	**1.8**	0.06	-

[a] Association constants $K_a \times 10^7$ M^{-1} in 2 mM $MgCl_2$, 90 mM KCl, 10 mM NaCl, 50 mM potassium phosphate at pH 7.4 and 37 °C;

[b] In 10 mM sodium phosphate buffer containing 1 mM $MgCl_2$, 50 mM NaCl and 0.1 mM EDTA at pH 7.4 and 37 °C;

[c] NB – no binding, $K_a < 10^3$;

[d] From ref. 5.