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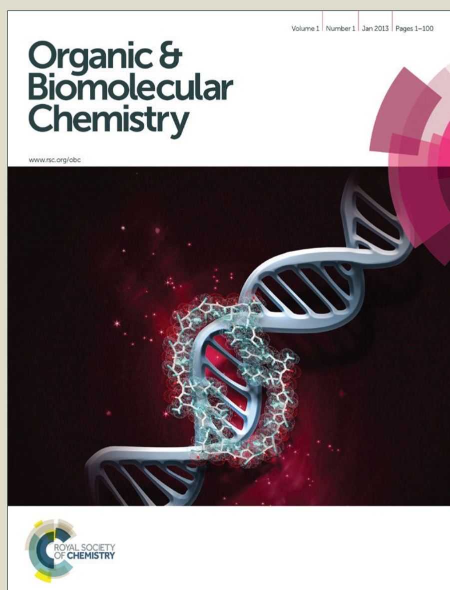

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Journal Name

COMMUNICATION

Clamping of RNA with PNA enables targeting of microRNA

Alice Ghidini[a], Helen Bergquist[b], Merita Murtola[a,c], Tanel Punga[b], Rula Zain[d,e] and Roger Strömberg[a]*

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To be able to target microRNAs also at stages where these are in a double stranded or hairpin form we have studied BisPNA designed to clamp the target and give sufficient affinity to allow for strand invasion. We show that BisPNA complexes are more stable with RNA than with DNA. In addition, 24-mer BisPNA (AntimiR) constructs form complexes with a hairpin RNA that is a model of the microRNA miR-376b, suggesting that PNA-clamping may be an effective way of targeting microRNAs.

In nucleic acid therapeutics, microRNA (miRNA) targeting is emerging as an alternative approach with high potential.[1,2] A number of stages in the miRNA biogenesis pathway can be considered as targetable and in most of these stages the miRNA is either present as a hairpin structure or as a double stranded RNA (dsRNA).[3] Use of a modified oligonucleotide to target the dsRNA or hairpin would then need to involve strand invasion, formation of a triple helix (triplex)[4] or both. For such purposes peptide nucleic acids (PNA)[5] may be highly suitable as these have been shown to be efficient in perturbing double stranded DNA[6, 7] and tail-clamp PNAs significantly increase binding to DNA[8,9]. There have been only a few reports on triplexes between RNA and PNA. Rozners et al. have shown that PNA can form stable triplexes with dsRNA[10] and that, with inclusion of 2-aminopyridine bases, these triplexes can be stable at physiological pH.[11] Strand invasion of a short hairpin RNA by PNA, where both 1:1 and 1:2 complexes, as identified by Maldi-Tof mass spectrometry, has been reported.[12] It has been shown that a PNA-clamp targeting mRNA can reduce translation of the RNA into protein.[13]

[a] Department of Biosciences and Nutrition, Karolinska Institutet,Novum, 141 83 Huddinge, Stockholm, Sweden
[b] Department of Medical Biochemistry and Microbiology, Uppsala University, BMC, 75123 Uppsala, Sweden
[c] Department of Chemistry, University of Turku, 20014 Turku, Finland
[d] Department of Laboratory Medicine, Clinical Research Center, Karolinska Institutet, 141 86 Huddinge, Stockholm, Sweden
[e] Centre for Rare Diseases, Karolinska University Hospital, 17176, Stockholm, Sweden.
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There have been quite a few reports on use of PNA for targeting of miRNA although generally by duplex formation[14-16], and often focused on promoting uptake of the PNA[17-19]. In order to see if there may a benefit of targeting double stranded or hairpin miRNA with PNA-clamps we have, in the present study, first investigated the stability of complexes of BisPNA clamps with RNA and compared these to complexes with similar DNA targets.

We started with an RNA target sequence derived from our studies on PNA based artificial nucleases (PNAzymes)[20, 21] where we altered the 5'-end to encompass 6 adenosines, thus allowing for TAT triplex formation (Figure 1). A BisPNA (BisPNA 1, Figure 1), where the additional thymine stretch was linked with two AEEPs (9-amino-4,7-dioxanonanoic acid), was synthesized and the thermal melting temperature of the complex with RNA was determined to be 80 °C. The melting point (T_m) for the corresponding duplex between the RNA and PNA 2 was 8 °C lower showing that the additional thymines added after the linker in BisPNA 1 did improve the affinity to the RNA target. Since the melting curve was on the borderline of being outside a recordable region for getting a complete curve (see Supporting information) we also made a construct where a GC base pair was swapped for a GT wobble pair to create the complex of the RNA with BisPNA 3 that has a lower T_m value (73°C, Figure 1).

It seems reasonable that the higher Tm with BisPNA 1 as compared to with PNA 2 is due to clamping by triplex formation with the additional thymine bases. Comparison of thermal melting with different ratios of PNA 2 to RNA target 1 show that an increase in ratio gives a higher Tm, although only with a PNA-RNA ratio 3:1 or higher and with 8:1 this goes up with 5 °C (see supporting information S4). It then seems reasonable to conclude that there is little or no triplex formation with equimolar amounts of PNA 2 to RNA target 1. The tethering of the Watson-Crick pairing sequence with the triplex forming T_6 appear to give a considerable intramolecular effect compared to having two separate PNA 2 molecules forming a triplex with the RNA. Binding of a shorter PNA with

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only Ts and one G (PNA 4, Figure 1) with the RNA target also resulted in a higher T_m at a PNA:RNA ratio of 3:1 compared to the T_m obtained at 1:1 ratio (Figure 1).

BisPNA is well known to bind DNA by combined Watson-Crick base-pairing and Hoogsteen-type triplex formation. This PNA-clamp invasion forms complexes that are considered as quite stable.[6-9]

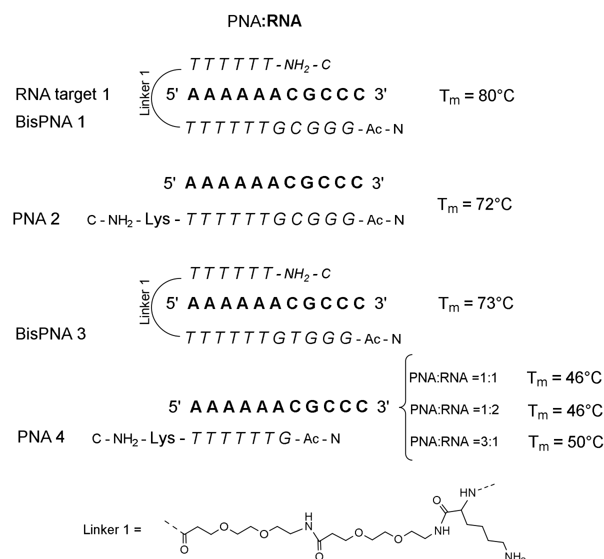

Figure 1. Schematic representation of BisPNA:RNA complexes and the corresponding melting temperatures.

When comparing the thermal melting of the complexes of RNA target 1 (Figure 1) and DNA target 1 (Figure 2) with BisPNA 1 it is clear that the complex with the PNA clamp is less readily dissociated from the RNA target. A similar difference in T_m was found for the complexes with BisPNA 3 that include a wobble GT pair (Figure 1 and 2). The difference between the T_m for the complexes formed using PNA 2 or BisPNA 1 is the same (8 °C) with the DNA as with the corresponding RNA target. The difference in melting point between the DNA and RNA duplexes with PNA 2 is the same (6 °C) as between BisPNA 1 complexes with DNA and RNA (Figure 1 and 2). This suggests that the stabilization with the clamp is similar for DNA and RNA, but that the BisPNA gives a more stable complex with RNA is due to the more stable Watson-Crick pairing in the duplex part.

To examine if a polypurine sequence composed of both guanosines and adenosines will form a reasonably stable complex with a BisPNA at physiological pH (where only one Hoogsteen H-bond would be formed in a CGC triplex) we investigated interaction of BisPNA 5 with RNA target 2 (Figure 3). The complex of RNA target 2 with BisPNA 5 gave a higher T_m than for the duplex with PNA 7 (Figure 3) showing a clear stabilization with the PNA clamp.

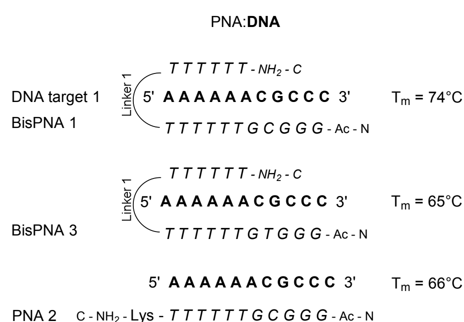

Figure 2. Schematic representation of BisPNA:DNA complexes and corresponding melting temperatures.

A PNA clamp construct, BisPNA 6, that theoretically could form an antiparallel triplex was also synthesized by switching direction of the PNA during synthesis and make use of a diaminopropionic acid branching point to link the two C-terminals of the duplex and triplex forming PNA parts (Figure 3). Although BisPNA 6 in theory could form both TAT and CGG triplexes with RNA target 2 the thermal melting point did not suggest stabilization as compared to the duplex of the RNA with PNA 7, but the potential triplex stretch is relatively short. We do not at this stage have direct proof that clamping to form a triplex is occurring with the studied BisPNAs but it seems like a reasonable hypothesis that this is the main reason for higher melting points than with the duplexes. We did perform CD analysis of several of the complexes and there are some differences in the CD spectra for the 1:1 vs 2:1 ratios of PNA 4 and RNA target 1. There are also differences in the CD spectra for complexes between RNA target 1 and PNA 2 vs BisPNA 1 and 3 (see Supporting information). Clearly there is some structural difference, but it is difficult to interpret the data and there seem to be no reported CD studies on PNA:RNA:PNA triplexes. We do, however, think that we can recognize some characteristics that resemble those found in CD analysis of PNA:DNA:PNA triplexes.[22]

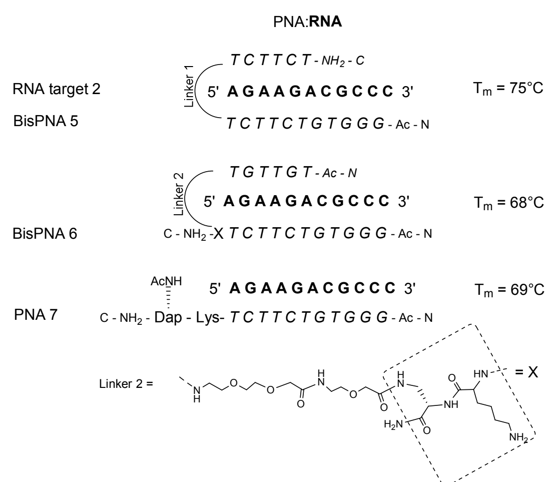

Figure 3. Schematic representation of the parallel and antiparallel BisPNA:RNA complexes and the corresponding melting temperatures.

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Encouraged by the results that PNA clamps can form even more stable complexes with RNA than with DNA it seemed appropriate to target double stranded RNA, especially since miRNAs are present in that form in most stages during their biogenesis. The most demanding would be to invade a hairpin structure and form a complex with one of the strands. An interesting target is miR-376b, a human miRNA that controls autophagy.[23] We chose to study an RNA target corresponding to a truncated version of miR-376b. Our model, "miR-376b", is a hairpin that includes the whole region (the so called 5p and 3p strands) that would remain in the mature miRNA after the Dicer processing in cells. This "miR-376b" 3p strand contains a 9 nucleotide polypurine stretch with 6 adenosines and 3 guanosines in the targeting region. Two AntimiR PNA clamps were synthesized, with the potential to form either parallel (AntimiR 1) or antiparallel (AntimirR 2) triplexes [a reference PNA (Ref PNA) without the triplex forming unit was also prepared] (Figure 4).

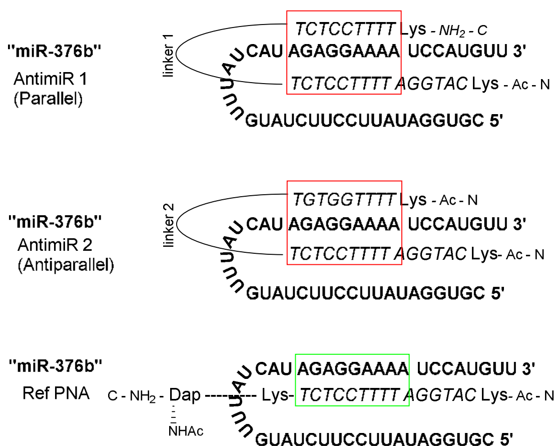

Figure 4. Structures of the parallel and antiparallel AntimiR:"miR-376b" complexes.

To investigate the interaction of the two AntimiRs and the Ref PNA with "miR-376b" we performed binding experiments of ^{32}P labelled target RNA in the presence of different concentrations of PNAs followed by analysis using an electrophoretic mobility gel shift assay (EMSA). Already after 1 h incubation, we were able to detect interaction with the RNA, in particular with AntimiR 1, as shown by the presence of bands with slower mobility (Figure 5, panel a, arrow 3 and 4) and a decrease in the bands for the non-bound target miRNA. After longer incubation time the main initial band (Figure 5, Panel a, arrow 1) from the microRNA is reduced in intensity while a shift to new bands appears (Figure 5, panel b, arrow 3 and 4). AntimiR 2, which is designed to form an antiparallel triplex, appears to also form a PNA complex as detected by the appearance of new bands (Figure 5, panel c, arrow 3 and 5). However, the intensity of the bands reveals a considerably lower amount of the formed PNA-RNA complex, which also seems to require longer incubation whereas AntimiR 1 presents a clear shift already after 24 h of incubation (Figure 5, panel b).

Our results show that AntimiR 1, with the ability to form a parallel triplex, has a clear affinity for the miRNA target at neutral pH. The gel shift assay alone doesn´t provide evidence that strand invasion and clamping, by formation of a triplex, is the mechanism behind complex formation. Together with the thermal melting experiments with the shorter constructs, it seems reasonable that the affinity of AntimiR 1 to "miR-376b" is mainly due to that the PNA-clamp utilizes both Watson-Crick pairing and triplex formation to invade the hairpin miRNA.

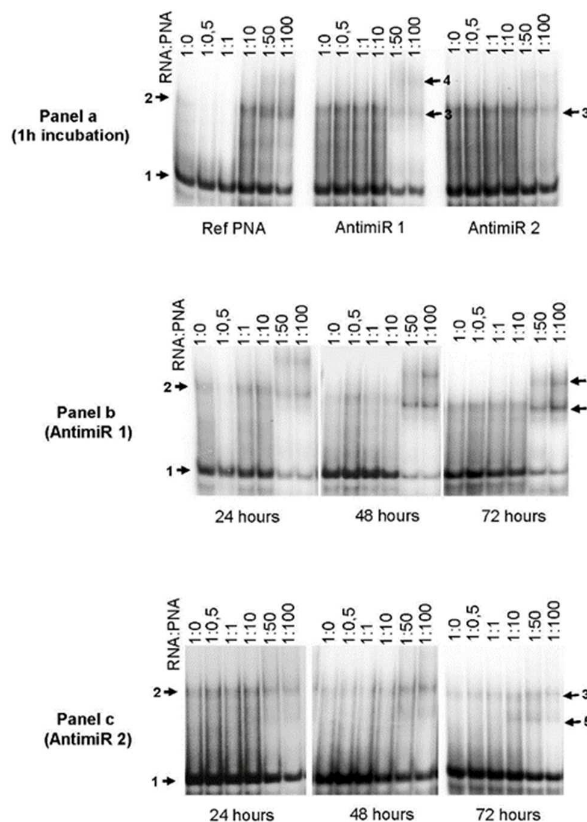

Figure 5. Complex formation between PNA and "miR-376b". Electrophoretic mobility shift assay displaying migration of ^{32}P-labelled "miR-376b" on PAGE after incubation during 1, 24, 48 or 72 hours at pH 7,1 in the presence of increasing concentrations of AntimiR 1, AntimiR 2 or Ref PNA.

With triplex formation being responsible for the higher affinity of AntimiR 1 to "miR-376b" we should expect an increase in affinity at lower pH where the cytosines are protonated and able to form additional hydrogen bonds in CGC^{+} triads. This was supported by the higher temperature required for melting of the BisPNA 5/RNA target 2 complex at lower pH (see Supporting information) and was also confirmed by binding experiments at pH 5,5 (Figure 6). The EMSA assay clearly displays a more complete binding of the "miR-376b" target than at neutral pH. A lower PNA concentration is required, complexation is complete (Figure 6, panel a and b, arrow 3 and 4) already after 24 hours and the main band from the native microRNA disappears (Figure 6, panel a and b, arrow 1). The control Ref PNA also appear to form a complex (possibly due

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to formation of a triplex by binding of two PNA molecules to the RNA) but this is incomplete even at higher concentration of PNA and longer incubation time (Figure 6, panel a and b, arrow 3).

The enhanced extent of complex formation at lower pH further supports that triplex formation is involved in the binding mechanism of AntimiR 1 leading to the formation of a high affinity PNA:RNA:PNA complex.

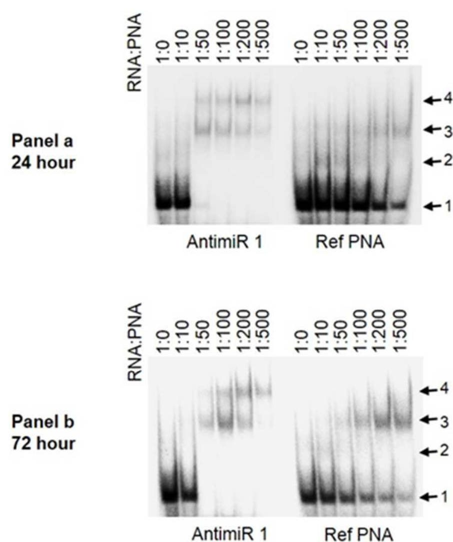

Figure 6. Complex formation between PNA and "miR-376b". Electrophoretic mobility shift assay (EMSA) displaying migration of ^{32}P-labelled "miR-376b" on PAGE after 24 and 72 hours incubation at pH 5,5 in the presence of increasing concentrations of AntimiR 1 or Ref PNA.

In the present study we show that PNA clamps with an additional triplex forming part forms more stable complexes with RNA than with the corresponding DNA target. We also show that a PNA-clamp with the possibility to form a parallel triplex is more efficient in binding to a miRNA hairpin structure than either the corresponding construct with a potential antiparallel triplex forming part or the non-clamping PNA. The apparent complexing of our BisPNA with a hairpin miRNA and the stability of the complex suggests that PNA-clamping may be more effective than classical duplex formation in targeting of microRNAs, as these can then be targeted at almost all stages of their biogenesis, as a hairpin, as a double stranded RNA as well as in its final single stranded form.

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