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Article

Comparison of 2-Aminopyridine and 4-Thiopseudisocytosine PNA Nucleobases for Hoogsteen Recognition of Guanosine in RNA

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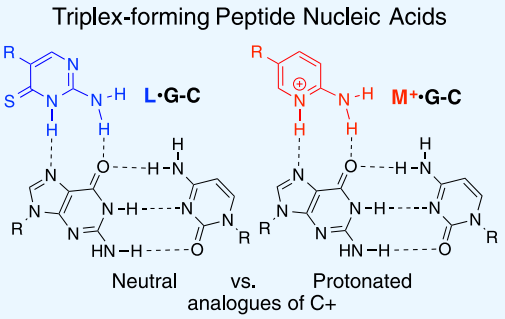
ABSTRACT: Peptide nucleic acid (PNA) is emerging as a promising ligand for triple-helical recognition of folded biologically relevant RNA. Chemical modifications are actively being developed to achieve high affinity and sequence specificity under physiological conditions. In this study, we compared two modified PNA nucleobases, 2-aminopyridine (M) and 4-thiopseudisocytosine (L), as alternatives to protonated cytosine (unfavorable under physiological conditions), to form more stable triplets than C+·G-C. Both nucleobases formed M+·G-C and L·G-C triplets of similar stability; however, the L-modified PNAs showed somewhat reduced sequence specificity. In conclusion, M and L represent two alternative solutions to the problem of cytosine protonation in triple-helical recognition of RNA. In M, the pK_a is increased to favor partial protonation, which improves solubility and cellular uptake of M-modified PNAs. In L, the sulfur substitution enhances favorable hydrophobic interactions, which may have advantages in avoiding off-target effects that may be caused by cationic modifications. However, our results showed that substituting Ms with Ls did not restore the sequence specificity of a PNA containing cationic groups.



INTRODUCTION

Peptide nucleic acid (PNA) was originally developed by Nielsen and co-workers as a triplex-forming ligand for Hoogsteen hydrogen bonding to double-stranded DNA (dsDNA).[1] The main design feature was the neutral pseudopeptide backbone (Figure 1) that was expected to enhance the kinetics and thermodynamics of binding to the negatively charged dsDNA by eliminating electrostatic repulsion that destabilized native DNA triplexes.[2] The unusually high affinity of PNAs for single-stranded DNA and RNA and the unique ability to invade dsDNA by clamping on the purine-rich strand and replacing the pyrimidine-rich strand of DNA as a single-stranded loop led to many practical applications of PNAs beyond the original intent.[3,4] Nevertheless, triplex-forming PNAs are still actively being developed for recognition of biologically important double-stranded RNAs (dsRNAs)[5] and as part of PNA tail-clamps to optimize invasion of dsDNA.[3]

In general, the RNA triple helices have been much less explored than the DNA counterparts.[6] Triple-helical binding of unmodified PNAs to dsRNA at pH 5.5 was first reported in 2010 by Rozners and co-workers.[7] The low pH was required to enable cytosine protonation (pK_a ∼ 4.5) to form the C+·G-C triplet (Figure 1) and stabilize the native Hoogsteen triplexes. Consequently, many research groups have developed modified nucleobases either to mimic protonated cytosine or to modulate the pK_a, facilitating protonation at physiological pH.[8] In the former strategy, Nielsen and co-workers adopted pseudoisocytosine (J in Figure 1),[9] originally developed by Kan and co-workers[10] for stabilizing DNA triplexes, as a PNA nucleobase displaying the hydrogen bonding scheme of protonated cytosine. Since then, J has become the modification of choice for removing the pH dependency of PNA-dsDNA triplexes and increasing the PNA triplex stability under physiological conditions.

Replacing C═O with C═S at the 2-position of pyrimidine nucleobases enhances the stability of Hoogsteen triplets in DNA and RNA triplexes.[11,12] These modifications have also been used to optimize triplex-forming PNAs as well. Chen and co-workers[13] adopted 4-thiopseudoisocytosine (L in Figure 1), a derivative of J previously developed by Sekine and co-workers[11] for DNA triplexes, as an improved nucleobase for triplex-forming PNAs binding to dsRNA. Replacement of the C═O with C═S group resulted in substantially stronger binding of L (compared to J) to the Hoogsteen face of guanosine.[13] The authors proposed that the enhanced affinity

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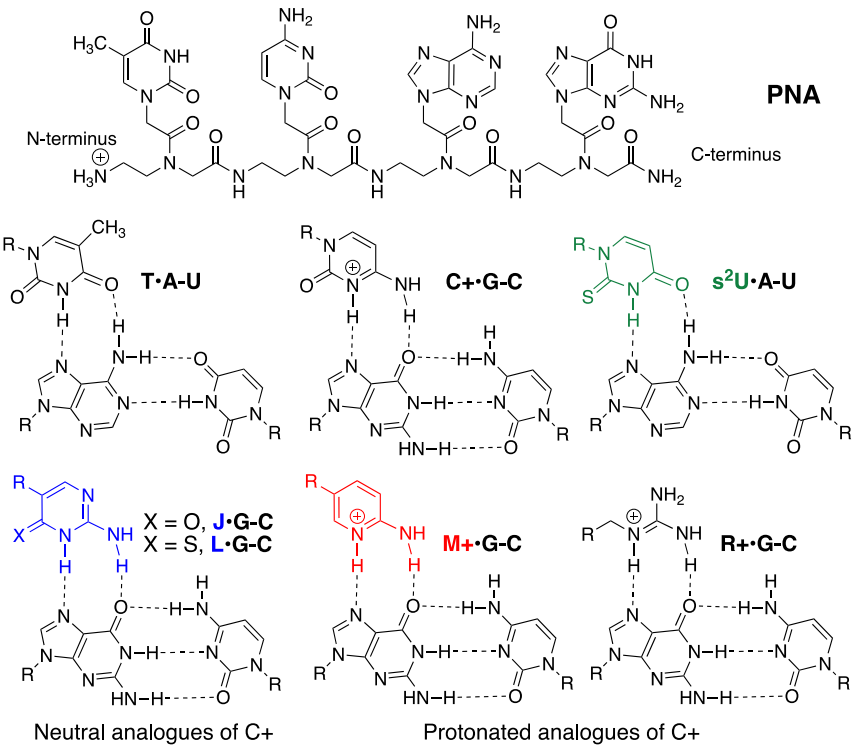

Figure 1. Structures of PNA, native Hoogsteen triplets (T·A-U and C+·G-C), and triplets formed by modified nucleobases.

of L resulted from favorable van der Waals interactions of C═S, improved base stacking, stronger hydrogen bonding of N−H (pK_a modulation by the neighboring C═S), and reduced dehydration penalty.[13] Compared to J, the C═S functionality prevented L from forming Watson−Crick base pairs with G, which greatly reduces off-target binding of L-modified PNAs to single-stranded nucleic acids (ssDNA and ssRNA).[13] Similar to L, substitution of C═O with C═S at the 2-position of uridine in PNAs resulted in more stable s^2U·A-U triplets (Figure 1) compared to the canonical T·A-U triplets.[14] Chen and co-workers demonstrated the potential of L-modified triplex-forming PNAs in stimulating ribosomal frameshifting[15] and suppressing replication of influenza A virus.[16] These authors also showed that PNAs having both L and s^2U modifications inhibited the dicing of pre-microRNA-198 in an in vitro assay. Taken together, these results suggest that sulfur substituted L and s^2U are promising nucleobases for future development of triplex-forming PNAs targeting biologically important dsRNAs.[14]

In DNA triplexes, several research groups explored derivatives of 2-aminopyridine (M in Figure 1) that are partially protonated under physiological conditions due to pK_a ~6.7.[17] While M modification gave modest results in DNA triplexes, Rozners and co-workers discovered that M was remarkably effective as a modified nucleobase in triplex-forming PNAs targeting dsRNA.[18] The high affinity and sequence specificity of M-modified PNAs are likely due to the partial cationic charge that provides favorable electrostatic attraction to negatively charged RNA. The partial charge allows placing several consecutive M nucleobases in a PNA sequence without notable unfavorable destabilization due to electrostatic repulsion between the adjacent partially protonated Ms. In our previous studies, PNAs having up to four consecutive M nucleobases were binding strongly and sequence specifically to the RNA targets.[19] Meanwhile, M nucleobases become fully protonated only upon forming the correct M+·G-C triplets (Figure 1), which minimizes non-specific electrostatic interactions. In other words, the partial protonation (due to pK_a of M being ~6.7) provides a fine balance of electrostatic interactions, resulting in excellent affinity and sequence specificity. Consistent with the partial protonation, lowering of the pH of binding buffer significantly increases the RNA binding affinity of M-modified PNAs.[20]

Similar to L, M did not form Watson−Crick base pairs with any of the natural nucleobases minimizing off-target binding to single stranded nucleic acids.[21] Moreover, the partial charge improves cellular uptake of M-modified PNAs,[22] most likely due to resemblance of arginine structure that stimulates endocytosis and may be enhancing endosomal escape through a proton sponge mechanism.[23] M-modified triplex forming PNAs are easy to synthesize[24] and have already been used by us and others to direct RNA-specific templated reactions,[25] suppress mRNA translation,[26] inhibit microRNA maturation,[27] and control conformation of dynamic structures of RNA bulges.[28] In our hands, M has become the most impactful PNA modification that both enables strong and selective binding to dsRNA and improves the cellular uptake of triplex-forming PNAs. As an alternative to M, Chen and co-workers used a guanidinium group (R, Figure 1) as a nucleobase surrogate to recognize guanosine in the R+·G-C triplet.[29] While PNAs having a single R modification had good RNA binding properties, two consecutive R modifications were destabilizing, which may be the result of reduced stacking interactions.

In a recent study, Rozners and co-workers demonstrated that 2-aminopyridine formed stronger M+·G-C triplets compared to the J·G-C triplets.[21] Since Chen and co-workers demonstrated that L had superior binding affinity and specificity over J, we were interested in a similar direct comparison of RNA binding properties of M- and L-modified triplex-forming PNAs. In the present study, we evaluated the

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advantages of sulfur-modified PNA nucleobases by comparing the stability of M+·G-C to that of L·G-C triplets in the context of PNA-dsRNA triplexes. We found that both M and L performed similarly in recognition of G-C base pairs in model RNA sequences, forming M+·G-C and L·G-C triplets of similar stability. However, L had a somewhat lower sequence specificity because of notably stronger off-target binding to the A-U base pairs. Taken together, our results show that M and L are two alternative approaches to triple-helical recognition of RNA under physiological conditions and may have their own context dependent advantages or disadvantages.

RESULTS AND DISCUSSION

To evaluate the sulfur-modified L and s^2U nucleobases, we used our previously established model system of four dsRNA hairpins having a variable base pair in the middle of the stem (Figure 2).[21] Placing the nucleobase to be evaluated in the middle of hairpin stem provides the most rigorous evaluation of modification's effect on binding affinity and sequence specificity. This model system enables the comparison of the binding affinity and sequence specificity of L with the previously studied M and T controls, PNA1 and PNA2. Fmoc-protected L monomers were synthesized following published procedures by Chen and co-workers, except that Fmoc-protected PNA backbone was used in the final synthesis step (for details see the Supporting Information) instead of the originally described Boc-protected PNA backbone.[13] Fmoc-protected M monomers were synthesized following our recently published procedures.[24] PNA3−PNA5 (Figure 2) were synthesized and purified using our previous developed methods.[30] Because of the additional Cbz and PMB protecting groups on L, the PNA deprotection conditions were modified as follows. A 10:1 mixture of trifluoroacetic acid (TFA) and trifluoromethanesulfonic acid (TFMSA) for 3 h was used to fully deprotect L-modified PNAs followed by precipitation with diethyl ether. L was incorporated either as a single modification facing the variable base pair (PNA3) or as a partial substitution of M (PNA4) or as a full L modification (PNA5). The binding affinity was measured using isothermal titration calorimetry (ITC) and UV thermal melting, as reported in our previous studies.[21] Earlier, we have demonstrated that UV thermal melting experiments at 300 nm allowed measurement of triplex to duplex transitions only (due to unique absorbance of M heterocycles) without interference from melting of RNA hairpins than had a negligible absorbance at 300 nm.[31]

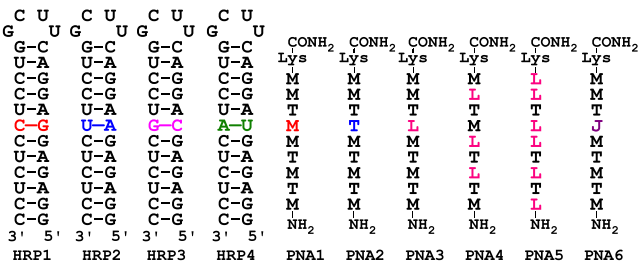

Figure 2. Sequences of RNA hairpins and nucleobase-modified PNAs.

Binding data showed that PNA3 having L at the variable position had similar affinity for the matched HRP1 than for the control PNA1 to HRP1 and PNA2 to HRP2 (Table 1). UV thermal melting suggested that the PNA3-HRP1 triplex was more stable by ΔT_m = 5−8 °C compared to PNA1-HRP1 and PNA2-HRP2. However, ITC results suggested that PNA3-HRP1 was somewhat less stable than PNA1-HRP1 (cf., K_a 16 vs 33 × 10^6 M^{-1}). On the other hand, L modification somewhat decreased the sequence specificity of PNAs with PNA3-HRP2 mismatch having K_a ~9 × 10^6 M^{-1} and T_m = 55 °C, which was significantly higher than K_a ~ 2 × 10^6 M^{-1} and T_m = 46 °C for PNA2-HRP1 (the highest affinity control

Table 1. Binding Affinity and Sequence Specificity of PNAs by ITC and UV Thermal Melting

PNA/dsRNA	Affinity [a]	HRP1	HRP2	HRP3	HRP4
PNA1 (M) [b]	K_a × 10^6 M^{-1}	33 ± 1	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
	T_m °C	66.5 ± 0.7	36.3 ± 0.4	36.8 ± 0.4	32.6 ± 0.4
PNA2 (T) [b]	K_a × 10^6 M^{-1}	1.9 ± 0.1	12 ± 1	0.9 ± 0.1	0.4 ± 0.1
	T_m °C	46.4 ± 0.5	69.6 ± 0.8	35.4 ± 0.4	34.6 ± 0.2
PNA3 (L)	K_a × 10^6 M^{-1}	16 ± 1	8.7 ± 0.5	2.8 ± 0.1	2.6 ± 0.2
	T_m °C	74.5 ± 0.4	55.4 ± 0.1	36.0 ± 0.1	35.2 ± 0.6
PNA4 (3×L)	K_a × 10^6 M^{-1}	9.1 ± 0.6	~5.1 [c]	1.6 ± 0.1 [c]	1.5 ± 0.2
	T_m °C	63.3 ± 0.3	40.1 ± 0.4	31.3 ± 0.3	35.8 ± 0.4
PNA5 (6×L)	K_a × 10^6 M^{-1}	15 ± 1	9.5 ± 1.2	1.5 ± 0.1 [c]	5.8 ± 0.1 [c]
	T_m °C	74.9 ± 0.3	61.6 ± 0.2	35.5 ± 0.2	47.7 ± 0.3
PNA6 (J) [b]	K_a × 10^6 M^{-1}	11 ± 1	1.6 ± 0.4	0.9 ± 0.1	0.4 ± 0.1
	T_m °C	60.7 ± 0.6	43.8 ± 0.6	39.1 ± 0.3	35.7 ± 0.5

[a]Association constants, K_a × 10^6 M^{-1}, average of three experiments ± stand. dev., for binding of PNAs to the respective RNA hairpins (10 μM) in 50 mM potassium phosphate buffer (pH 7.4), containing 2 mM $MgCl_2$, 90 mM KCl, 10 mM NaCl at 25 °C. UV thermal melting temperatures, T_m °C, average of five experiments ± stand. dev. measured at 300 nm and 18 μM of each dsRNA (or dsDNA) and PNA in the ITC buffer as above (for details, see the Supporting Information). [b]Previously studied control PNAs from ref. 21. [c]Approximate value based on two replicates.

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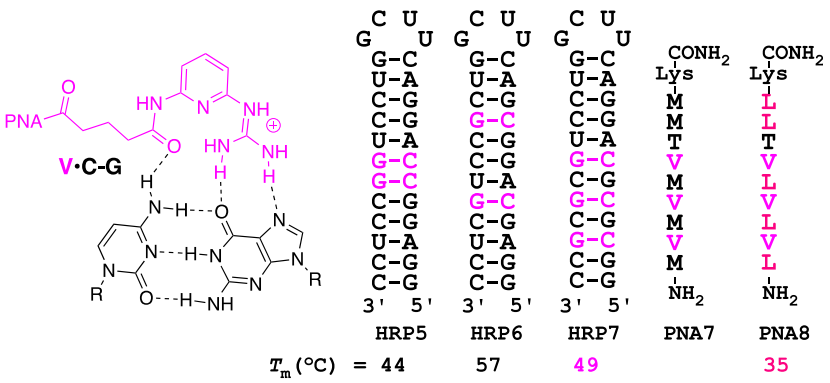

Figure 3. Sequences of RNA and PNAs that recognize three cytosine interruptions (highlighted in pink) in the polypurine tract of HRP7; the related HRP5 and HRP6 served as mismatched controls. T_m values are shown for PNA8 with HRP5−HRP7 and PNA8 self-melting.

mismatch) and $K_a \sim 1 \times 10^6$ M^{-1} and T_m ~35 °C for typical PNA-dsRNA mismatched triplexes. Increasing the number of L modifications did not further increase the stability of triplexes and showed a similar trend of lower sequence specificity. The affinity of PNA4 having three L substitutions somewhat decreased, while full substitution in PNA5 restored but did not exceed the stability of singly substituted triplex. PNA5 having only L and T nucleobases showed the lowest sequence specificity, with L·A-U and L·U-A mismatches notably more stable than typical mismatches by control PNA1 and PNA2.

Consistent with previous studies by Chen and co-workers,[13] L·G-C triplets were significantly more stable than J·G-C triplets both as single substitutions (cf., PNA3 and PNA6 in Table 1) and as fully modified PNAs. In our previous study comparing M+·G-C and J·G-C triplets, triplexes formed by fully J-modified PNAs were not stable enough to be studied under our common experimental conditions.[21] The results in Table 1 demonstrate that in the context of PNA·dsRNA triplexes, L·G-C triplets had stability similar to that of M+·G-C triplets. However, compared to J-modified PNAs, the gain in affinity achieved by substitution of C═O with C═S was accompanied by notably reduced sequence specificity (cf., PNA3 and PNA6).

An important difference between L and M, and a potential advantage of L, is the lack of a positive charge. In our previous study on 2-guanidyl pyridine (V) nucleobase for recognition of cytosine interruptions of polypurine tracts using V·C-G triplets (Figure 3), we found that PNA7 having three Vs and five Ms was binding indiscriminately to the mismatched HRP5 as well as to the matched hairpin HRP7 with stoichiometry >2, which strongly suggested nonsequence specific interactions.[32] We proposed that the loss of sequence specificity was most likely caused by the highly cationic nature of PNA7, where the charged Vs were interspaced between the partially charged Ms. We hypothesized that the sequence specificity might be restored by using L instead of M. To test this hypothesis, we synthesized PNA8 having three Vs interspaced between five Ls (Figure 3) and studied the binding affinity and sequence specificity of PNA8 for the matched HRP7 and related but mismatched HRP5 and HRP6.

UV thermal melting experiments showed that PNA8 had a good affinity for the matched HRP7 (Figure 3, T_m = 49 °C). However, PNA8 was also binding to the mismatched HRP5 and HRP6 with an affinity for HRP6 (T_m = 57 °C) even higher than the affinity for the matched HRP7. Interestingly, melting of PNA8 alone also showed a transition with an apparent T_m ~35 °C, suggesting that PNA8 may have some self-structure. While the nonspecific binding of PNA8 is most likely driven by the cationic guanidine groups of V bases, it is conceivable that the strong hydrophobic interactions of L, that may have caused lower discrimination against L·A-U mismatches for L-modified PNAs in Table 1, are also contributing to overall lack of sequence specificity.

In parallel to L-modified PNAs, we also attempted to evaluate 2-thiouridine (s^2U) in triplex-forming PNAs (Figure S1). In our hands, the synthesis of s^2U containing M-modified PNAs was complicated by an apparent instability of crude PNA synthesis materials. Purified s^2U-modified PNAs were stable and showed correct molecular weight on LCMS (for details, see the Supporting Information); however, UV melting showed no detectable transitions, and we could not measure stable association with the target matched HRP2. This was not due to the formation of stable self-structures because the UV melting of s^2U-modified PNAs alone showed no detectable transition (Figure S22). While these data are too preliminary to make conclusions, it appeared that in our experiments s^2U modification was not compatible with M-modified PNAs.

CONCLUSIONS

In the present study, we compared 2-aminopyridine (M) and 4-thiopseudisocytosine (L) PNA nucleobases for triple-helical recognition of G-C base pairs in dsRNA. These modified nucleobases present two alternative and complementary approaches to solving the problem of low pK_a of cytosine that needs to be protonated to form stable C+·G-C triplets. Our results in our model system indicated that the stability of L·G-C triplets was similar to that of M+·G-C triplets. However, L modifications resulted in somewhat reduced sequence specificity, as the L·A-U mismatched triplets were notably more stable than any of the mismatches formed by M. This trend was most pronounced in a fully L-modified PNA that showed the lowest sequence specificity. In our hands, M-modified PNAs were easier to synthesize, especially, following our recently optimized synthetic procedures.[24] The high affinity of M-modified PNAs is driven by ionic interactions between partially protonated Ms and negatively charged phosphates. While these interactions do not reduce the sequence specificity of RNA recognition, the cationic nature of M-modified PNAs may be a liability in certain biological contexts. On the other hand, the high affinity of L-modified PNAs is suggested to be due to favorable hydrophobic interactions.[13] However, in the V-modified PNA system (Figure 3, PNA7 and PNA8), replacing M with L did not

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restore the sequence specificity of PNA that was compromised by cationic modifications. In conclusion, M and L modifications are complementary and may have advantages or disadvantages depending on the specific application.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09775.

Synthesis of PNA monomers; synthesis, purification, and LC−MS characterization of PNA oligomers; UV melting results; ITC results and representative titration images; copies of ^{1}H and ^{13}C NMR spectra of synthetic intermediates and products (PDF)

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Notes

The authors declare no competing financial interest.

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