Recognition of A-U and U-A base pairs in RNA duplexes by modified peptide nucleic acids

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**Recognition of A-U and U-A base pairs in RNA
duplexes by modified peptide nucleic acids**

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requirement for the degree of Doctor of Philosophy

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List of Abbreviations

Ade	Adenine
ACN	Acetonitrile
Cyt	Cytosine
CHCA	α -cyano-4-hydroxycinnamic acid
DFT	Density functional theory
dHP	Deoxyribonucleic acid hairpin
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
E	3-oxo-2,3-dihydropyridazine
EtBr	Ethidium bromide
Gua	Guanine
HBTU	3-[Bis(dimethylamino)methylumyl]-3 <i>H</i> -benzotriazol-1-oxide hexafluorophosphate
HCl	Hydrochloric acid
K ₂ CO ₃	Potassium Carbonate
K _d	Dissociation constant between RNA and PNA
K _c	Dissociation constant between competitor RNA and PNA
L	Thio-pseudoisocytosine
LiOH	Lithium hydroxide
M	2-aminopyridine
MBHA•HCl	4-methylbenzhydramine hydrochloride
MgCl ₂	Magnesium Chloride

NaCl	Sodium Chloride
NMM	4-Methylmorpholine
PAGE	Polyacrylamide gel electrophoresis
PNA	Peptide nucleic acid
rHP	Ribonucleic acid hairpin
RNA	Ribonucleic acid
S	N-(4-(3-aminophenyl)thiazol-2-yl)acetamide
ssRNA	Single-stranded ribonucleic acid
Thy	Thymine
TFA	Trifluoroacetic acid
TFMSA	Trifluoromethanesulfonic acid
TFO	Triplex-forming oligonucleotide
TLC	Thin-layer chromatography
Ura	Uracil
UV-vis	Ultraviolet-visible

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Abstract

RNA triplex structures play vital roles in regulating various biological processes, and have attracted a lot of interest in designing TFOs (triplex-forming oligomers) that could selectively and specifically bind to the nucleic acid duplex. The PNAs (peptide nucleic acids), with the natural nucleic acids' entire negatively charged phosphate backbone replaced with a neutral N- (2-aminoethyl)glycine-based structure, could bind to nucleic acids better than their natural counterparts. We tested various modified bases derived from thymine to probe how the hydrogen bonding (acidity of the N3 atom) and base stacking may affect the recognition of an A-U pair by PNA-RNA₂ triplex formation. Our non-denaturing polyacrylamide gel electrophoresis and thermal melting results suggest that it is promising to incorporate modified thymine residues into PNAs for selective and sequence-specific recognition of RNA duplexes.

The stem loop structure present in the tau pre-mRNA splice site is crucial for the splicing process and contains two U-A base pairs. Modified nucleobases E and S are incorporated into PNAs for recognizing U-A pairs present in the wild type, as well as disease-causing mutated stem loop structure of tau pre-mRNA. Fluorescence binding studies were carried out to measure the binding affinity at physiological pH conditions. The E and S incorporated PNAs demonstrate similar binding affinity towards the target RNA hairpin structures.

Chapter 1. Recognition of A-U base pairs in RNA duplexes by modified peptide nucleic acids

Introduction

Nucleic acid structures serve crucial roles in various biological processes, such as genetic information storage and gene expression. The nucleobase recognition via Watson-Crick base pairing rules ensures the formation of stable duplex structures for the genetic information storage, replication, and gene expression.

Any trivial mistakes such as chemical damages and mutations in the nucleic acids could lead to severe dysfunction in the living systems. Many diseases which are fatal or bring the patients with life-long suffering could also find their genetic causes. Therefore, scientists have taken much effort in studying the structural properties of nucleic acids, and developing methods that could restore biological functions. Among all the methods, nucleic acid analogs appear to be promising candidates for modulating functions. The differences between DNA and RNA (**Figure 1-1**) is only the sugar building block of the backbone, and one nucleobase differs among four (T versus U). The similarities in composition and structural conformation ensure that the DNA and RNA could still recognize each other following the Watson-Crick base pairing pattern. On the other hand, minor modifications in nucleic acids could result in distinct changes in structures, properties and biological functions (1-4).

Therefore, for the design of the nucleic acid analogs, one should maintain the structural comparability with natural nucleic acids. Currently, there are two major courses for the synthesis of novel nucleic acids. One is focused on the modification in

backbone, and the other is devoted to novel nucleobase synthesis. The primary and ultimate purpose for all these modifications is to restore the balance between stability and flexibility as normal nucleic acid may do. As evidenced by the naturally occurring chemical modifications (1-4), any minor modification in backbone building block would cause ineligible change in overall conformation. Compared to DNA and RNA, PNA has a neutral peptide-like backbone instead of a negatively-charged sugar-phosphate backbone (**Figure 1-1**). PNA has higher binding affinity than its natural counterparts. Other than the binding affinity enhancement, the binding specificity enhancement is also observed (5).

PNA oligomers are stable even in a strongly acidic condition. PNA also shows sufficient tolerance towards weak bases. Thus, PNA has a great potential to function in various biological environments. The neutral backbone, however, results in a decreased solubility and self-aggregation in aqueous solutions. PNA solubility drops with increasing length and purine: pyrimidine ratio. Introduction of charged groups into the PNA can improve the solubility greatly without much compromise on the binding affinity. Therefore attachment of lysine at the termini of PNAs could be a simple solution for improving the solubility.

Figure 1-1: Chemical structures of PNA, DNA (X=H), RNA (X=OH) and peptide. The amide bond characteristic for peptide is highlighted in a box for the PNA and peptide, respectively. The letters A, T, C and G represent the nucleobases. The side chains of the peptide are indicated by the letter "R".

PNAs bind to nucleic acids strictly following the Watson-Crick pairing rule. However, unlike natural nucleic acids, PNA could bind in both parallel and antiparallel orientations. The PNA C-terminus and N-terminus correspond to the 3' end, and 5' end of natural nucleic acids, respectively. Nevertheless, the antiparallel orientation is still favoured (6). As a binding partner, PNA could adopt well in both A- and B-form conformations (7). PNA can form a distinct conformation different from either A-form or B-form.

RNA plays important roles (8) in catalysis and gene expression processes. RNA can form complex secondary and tertiary structures, and in turn, can have interactions with

the surrounding factors including proteins and metabolites (9,10). Therefore, the sequence and the structure of RNAs share equal importance in the recognition process. RNA triplex structures are naturally occurring and emerging as an important tertiary structure motif (11). Previous studies have shown that it is promising to target RNA duplexes through sequence-specific PNA-RNA₂ triplex formation (11,12). To recognize a G-C base pair in an RNA duplex by C⁺•G-C triple formation (**Figure 1-2**), a relatively low pH (<6) is required for the protonation of the N3 atom of the cytosine nucleobase. Modified PNA nucleobases such as thio-pseudoisocytosine (L) and 2-aminopyridine (M) have close-to-neutral pK_a for the nitrogen atoms and, thus show enhanced recognition of a G-C pair in RNA duplexes through L•G-C and M•G-C base triple formation, respectively (13-15).

Figure 1-2: Nucleobase triple structures of T•A-U, U•A-U, and ^XU•A-U, C⁺•G-C. The letter X represents fluorine, chlorine, bromine or iodine. The letter R represents the sugar-phosphate backbone of RNA.

Previous studies suggest that the binding of the third strand to a RNA/DNA duplex induces a conformational change, and the resulting structure of the RNA/DNA duplex is different from either A-form or B-form structure (16,17), although the base-base Watson-Crick bonding interactions remain the same. The conformational changes in the parental DNA/RNA duplexes may cause changes in Hoogsteen pairing and base stacking interactions (**Figure 1-2**). It is thus conceivable that base-base stacking interactions may play an important role in stabilizing triplex structures. As shown in

Figure 1-2, we tested various modified bases derived from thymine to probe how the hydrogen bonding (acidity of the N3 atom) and base stacking may affect the recognition of the A-U pair by PNA-RNA₂ triplex formation (14).

Materials and Methods

Materials and Monomer Synthesis

The PNA monomers thymine and cytosine were purchased from ASM. All the HPLC-purified DNA and RNA oligonucleotides were purchased from Sigma Aldrich.

The modified monomers uracil and 5-iodouracil were synthesized (**Scheme 1-1**) similar to the method for PNA T monomer synthesis (18,19). A series of PNAs (**Table 1-1**) were synthesized, purified, and characterized by MALDI-TOF (**Appendix i**).

Scheme 1-1: Reagents and conditions: a) Et₃N, ethylbromoacetate, CH₃CN, 0°C to rt., 60%. b) THF, 1M LiOH, diluted HCl, 0°C to rt, 76%. c) HBTU, NMM in DMF, 1 M LiOH, diluted HCl, 0°C to rt, 76%. d) Bromoacetic acid, KOH, in water, 0°C to rt, 76% (20-22).

PNA Synthesis

All the PNAs (**Table 1-1**) were synthesized via solid phase synthesis method. The solid support resin is polystyrene beads carrying 4-methyl benzhydrylamine groups (MBHA resin). The finished sequences were cleaved from the solid support with trifluoromethanesulfonic acid (TFMSA). PNA purification was carried out by reverse-phase HPLC.

Table 1-1: Summary of PNA sequences

PNA	Sequences	Chemical Formula	Calculated Mass (Da)	Measured Mass (Da)*
P8	LysNH-TCTCTTTC-CONH ₂	C ₉₁ H ₁₂₄ N ₃₈ O ₃₀	2228.93	2230.33
U1-3	LysNH-TCUCTTTC-CONH ₂	C ₉₀ H ₁₂₂ N ₃₈ O ₃₀	2214.92	2216.14
U2-3,5	LysNH-TCUCUTTC-CONH ₂	C ₈₉ H ₁₂₀ N ₃₈ O ₃₀	2200.90	2202.13
^F U1-3	LysNH-TC ^F UCTTTC-CONH ₂	C ₉₀ H ₁₂₁ N ₃₈ O ₃₀ F	2340.82	2342.03
^F U2-3,5	LysNH-TC ^F UC ^F UTTC-CONH ₂	C ₈₉ H ₁₁₈ N ₃₈ O ₃₀ F ₂	2236.88	2235.83
^{Cl} U1-3	LysNH-TC ^{Cl} UCTTTC-CONH ₂	C ₉₀ H ₁₂₁ N ₃₈ O ₃₀ Cl	2248.88	2252.48
^{Cl} U2-3,5	LysNH-TC ^{Cl} UC ^{Cl} UTTC-CONH ₂	C ₈₉ H ₁₁₈ N ₃₈ O ₃₀ Cl ₂	2268.83	2272.73
^{Br} U1-3	LysNH-TC ^{Br} UCTTTC-CONH ₂	C ₉₀ H ₁₂₁ N ₃₈ O ₃₀ Br	2292.83	2295.70
^{Br} U2-3,5	LysNH-TC ^{Br} UC ^{Br} UTTC-CONH ₂	C ₈₉ H ₁₁₈ N ₃₈ O ₃₀ Br ₂	2356.72	2360.35
^I U1-3	LysNH-TC ^I UCTTTC-CONH ₂	C ₉₀ H ₁₂₁ N ₃₈ O ₃₀ I	2340.82	2342.03
^I U2-3,5	LysNH-TC ^I UC ^I UTTC-CONH ₂	C ₈₉ H ₁₁₈ N ₃₈ O ₃₀ I ₂	2352.70	2353.93

* MALDI-TOF spectrums details are summarized in Appendix i.

Theoretical calculation of gas-phase pK_a values

Density Functional Theory (DFT) calculations were carried out with the Gaussian 09 package (23). The base geometries were fully optimized by B3LYP method with LANL2DZ basis set. Ultrafine integration grids were used in the calculation with tight convergence criteria. Harmonic frequency calculations were carried out to calculate the free energy in 298.15 K.

Table 1-2: The pK_a values calculated for the deprotonation process in the gas phase. $^*\Delta G^\ominus = 2.303RTpK_a$

Deprotonation process	pK_a^*	Reported pK_a in aqueous phase (24)
$T \rightarrow T^- + H^+$	254.38	9.75
$U \rightarrow U^- + H^+$	253.78	9.42
$^F U \rightarrow ^F U^- + H^+$	247.49	7.93
$^{Cl} U \rightarrow ^{Cl} U^- + H^+$	247.14	7.91
$^{Br} U \rightarrow ^{Br} U^- + H^+$	247.42	7.92
$^I U \rightarrow ^I U^- + H^+$	247.58	8.13

* pK_a value in gas form.

Gel Electrophoresis

12% poly-acrylamide gel electrophoresis was used to examine the binding affinity. All gels were run under 250 V for 5 hours. The running buffer is 1X TBE (Tris-borate EDTA) buffer. The gels were stained via ethdium bromide. A Typhoon scanner machine was used for gel visualisation.

Fluorescence Binding Study

In this study, we choose to incorporate fluorescent 2-aminopurine base (**Figure 1-3**, denoted as “2” in **Figure 1-4**) into the RNA sequences for binding studies.

Figure 1-3: Nucleobase structures of 2-aminopurine and adenine.

Similar to adenine (6-aminopurine), 2-aminopurine could also form a base pair with uracil or thymine. Therefore, 2-aminopurine incorporation does not significantly affect

the conformation and stability. Fluorescence intensity was measured by on a fluorescence spectrophotometer Cary Eclipse with a thermostat. In the study of the PNA binding to the 2-aminopurine labelled RNA, the samples were excited at 303 nm, and the intensities at 370 nm were monitored. For each sample, three measurements were taken and averaged for analysis. All samples were prepared in a buffer containing 100 mM NaCl, 0.5 mM EDTA, 20 mM HEPES at pH 7.0.

Steady state fluorescence experiments were done in determining the binding parameters, especially dissociation constant, with the following equations (25-27)

$$i = i_0 + \frac{(i_{\infty} - i_0) \left(\left\{ [PNA]_0 + [RNA]_0 + K_d \right\} - \left\{ \left([PNA]_0 + [RNA]_0 + K_d \right)^2 - 4[PNA]_0[RNA]_0 \right\}^{\frac{1}{2}} \right)}{2[RNA]_0} \quad (1-1)$$

Here, i , i_{∞} , and i_0 are the fluorescence intensities of the labeled RNA partially in complex with PNA, with PNA fully bound RNA, and with no PNA bound, respectively. $[PNA]_0$ is the total PNA concentration. $[RNA]_0$ is the total labeled RNA concentration, K_d is the dissociation constant.

UV Melting Study

In our study, the thermal melting experiment was conducted using Shimadzu UV-vis spectrophotometer UV-2550. The samples were prepared in a buffer of 20 mM HEPES, 200 mM NaCl and 0.5 mM EDTA at pH 7.5. The path length of the cell is 1 cm. The sample concentration was 5 μ M for both RNAs and PNAs. The scan rate of the melting experiments is 0.5 $^{\circ}$ C/min from 15 $^{\circ}$ C to 95 $^{\circ}$ C. The absorbance of the samples was monitored at wavelengths 260 nm and 280 nm.

Results and Discussion

Figure 1-4: Structures studied in this project. Incorporation of modified nucleobases into PNAs selectively stabilises an (RNA)₂-PNA triplex structure shown in panel (a). (a) A model (RNA)₂-PNA triplex formed between a PNA and rHP1. (b) A model (RNA)₂-PNA triplex formed between a PNA and rHP2 with one mismatch. (c) A model (RNA)₂-PNA triplex formed between a PNA and rHP3 with one mismatch. (d) A model (RNA)₂-PNA triplex formed between a PNA and rHP4 with one mismatch. (e) A triplex formed between a PNA and an RNA duplex labeled with 2-aminopurine (indicated with the number “2”), (f) A model (DNA)₂-PNA triplex formed between a PNA and dHP1. dHP1 is homologous to rHP1. (g) A parallel PNA-RNA duplex.

Effect of Chemical modifications on binding affinity

Figure 1-5: Binding of synthesized PNAs towards rHP1 measured by PAGE. Samples were incubated in buffer of 200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.5. Non-denaturing PAGE (12%) was running with a buffer of 1×TBE, pH 8.3 for 6 h at 250 V. The RNA concentration remains at 0.25 μM, and the PNA concentrations from left to right are 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 1, 2, 4, 10, and 20 μM respectively. Each loaded sample is 25 μL. The obtained K_d values are summarized in Table 1-3.

a) P8 with dsRNA1

b) U2-3,5 with dsRNA1

c) ¹⁹FU2-3,5 with dsRNA1

d) ¹³CU2-3,5 with dsRNA1

e) ¹⁸BrU2-3,5 with dsRNA1

f) ¹⁵IU2-3,5 with dsRNA1

Figure 1-6: Fluorescence binding studies for all doubly modified PNAs towards duplex RNA. Samples were incubated in buffer of 200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.5. The RNA concentration remains at 0.25 μ M, and the PNA concentrations are 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 1, 2, 4, 10, and 20 μ M, respectively. Each sample is 70 μ L. The K_d values (Appendix ii) obtained are (0.9 ± 0.1) , (0.7 ± 0.3) , (0.6 ± 0.2) , (0.7 ± 0.3) , (0.3 ± 0.1) , and (0.3 ± 0.1) μ M, respectively.

The non-denaturing PAGE binding studies were carried between the PNAs and a series of RNA/DNA structures as shown in Figure 1-3 and Table 1-3. The unmodified P8 PNA could bind to rHP1 with K_d value of 5.2 μM at pH 7.5 (12) and 15.4 μM at pH 7.7. The singly modified sequence U1-3 showed weakest binding affinity among all modified PNA sequences ($K_d = 1.2 \mu\text{M}$ at pH 7.5 and $K_d = 2.9 \mu\text{M}$ at pH 7.7). Thus, all modified PNAs show stronger binding affinity than the unmodified PNA.

The results show that with a single replacement of uracil for thymine, it could lead to the enhancement in binding affinity towards the target duplex region. Upon the halogenation modifications, the K_d values are generally further reduced. Among the six substituted groups in the six bases (thymine, uracil and 5-halogenated uracil), the halogen atoms are more electronegative compared to hydrogen atom in uracil and the methyl group in thymine. The electronegativity of the substituted groups at 5 position affects the pK_a of N3 atom (**Table 1-2**) (22). The calculated pK_a values in gas phase are significantly higher than experimentally determined values in aqueous solution (**Table 1-2**). The trends of the theoretical and experimental values, however, are the same and show the effect of 5-position substitution on the pK_a of N3 atom. Thus, lowering the N3 pK_a value towards neutrality enhances the Hoogsteen hydrogen bond formation. Although 5-fluorouracil nucleobase has a similar pK_a value with 5-chlorouracil, 5-bromouracil and 5-iodouracil, the PAGE results (**Figure 1-4, 1-5** and **Table 1-3**) indicates that the enhancement effect for 5-fluorouracil is not as strong as the other modified uracil bases. It is likely due to the fact that chlorine, bromine and iodine atoms have a relatively larger size compared to fluorine atom, and are expected to enhance base stacking with adjacent bases.

Table 1-3: Summary of K_d values for PNA binding to RNA or DNA duplexes obtained by non-denaturing PAGE^a

	rHP1			rHP2	rHP3	rHP4	dHP1
	pH 7.5	pH 7.7	pH 8.0	pH 7.7			
P8	5.23±1.3 μM^b	15.4±4.4 μM	> 20 μM	NB	NB	NB	NB
U1-3	1.2±0.3 μM	2.9±0.5 μM	-	NB	NB	NB	NB
U2-3,5	1.0±0.3 μM	3.2±0.7 μM	2.9±0.4 μM	NB	NB	NB	NB
^F U1-3	1.1±0.2 μM	2.8±0.5 μM	-	NB	NB	NB	NB
^F U2-3,5	0.3±0.1 μM	0.6±0.1 μM	4.0±0.4 μM	NB	NB	NB	NB
^{Cl} U1-3	0.6±0.2 μM	0.9±0.2 μM	-	NB	NB	NB	NB
^{Cl} U2-3,5	0.6±0.1 μM	0.5±0.1 μM	2.5±0.3 μM	NB	NB	NB	NB
^{Br} U1-3	0.4±0.1 μM	0.95±0.19 μM	-	NB	NB	NB	NB
^{Br} U2-3,5	0.3±0.1 μM	0.4±0.1 μM	1.5±0.1 μM	NB	NB	NB	NB
^I U1-3	0.8±0.2 μM	1.4±0.2 μM	-	NB	NB	NB	NB
^I U2-3,5	0.5±0.1 μM	0.9±0.2 μM	2.9±0.4 μM	NB	NB	NB	NB

^aThree incubation buffer were used: pH 7.5 (200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.5), pH 7.7 (200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.7), pH 8.0 (200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 8.0). “-” indicates that the data were not measured in this study. “NB” indicates that no binding was observed in PAGE.

^bThe K_d value was obtained from previously reported work (14).

Binding Affinity at Various Buffer Conditions

Figure 1-7: pH dependence study for all doubly modified PNAs with rHP1 at pH 7.7. Non-denaturing PAGE (12%) with a running buffer of 1×TBE, pH 8.3 for 6 h at 250 V. The incubation buffer is 200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.7. For a-d and j & k, the loaded hairpins are at 1 μ M, and the PNA concentrations in lanes from left to right are 0, 0.05, 0.1, 0.2, 0.4, 1, 1.6, 2, 4, 10, 16, 20, 28, and 50 μ M, respectively. For e-i, the loaded hairpins are at 0.25 μ M in 25 μ l. PNA concentrations in lanes from left to right are 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 1, 2, 4, 10, and 20 μ M, respectively. The K_d values obtained are summarized in Table 1-3.

Figure 1-8: pH dependence study for all double modified PNAs with rHP1 at pH 8.0. The non-denaturing PAGE (12%) was run with a running buffer of 1×TBE, pH 8.3 for 6 h at 250 V. The incubation buffer are 200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 8.0. The loaded hairpins are at 0.25 μ M, and the PNA concentrations in lanes from left to right are 0, 0.05, 0.1, 0.2, 0.4, 1, 1.6, 2, 4, 10, 16, 20, 28, and 50 μ M, respectively. The K_d values obtained are summarized in Table 1-3.

The PNA sequences form triplexes with rHP1 at pH up to 8.0 (**Figures 1-5, 1-7 and 1-8**). The binding affinity drops with increasing pH (**Table 1-3**), which is consistent with the fact that all PNAs have three cytosine residues, because the $C^+ \cdot G-C$ base triple formation is pH dependent. Thymine (monomer $pK_a = 9.75$), uracil (monomer $pK_a = 9.42$) and 5-iodouracil (monomer $pK_a = 8.1$) residues (see **Table 1-2** for the free base pK_a values) are expected to have significantly increased pK_a when incorporated into triplexes (28,29), and thus are able to form base triples with A-U base pair in a relatively pH independent manner.

Figure 1-9: Fluorescence binding study of $BrU_{2-3,5}$ with rHP1 at various pH. For a-d, samples are incubated in buffer of 200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, with the pH ranging from 7.0 to 8.0. The RNA concentration remains at 0.25 μM , and the PNA concentrations are 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 1, 2, 4, 10, and 20 μM , respectively for pH 7.0, 7.5 and 7.7. For pH 8.0, the PNA concentrations are 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 16, 20, 28, and 50 μM respectively. Each sample is 70 μL . The K_d value obtained are (0.3 ± 0.1) , (0.3 ± 0.1) , (0.4 ± 0.1) , and (0.6 ± 0.1) μM , respectively.

The binding of the PNAs to the RNA duplexes was further confirmed by fluorescence titration assay. A 2-aminopurine labeled RNA duplex was used. The 2-aminopurine residue (**Figure 1-3**) is present adjacent to the PNA binding site (**Figure 1-4e**). The fluorescence titration data (**Figures 1-6** and **1-9**) show that the 2-aminopurine fluorescence intensity decreases upon the PNA binding and PNA-RNA2 triplex formation. Fitting of the fluorescence intensity at 370 nm versus PNA concentration gives the K_d values (**Table 1-4**), which are generally consistent with the data obtained by PAGE (**Table 1-3**).

Table 1-4: Summary of K_d values for PNA binding to RNA or DNA duplexes obtained by fluorescence binding study^a

PNA	pH 7.0	pH 7.5	pH 7.7	pH 8.0
P8	-	0.9 ± 0.1 μM	-	-
U2-3,5	-	0.7 ± 0.3 μM	-	-
^F U2-3,5	-	0.6 ± 0.2 μM	-	-
^{Cl} U2-3,5	-	0.7 ± 0.3 μM	-	-
^{Br} U2-3,5	0.3 ± 0.1 μM	0.3 ± 0.1 μM	0.4 ± 0.1 μM	0.6 ± 0.1 μM
^I U2-3,5	-	0.3 ± 0.1 μM	-	-

^aThree incubation buffer were used: pH 7.0 (200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.0), pH 7.5 (200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.5), pH 7.7 (200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.7), pH 8.0 (200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 8.0). “-” indicates that the data were not measured in this study.

Binding Selectivity Study

Figure 1-10: The binding study for doubly modified PNAs with hairpin RNAs which have one mismatch in sequences. The non-denaturing PAGE (12%) was run with a running buffer of 1XTBE, pH 8.3 for 6 h at 250 V. The incubation buffer are 200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.7. The loaded hairpins are at 1 μM in 10 μL. PNA concentrations in lanes from left to right are 0, 0.05, 0.1, 0.2, 0.4, 1, 1.6, 2, 4, 10, 16, 20, 28, and 50 μM, respectively. rHP2, rHP3 and rHP4 show no binding towards all PNAs.

Upon changing the A-U base pair in model hairpin RNA (rHP1) to a U-A base pair (rHP2), C-G base pair (rHP3), G-C base pair (rHP4), all 8-mer PNAs including both non-modified and modified-PNA sequences show no triplex formation (**Figure 1-10**). All the gel electrophoresis experiments were carried out at pH 7.7 conditions.

Although compared to rHP1, only one base pair is changed in rHP2, rHP3 and rHP4, the binding affinity is compromised severely. The results suggest that besides the enhancement in binding affinity, the modified PNA sequences still reserve a high selectivity as the non-modified PNA. Therefore, it is promising to incorporate modified thymine residues into PNAs for the enhanced recognition of A-U pairs in RNA duplexes.

Figure 1-11: The binding study of all PNA sequences towards a DNA hairpin (dHP1). The non-denaturing PAGE (12%) was run with a running buffer of 1XTBE, pH 8.3 for 6 h at 250 V. The incubation buffer are 200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.7. The loaded hairpins are at 1 μ M in 10 μ L. PNA concentrations in lanes from left to right are 0, 0.05, 0.1, 0.2, 0.4, 1, 1.6, 2, 4, 10, 16, 20, 28, and 50 μ M, respectively. dHP1 is homologous to rHP1, and shows no binding to all PNAs.

The similar results (**Figure 1-11**) are also observed with binding study towards model hairpin DNA (dHP1) (**Figure 1-4**). As a homologous DNA hairpin (dHP1) of rHP1, the non-modified PNA and modified PNA show no triplex formation even with 50 μ M PNA present at pH 7.7. It demonstrated the superior selectivity for RNA duplex over

DNA duplex for both non-modified and modified PNA. As PNA binds to nucleic acid duplexes in the major groove, the preference towards RNA duplex over DNA duplex may suggest that the relatively deep and narrow RNA duplex groove has better compatibility in structure with PNA.

Figure 1-12: Thermal melting results for RNA-PNA duplexes. Normalized thermal melting curves for ssRNA-PNA duplexes at 200 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.5. The samples contain 5 μ M ssRNA and 5 μ M PNA in 130 μ L.

The thermal melting studies of PNA-RNA duplexes is carried out at pH 7.5. All samples contain 5 μ M single strand RNA (ssRNA) and 5 μ M PNA. The thermal melting of raw data was also presented in Appendix iii, and the melting temperature was approximated as the Gaussian peak of the first derivative curve of the melting data. As expected, all PNA sequences could form stable duplex structure with the target single strand RNA (ssRNA) (**Figure 1-12**). The singly and doubly modified PNA sequences have higher melting temperatures than the non-modified PNA (P8). The chloro- and bromo- modified sequences show highest melting temperatures. Taken together, the PAGE and thermal melting studies suggest that incorporation of the modified T residues in PNAs can enhance both PNA-RNA₂ triplex and PNA-RNA duplex formation. At the conditions studied, the chemical modifications do not induce the formation of strand invasion complex. As we learned from previous studies that

the strand invasion complex would move much slower than the triplex in a non-denaturing PAGE (14), and no band other than hairpin and duplex was observed in all electrophoresis gel images.

In summary, as we discussed above, the modifications are all located at 5-position of T, which is not involved in forming Hoogsteen hydrogen bonds. However, the modified PNAs show enhanced binding affinity (compared to the unmodified PNA) towards the target RNA hairpin structure. The theoretical calculations and previous reported experimental pK_a values of the modified nucleobase structures indicate that due to the electronegativity of the attached functional group, the pK_a value of N1 nitrogen atom can shift towards neutral upon the modification, and thus the strength of the hydrogen bond with N7 nitrogen of adenine is enhanced within the triplex. At the same time, the larger atom size of the substituted atoms can result in enhanced base stacking with the adjacent base triples.

For further addressing these two factors' effects on triplex formation with RNA duplex target, as well as obtaining better understanding of various functional groups attached to 5-position, there are two main directions to explore. The first would be including some electron-donating groups. In this way, we may have a more comprehensive understanding towards how electronegativity of the substituted atom on 5-position can affect the triplex formation. The second would be attaching planar functional group such as six-member ring to reveal the role of base stacking in triplex formation process. In addition, computational modeling will be quite helpful in providing insights into how various factors can affect the binding strength of a PNA towards RNA duplex.

Chapter 2. Recognition of U-A base pairs in tau pre-mRNA splice site structures by modified peptide nucleic acids

Introduction

The microtubule-associated protein tau is mainly present in neurons and plays various vital roles such as promoting microtubule assembly, maintaining neuronal integrity, and assisting axonal transport (30). The protein tau is identified to be associated with numerous neurodegenerative diseases such as Alzheimer's disease, Parkinsonism, and other familial frontotemporal dementia (31,32). A new term "tauopathies" is used to describe the neurodegenerative diseases associated with tau protein aggregation (33).

The tau proteins mainly consist of six isoforms (34), which are produced from alternative splicing of 16 exons (35). Two classes of isoforms, 3R and 4R, are classified based on the inclusion or exclusion of exon 10 (36,37). Naturally, the production ratio of these two isoforms is close to unity. However, the deviation from a 1:1 ratio is observed in some of the dementia patients (38). A number of mutations have been identified in the tau gene that cause familial frontotemporal dementia and Parkinsonism (32,39,40). Not all of these mutations are located in the coding regions (32,39-41).

Figure 2-1 Sequences and secondary structures of wild type and mutants of human tau exon 10 splicing regulatory elements. Red color letters denote possible destabilizing mutations observed in patients (38).

A few disease-causing mutations are present near the splice-donor site at exon 10-intron 10 junction (**Figure 2-1**). Inclusion of exon 10 in translation alters the produced protein isoform from 3R tau to 4R tau. The observed mutations are present within an RNA stem loop structure at the exon 10-intron junction (**Figure 2-1**), and the stability of the RNA stem loop is affected by these mutations (39,42,43). It could suggest that the RNA stem loop's stability is important to maintain the balance of the two tau isoforms' production (39). There have been attempts to use small molecules to bind to the RNA stem loop with mutations to restore the stability to that of the wild type structure (44). Small molecules may lack the selectivity and specificity in binding to a small RNA.

Given the target as an RNA stem loop in the pre-mRNA, PNA may be a promising therapeutic candidate with high affinity and selectivity. PNA has supreme binding affinity and resistance towards nucleases and proteases. Short PNAs have been shown to selectively bind to RNA duplex over DNA duplex and single-stranded RNA or DNA (11,12,14).

One of the major obstacles is that a stable triplex structure could be established with only the purine strand as the middle strand. There is no natural nucleobase available to recognize the pyrimidine nucleobase and form stable base triples.

Figure 2-2: Base triple structures of S•U-A and E•U-A (45,46). The letter “R” represents the sugar-phosphate backbone of RNA.

There are two U-A base pairs present in the middle of the tau pre-mRNA splice site structure (**Figure 2-1**). A mismatched triple in the middle position of triplexes has a strong destabilizing effect (see chapter 1). Therefore, the first and most critical issue to address is to design modified nucleobases, which could form a stable base triple with a U-A base pair.

In literature, there are quite a number of novel nucleobases reported in targeting DNA T-A base pair. In 2003, one research group incorporated into DNA sequences with S nucleobase (see the base structure in **Figure 2-2**), which can recognize a T-A base pair in the target DNA duplex (46-48), and the formed triplex structure has moderate thermal stability. Therefore in our study, the S-nucleobase is incorporated into the neutral backbone of PNA.

The PNA monomer 3-oxo-2,3-dihydropyridazine (E) (**Figure 2-2**) was originally designed for targeting DNA duplexes by forming PNA-DNA-PNA triplexes (49). The E base has an elongated linkage between the nucleobase and PNA backbone to minimize the steric hindrance with the methyl group present in thymine nucleobase. PNAs with the E nucleobase incorporated can bind to the target RNA duplex with U-A base pair (12). E base, however, shows moderate sequence selectivity probably due to the fact that it forms only one hydrogen bond with a T-A or U-A pair (**Figure 2-2**).

Figure 2-3: Base triple structures of L•G-C and C⁺•G-C. The letter “R” represents the sugar-phosphate backbone of RNA.

The PNA monomer thio-pseudocytosine (L) developed by our group, when incorporated into PNAs, can form a stable base triple with a G-C base pair at physiological pH condition (**Figure 2-3**) (14). Therefore, we incorporated the L monomer into our PNA sequences to bind to the tau pre-mRNA stem loop, which contains G-C pairs (**Figure 2-1**).

Materials and Methods

Materials and PNA monomer Synthesis

All reagents were obtained from commercial sources and were used without further purification. TLC was performed on silica 60 (Merck 5554 aluminium sheet), column chromatography on silica 60 (Merck 9385). The PNA S monomer was synthesized as described in **scheme 2-1** and **Appendix iii**.

Scheme 2-1: Reagents and conditions: a) $(\text{Boc})_2\text{O}$, THF, 0°C to rt., 60%. b) Et_3N , ethylbromoacetate, CH_3CN , 0°C to rt., 60%. c) H_2O , EtOH, reflux, 81%. d) Acetic anhydride, reflux, 92%. e) EtOH, MeOH, 10% Pd/C, hydrogen pressure, rt. 70%. f) Et_3N , chloroacetyl chloride, DCM, 0°C to rt., 80%. g) K_2CO_3 , DMF, rt. 8%. h) THF, LiOH, HCl, 0°C to rt. 76%.

PNA Synthesis

All the PNAs (Table 2-1) were synthesized via solid phase synthesis method. The solid support resin is polystyrene beads carrying 4-methyl benzhydrylamine groups (MBHA resin). The finished sequences were cleaved from the solid support with trifluoroacetic acid and trifluoromethanesulfonic acid, and purification was carried out by reverse-phase HPLC. All purified PNAs (Table 2-1) were characterized by MALDI-TOF (Appendix i).

Table 2-1: Summary of PNA sequences

PNA	Sequences	Chemical Formula	Calculated Mass (Da)	Measured Mass (Da)*
E-1-Tau	LysNH-LELELTC- CONH ₂	C ₇₉ H ₁₁₁ N ₃₇ O ₂₀ S ₃	1993.80	1995.46
E-2-Tau	LysNH-CELELTC- CONH ₂	C ₇₉ H ₁₁₁ N ₃₇ O ₂₁ S ₂	1977.82	1979.47
S-1-Tau	LysNH-LSLSLTC-CONH ₂	C ₉₁ H ₁₁₉ N ₃₇ O ₂₀ S ₅	2209.80	2212.28
S-2-Tau	LysNH-CSLSLTC-CONH ₂	C ₉₁ H ₁₁₉ N ₃₇ O ₂₁ S ₄	2193.83	2195.87

* MALDI-TOF spectrums details are summarized in Appendix i.

The fluorescent direct binding assay was carried out similar to the method described in chapter 1.

In the competitive binding assay, the affinity of PNA towards non-labelled RNA sequences could be determined with the labelled RNA present. With the assumption that the PNA could bind to two RNA sequences independently in a 1:1 binding ratio, by forming triplexes. The binding dissociation constants between non-labeled RNA and PNA (K_c) can be determined using the following equation as described previously (50,51).

$i =$

$$i_0 + \frac{i_\infty - i_0}{2[RNA]_0} ([PNA]_0 + [RNA]_0 + K_d + \frac{K_d}{K_c} [RNA]_c - \sqrt{([PNA]_0 + [RNA]_0 + K_d + \frac{K_d}{K_c} [RNA]_c)^2 - 4[PNA]_0[RNA]_0}) \quad (1-2)$$

where i , i_∞ , and i_0 are the fluorescence intensities of the samples with the labeled RNA partially bound by PNA, with PNA fully bound, and with no PNA bound, respectively. $[PNA]_0$ is the total PNA concentration (20 μ M). $[RNA]_0$ is the total labeled RNA concentration (1 μ M). $[RNA]_c$ is the total unlabeled competitor RNA concentration (0-50 μ M). K_d is the dissociation constant of the binding between labeled RNA and PNA. K_c is the dissociation constant of the binding between unlabeled competitor RNA and PNA. i_∞ , i_0 , and K_d are determined by direct binding assay (see eq. 1-1).

Results and Discussion

Figure 2-4: Structures studied in this project. The mutated base pairs (Figure 2-1) are highlighted. (a) A tau pre-mRNA hairpin fragment rHP1 with the A bulge residue replaced by 2-aminopurine (denoted as “2”). (b) A model (RNA)₂-PNA triplex formed between a PNA and rHP2. (c) A model (RNA)₂-PNA triplex formed between a PNA and rHP3. (d) A model (RNA)₂-PNA triplex formed between a PNA and rHP4.

Direct Binding Study

a) E-1-tau with rHP1

b) E-2-tau with rHP1

c) E-1-tau with rHP1

d) E-2-tau with rHP1

Figure 2-5: Direct binding study for E-modified PNAs. All samples were prepared in the buffer of 20 mM HEPES, 0.5 mM EDTA, 100 mM NaCl at pH 7.0. The rHP1 concentration remains constant at 1 μM in 70 μL for all samples. a) E-1-Tau PNA binds to rHP1. b) E-2-Tau PNA binds to rHP1. c) 2AP fluorescence intensity at 370 nm is quenched upon E-1-Tau binding towards rHP1. The obtained K_d is 16.1 ± 6.7 μM. d) 2AP fluorescence intensity at 370 nm is quenched upon E-2-Tau binding towards rHP1. The obtained K_d is 17.8 ± 3.4 μM.

In this study, we put the 2-aminopurine residue (**Figure 1-3**) right next to the binding site (**Figure 2-4**). Because the 2-aminopurine residue is located at the bulge position, this replacement should cause minimal changes in three-dimensional structure and thermodynamic stability.

The excitation wavelength for 2-aminopurine is around 303 nm, and the observed emission peak wavelength is at 370 nm. The maximum excitation/emission wavelengths vary slightly because of the sequences and other surrounding factors. However, in the steady state fluorescence experiment, we observe another peak

located at 470 nm, with its intensity increasing with the increase of the PNA concentration. Our measurements for PNA (S-2-tau) alone samples (**Appendix iv**) confirmed that the emission peak at 470 nm is due to the PNA. Since these two peaks are well separated, the peak at 470 nm is not expected to significantly affect 2-aminopurine fluorescence signal changes.

As shown in **Figure 2-5**, it is quite obvious that the 2-aminopurine fluorescence is quenched upon the addition of PNA, and a slight red shift was also observed. It could suggest that with the PNA strand binding to rHP1, the base stacking of 2-aminopurine with PNA and/or RNA is enhanced, which causes the fluorescence quenching to occur.

The quenching effect could be used as the indicator for monitoring the triplex forming process. By recording the peak intensity of each sample and fitting to equation 1-1, we could obtain the dissociation constant of each PNA towards the rHP1 (**Figure 2-4**), which is a mimic of wild type of human tau pre-mRNA stem loop structure.

The fluorescence binding data for PNA E-1-tau and E-2-tau are only different at N-terminal position (**Table 2-1**). Previous study suggests that L monomer could form more stable base triple with G-C base pair. However, this stabilizing effect is more significant when L monomer is present in the middle of the binding site (12). Consistently, in our studies, these two PNAs show comparable binding affinity towards rHP1 with K_d values of 16.1 versus 17.8 μM , respectively (**Figure 2-5**).

Figure 2-6: Direct binding study for E-2-tau PNA at various buffer conditions. The rHP1 concentration remains constant at 1 μM in 70 μL for all samples. a, b) E-2-tau PNA binds to rHP1 at 20 mM MES, 0.5 mM EDTA, 100 mM NaCl at pH 6.0. The obtained K_d is $2.8 \pm 1.4 \mu\text{M}$. c, d) E-2-tau PNA binds to rHP1 20 mM HEPES, 0.5 mM EDTA, 100 mM NaCl at pH 8.0. The obtained K_d is $64.7 \pm 20.4 \mu\text{M}$. e, f) E-2-tau PNA binds to rHP1 at 20 mM HEPES, 0.5 mM EDTA, 100 mM NaCl 2 mM MgCl_2 at pH 7.0. The obtained K_d is $56.3 \pm 20.1 \mu\text{M}$.

The results shown in **Figure 2-6** clearly show that the E-2-tau PNA (**Table 2-1**) can bind to rHP1 in the pH ranging from pH 6.0 to 8.0. However, with the pH increasing, the K_d value drops significantly. With the presence of MgCl_2 at pH 7.0, the observed binding affinity is weakened significantly. The results suggests that the presence of Mg^{2+} destabilizes the triplex formation, presumably due to the fact that the PNA has a positively charged lysine residue.

Figure 2-7: Direct binding study for S-modified PNAs. All samples are prepared in the buffer of 20 mM HEPES, 0.5 mM EDTA, 100 mM NaCl at pH 7.0. The rHP1 concentration remains constant at 1 μ M in 70 μ L for all samples. a) S-1-tau PNA binds to rHP1; b) S-2-tau PNA binds to rHP1; c) 2AP fluorescence intensity at 370 nm is quenched upon S-1-tau binding towards rHP1. The obtained K_d is 17.2 ± 3.3 μ M; d) 2AP fluorescence intensity at 370 nm is quenched upon S-2-tau binding towards rHP1. The obtained K_d is 16.6 ± 4.7 μ M.

Our fluorescence binding studies show that both S-1-tau and S-2-tau PNAs bind to rHP1 and form triplex structures at near physiological pH condition (**Figure 2-7**). Similar to the E-modified PNAs (**Figure 2-5** and **2-6**), observed that the fluorescence intensity at wavelength 470 nm increases as the PNA concentration increases. The magnitude of the increase in fluorescence intensity varies for different sequences, which suggests that the PNA sequence affects the fluorescence properties of monomers incorporated into the oligomers.

Compared to the E-modified triplex-forming PNAs, S-1-Tau and S-2-Tau PNAs have similar binding affinity towards rHP1. The results suggest that although S monomer

could potentially form three Hoogsteen hydrogen bonds with a U-A base pair (**Figure 2-2**), it does not show significant enhancement in binding compared to the E-modified PNAs. The possible reason for this unexpected results may be due to the fact that, in order to establish all three Hoogsteen hydrogen bonds, the S base containing multiple single bonds (**Figure 2-2**) needs to orientate itself within the oligomers, which is entropically unfavorable.

Although the binding affinity did not show significant enhancement by replacing the E monomer with S monomer, the PNA with S-monomer incorporated may show better specificity. The binding specificity measurement is in progress.

Table 2-2: Summary of K_d Values for PNA binding to rHP1 obtained by fluorescence studies^a.

PNA	pH 6.0	pH 7.0	pH 8.0	Mg ²⁺
E-1-tau	-	16.1±6.7 μM	-	-
E-2-tau	2.8±1.4μM	17.8±3.4 μM	64.7±20.4 μM	56.3±20.1 μM
S-1-tau	-	17.2±3.3 μM	-	-
S-2-tau	-	16.6±4.7 μM	-	-

^aFour incubation buffers were used: pH 7.0 (100 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 7.5), pH 6.0 (100 mM NaCl, 0.5 mM EDTA, 20 mM MES, pH 6.0), pH 8.0 (100 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, pH 8.0), and Mg²⁺ (100 mM NaCl, 0.5 mM EDTA, 20 mM HEPES, 2mM MgCl₂, pH 7.5). “-” indicates that the data were not obtained in this study.

Competitive Binding Study

Figure 2-8: Competitive binding for E-modified PNA (E-1-tau). All samples were prepared in the buffer of 20 mM HEPES, 0.5 mM EDTA, 100 mM NaCl at pH 7.0. The rHP1 concentration remains constant at 1 μ M, and E-1-tau PNA concentration remains at 20 μ M. a) rHP2 and rHP1 binding to E-1-tau PNA. b) rHP3 and rHP1 binding to E-1-tau PNA.

Next, we tested whether the PNAs bind to the mutated RNA hairpins. The results of the competitive steady state fluorescence binding studies are shown in **Figures 2-8** and **2-9**. The fluorescence intensity at 370 nm increases as the concentration of mutated RNA sequence increases in the sample, suggesting that the mutated RNA can compete with 2-aminopurine-labeled RNA in binding to the PNAs. The intensity at 470 nm, however, does not change significantly upon the addition of the competing unlabeled RNA, suggesting that the fluorescence of the PNAs is not affected by the presence of 2-aminopurine in a PNA-RNA₂ triplex.

The fluorescence intensity at 370 nm does not reach the same level as the rHP1 alone in the solution (**Figures 2-5, 2-8**). Therefore, with the mutated RNA's concentration at 50 μ M, it still could not compete out rHP1 completely in binding to PNA E-1-tau. Thus, we didn't fit the competitive binding data for PNA E-1-tau.

Figure 2-9: Competitive binding for S-modified PNA (S-1-tau). All samples were prepared in 20 mM HEPES, 0.5 mM EDTA, 100 mM NaCl at pH 7.0. The rHP1 concentration remains constant at 1 μM, and S-1-Tau PNA concentration remains at 20 μM in all samples. a, b) rHP2 and rHP1 binding to S-1-Tau PNA. c, d) rHP3 and rHP1 binding to S-1-Tau PNA. e, f) rHP4 and rHP1 binding to S-1-Tau PNA.

Compared to PNA E-1-tau, PNA S-1-tau shows much higher fluorescence recovery in the fluorescence competitive binding studies (**Figure 2-9**). When the mutated RNA (rHP2, rHP3, and rHP4) concentration reaches 50 μM, the fluorescence intensity is comparable to that of rHP1 alone in the solution (**Figures 2-7 and 2-9**). The K_c values obtained (**Figures 2-9**) suggest that rHP2 and rHP3 bind to PNA S-1-tau more tightly than 2-aminopurine labeled rHP1 (**Table 2-2**). The enhanced binding of rHP2 and

rHP3 compared to rHP1 might be due to the facts that rHP2 and rHP3 have a G-C pair mutated to a G-U pair, respectively. In addition, replacing 2-aminopurine with adenine may enhance the PNA binding. Thus, the S-modified PNA is promising in targeting the disease-causing mutants of tau pre-mRNA splice site RNA. The quantitative fitting and extraction of the dissociation constants from the competitive binding are undergoing.

References

1. Qi, L., Chan, T.H., Tenen, D.G. and Chen, L. (2014) RNA editome imbalance in hepatocellular carcinoma. *Cancer Res*, **74**, 1301-1306.
2. Zipeto, M.A., Jiang, Q., Melese, E. and Jamieson, C.H.M. (2015) RNA rewriting, recoding, and rewiring in human disease. *Trends Mol Med*, **21**, 11.
3. Ge, J. and Yu, Y.-T. (2013) RNA Pseudouridylation: New Insights into an Old Modification. *Trends Biochem Sci*, **38**, 9.
4. Yue, Y., Liu, J. and He, C. (2015) RNA N6-methyladenosine methylation in post-transcriptional gene expression regulation. *Genes Dev*, **29**, 1343-1355.
5. Finn, P.J., Gibson, N.J., Fallon, R., Hamilton, A. and Brown, T. (1996) Synthesis and properties of DNA-PNA chimeric oligomers. *Nucleic Acids Res*, **24**, 3357-3363.
6. Uhlmann, E., Peyman, A., Breipohl, G. and Will, D.W. (1998) PNA: Synthetic polyamide nucleic acids with unusual binding properties. *Angew Chem Int Edit*, **37**, 2797-2823.
7. Betts, L., Josey, J.A., Veal, J.M. and Jordan, S.R. (1995) A nucleic acid triple helix formed by a peptide nucleic acid-DNA complex. *Science*, **270**, 1838-1841.
8. Meister, G. and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature*, **431**, 343-349.
9. Doudna, J.A. (2000) Structural genomics of RNA. *Nat Struct Biol*, **7 Suppl**, 954-956.
10. Batey, R.T., Rambo, R.P. and Doudna, J.A. (1999) Tertiary Motifs in RNA Structure and Folding. *Angew Chem Int Ed*, **38**, 2326-2343.

11. Devi., G., Zhou., Y., Zhong, Z., Toh, D.-F.K. and Chen, G. (2015) RNA triplexes: from structural principles to biological and biotech applications. *Wiley Interdiscip Rev RNA*, **6**, 111-128.
12. Gupta, P., Zengeya, T. and Rozners, E. (2011) Triple helical recognition of pyrimidine inversions in polypurine tracts of RNA by nucleobase-modified PNA. *Chem Commun*, **47**, 11125-11127.
13. Cassidy, S.A., Slickers, P., Trent, J.O., Capaldi, D.C., Roselt, P.D., Reese, C.B., Neidle, S. and Fox, K.R. (1997) Recognition of GC base pairs by triplex forming oligonucleotides containing nucleosides derived from 2-aminopyridine. *Nucleic Acids Res*, **25**, 4891-4898.
14. Devi., G., Yuan., Z., Lu., Y., Zhao., Y. and Chen., G. (2014) Incorporation of thio-pseudoisocytosine into triplex-forming peptide nucleic acids for enhanced recognition of RNA duplexes. *Nucleic Acids Res*, **42**, 4008-4018.
15. Acharya, P., Cheruku, P., Chatterjee, S., Acharya, S. and Chattopadhyaya, J. (2004) Measurement of nucleobase pKa values in model mononucleotides shows RNA-RNA duplexes to be more stable than DNA-DNA duplexes. *J Am Chem Soc*, **126**, 2862-2869.
16. Radhakrishnan, I., de los Santos, C. and Patel, D.J. (1991) Nuclear magnetic resonance structural studies of intramolecular purine.purine.pyrimidine DNA triplexes in solution. Base triple pairing alignments and strand direction. *J Mol Biol*, **221**, 1403-1418.
17. Radhakrishnan, I. and Patel, D.J. (1994) DNA triplexes: solution structures, hydration sites, energetics, interactions, and function. *Biochemistry*, **33**, 11405-11416.

18. Nielsen, P.E., Egholm, M., Berg, R.H. and Buchardt, O. (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science*, **254**, 1497-1500.
19. Nielsen, P.E. (1991) Sequence-selective DNA recognition by synthetic ligands. *Bioconjugate Chem*, **2**, 1-12.
20. Ferrer, E., Shevchenko, A. and Eritja, R. (2000) Synthesis and hybridization properties of DNA-PNA chimeras carrying 5-bromouracil and 5-methylcytosine. *Bioorg Med Chem*, **8**, 291-297.
21. Jiang, B., Cao, J., Zhao, J., He, D., Pan, J., Li, Y. and Guo, L. (2012) Dual-targeting delivery system for bone cancer: synthesis and preliminary biological evaluation. *Drug Deliv*, **19**, 317-326.
22. Lohse, B., Ramanujam, P.S., Hvilsted, S. and Berg, R.H. (2005) Photodimerization in pyrimidine-substituted dipeptides. *J Pept Sci*, **11**, 499-505.
23. Frisch, M.J.T., G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, J. A., Jr.; Peralta, J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, J. M.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R.

- L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. (2009). Gaussian, Inc., Wallingford CT.
24. Privat, E.J. and Sowers, L.C. (1996) A proposed mechanism for the mutagenicity of 5-formyluracil. *Mutat Res*, **354**, 151-156.
25. Wang, Y., Hamasaki, K. and Rando, R.R. (1997) Specificity of aminoglycoside binding to RNA constructs derived from the 16S rRNA decoding region and the HIV-RRE activator region. *Biochemistry*, **36**, 768-779.
26. Tok, J.B., Cho, J. and Rando, R.R. (1999) Aminoglycoside antibiotics are able to specifically bind the 5'-untranslated region of thymidylate synthase messenger RNA. *Biochemistry*, **38**, 199-206.
27. Cho, J. and Rando, R.R. (2000) Specific binding of Hoechst 33258 to site 1 thymidylate synthase mRNA. *Nucleic Acids Res*, **28**, 2158-2163.
28. Asensio, J.L., Lane, A.N., Dhesi, J., Bergqvist, S. and Brown, T. (1998) The contribution of cytosine protonation to the stability of parallel DNA triple helices. *J Mol Biol*, **275**, 811-822.
29. Theimer, C.A., Blois, C.A. and Feigon, J. (2005) Structure of the human telomerase RNA pseudoknot reveals conserved tertiary interactions essential for function. *Mol Cell*, **17**, 671-682.
30. Hirokawa, N. (1994) Microtubule organization and dynamics dependent on microtubule-associated proteins. *Curr Opin Cell Biol*, **6**, 74-81.
31. Kertesz, A. and Munoz, D. (1998) Pick's disease, frontotemporal dementia, and Pick complex: emerging concepts. *Arch Neurol*, **55**, 302-304.

32. Spillantini, M.G., Murrell, J.R., Goedert, M., Farlow, M.R., Klug, A. and Ghetti, B. (1998) Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. *Proc Natl Acad Sci USA*, **95**, 7737-7741.
33. Lee, V.M., Goedert, M. and Trojanowski, J.Q. (2001) Neurodegenerative tauopathies. *Annu Rev Neurosci*, **24**, 1121-1159.
34. Goedert, M., Spillantini, M.G., Jakes, R., Rutherford, D. and Crowther, R.A. (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron*, **3**, 519-526.
35. Andreadis, A., Brown, W.M. and Kosik, K.S. (1992) Structure and novel exons of the human tau gene. *Biochemistry*, **31**, 10626-10633.
36. Goode, B.L. and Feinstein, S.C. (1994) Identification of a novel microtubule binding and assembly domain in the developmentally regulated inter-repeat region of tau. *J Cell Biol*, **124**, 769-782.
37. Gustke, N., Trinczek, B., Biernat, J., Mandelkow, E.M. and Mandelkow, E. (1994) Domains of tau protein and interactions with microtubules. *Biochemistry*, **33**, 9511-9522.
38. Zheng, S., Chen, Y., Donahue, C.P., Wolfe, M.S. and Varani, G. (2009) Structural basis for stabilization of the tau pre-mRNA splicing regulatory element by novantrone (mitoxantrone). *Chem Biol*, **16**, 557-566.
39. Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., Petersen, R.C., Stevens, M., de Graaff, E., Wauters, E., van Baren, J., Hillebrand, M., Joosse, M., Kwon, J.M., Nowotny, P., Che, L.K., Norton, J., Morris, J.C., Reed, L.A.,

- Trojanowski, J., Basun, H., Lannfelt, L., Neystat, M., Fahn, S., Dark, F., Tannenberg, T., Dodd, P.R., Hayward, N., Kwok, J.B., Schofield, P.R., Andreadis, A., Snowden, J., Craufurd, D., Neary, D., Owen, F., Oostra, B.A., Hardy, J., Goate, A., van Swieten, J., Mann, D., Lynch, T. and Heutink, P. (1998) Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature*, **393**, 702-705.
40. Morris, H.R., Perez-Tur, J., Janssen, J.C., Brown, J., Lees, A.J., Wood, N.W., Hardy, J., Hutton, M. and Rossor, M.N. (1999) Mutation in the tau exon 10 splice site region in familial frontotemporal dementia. *Ann Neurol*, **45**, 270-271.
41. Goedert, M., Spillantini, M.G., Crowther, R.A., Chen, S.G., Parchi, P., Tabaton, M., Lanska, D.J., Markesbery, W.R., Wilhelmsen, K.C., Dickson, D.W., Petersen, R.B. and Gambetti, P. (1999) Tau gene mutation in familial progressive subcortical gliosis. *Nat Med*, **5**, 454-457.
42. Donahue, C.P., Muratore, C., Wu, J.Y., Kosik, K.S. and Wolfe, M.S. (2006) Stabilization of the tau exon 10 stem loop alters pre-mRNA splicing. *J Biol Chem*, **281**, 23302-23306.
43. Varani, L., Hasegawa, M., Spillantini, M.G., Smith, M.J., Murrell, J.R., Ghetti, B., Klug, A., Goedert, M. and Varani, G. (1999) Structure of tau exon 10 splicing regulatory element RNA and destabilization by mutations of frontotemporal dementia and parkinsonism linked to chromosome 17. *Proc Natl Acad Sci USA*, **96**, 8229-8234.
44. Varani, L., Spillantini, M.G., Goedert, M. and Varani, G. (2000) Structural basis for recognition of the RNA major groove in the tau exon 10 splicing

- p>regulatory element by aminoglycoside antibiotics.
- Nucleic Acids Res*
- ,
- 28**
- , 710-719.
45. Eldrup., A.B., Dahl., O. and Nielsen., P.E. (1997) A Novel Peptide Nucleic Acid Monomer for Recognition of Thymine in Triple-Helix Structures. *J Am Chem Soc*, **119**, 2.
  46. Guianvarc'h., D., Fourrey., J.-L., Maurisse., R., Suna., J.-S. and Benhida., R. (2003) Design of Artificial Nucleobases for the Recognition of the AT Inversion by Triple-Helix Forming Oligonucleotides: A Structure–Stability Relationship Study and Neighbour Bases Effect. *Bioorg Med Chem*, **11**, 9.
  47. Guianvarc'h, D., Benhida, R., Fourrey, J.L., Maurisse, R. and Sun, J.S. (2001) Incorporation of a novel nucleobase allows stable oligonucleotide-directed triple helix formation at the target sequence containing a purine.pyrimidine interruption. *Chem Commun*, 1814-1815.
  48. Guianvarc'h., D., Fourrey., J.-L., Maurisse., R., Sun., J.-S. and Benhida., R. (2002) Synthesis, incorporation into triplex-forming oligonucleotide, and binding properties of a novel 2'-deoxy-C-nucleoside featuring a 6-(thiazolyl-5)benzimidazole nucleobase. *Org Lett*, **4**, 4209-4213.
  49. Schmidt, J.G., Nielsen, P.E. and Orgel, L.E. (1997) Enantiomeric cross-inhibition in the synthesis of oligonucleotides on a nonchiral template. *J Am Chem Soc*, **119**, 1494-1495.
  50. Weeks, K.M. and Crothers, D.M. (1992) RNA binding assays for Tat-derived peptides: implications for specificity. *Biochemistry*, **31**, 10281-10287.
  51. Ryder, S.P., Recht, M.I. and Williamson, J.R. (2008) Quantitative analysis of protein-RNA interactions by gel mobility shift. *Methods Mol Biol*, **488**, 99-115.

Appendix i. MALDI-TOF Characterization of Molecular Weight (Da) for Peptide Nucleic Acids

LysNH-TCUCTTTC-CONH₂

LysNH-TCUCUTTTC-CONH₂

LysNH-TC^FUCTTTC-CONH₂

LysNH-TC^FUC^FUTTC-CONH₂

LysNH-TC^{Br}UCTTTC-CONH₂

LysNH-TC^{Br}UC^{Br}UTTC-CONH₂

LysNH-TC'UCTTTC-CONH₂

LysNH-TC'UC'UTTTC-CONH₂

LysNH-LELELTC-CONH₂

LysNH-CELELTC-CONH₂

LysNH-LSLSLTC-CONH₂

LysNH-CSLSLTC-CONH₂

Appendix ii. K_d fitting for PAGE and Fluorescence studies

K_d fitting for PAGE at various pH

U1-3 with rHP1 at pH7.5

U2-3,5 with rHP1 at pH7.5

^FU1-3 with rHP1 at pH7.5

^FU2-3,5 with rHP1 at pH7.5

³²P U1-3 with rHP1 at pH7.5

³²P U2-3,5 with rHP1 at pH7.5

U1-3 with rHP1 at pH7.0

U2-3,5 with rHP1 at pH7.0

FU1-3 with rHP1 at pH7.0

FU2-3,5 with rHP1 at pH7.0

¹²⁵IU1-3 with rHP1 at pH7.0

¹²⁵IU2-3,5 with rHP1 at pH7.0

³²PU1-3 with rHP1 at pH7.0

³²PU2-3,5 with rHP1 at pH7.0

U1-3 with rHP1 at pH7.7

U2-3,5 with rHP1 at pH7.7

³²P-U1-3 with rHP1 at pH7.7

³²U2-3,5 with rHP1 at pH7.7

³HU1-3 with rHP1 at pH7.7

³HU2-3,5 with rHP1 at pH7.7

³HU1-3 with rHP1 at pH7.7

³HU2-3,5 with rHP1 at pH7.7

¹²⁵IU1-3 with rHP1 at pH7.7

¹²⁵IU2-3,5 with rHP1 at pH7.7

P8 with rHP1 at pH8.0

$^{\text{F}}$ U2-3,5 with rHP1 at pH 8.0

$^{\text{C}}$ U2-3,5 with rHP1 at pH 8.0

$^{\text{Br}}$ U2-3,5 with rHP1 at pH 8.0

³²P-UTP with rHP1 at pH 8.0

K_d Fitting for Fluorescence Study

U2-3,5 with dsRNA at pH 7.5

^FU2-3,5 with dsRNA at pH 7.5

^CU2-3,5 with dsRNA at pH 7.5

^IU2-3,5 with dsRNA at pH 7.5

^{Br}U2-3,5 with dsRNA at pH 7.5

P8 with dsRNA at pH 7.5

BrU2-3,5 with dsRNA at pH7.0

BrU2-3,5 with dsRNA at pH7.7

BrU2-3,5 with dsRNA at pH8.0

Appendix iii Thermal Melting of PNA-RNA duplex raw data curves

Singly modified PNA-RNA duplex melting at pH 7.5

Doubly modified PNA-RNA duplex melting at pH 7.5

Appendix iv S-Monomer Synthesis Details

Tert-butyl (2-aminoethyl)carbamate (2): Ethylenediamine (0.524 mol, 35mL) was dissolved in THF (300mL), and the solution was cooled in ice bath. Boc-Anhydride (0.0435 mol, 10mL) was diluted with THF (40mL), and the solution was added drop-wise at 0°C. After addition, the reaction mixture was stirred overnight at room temperature. THF was removed via rotavap, and the residue was dissolved in water (150mL). White precipitate was formed in around one hour, and was filtered out. The remain filtrate residue was extracted with ethyl acetate (150mL×4). The combined organic layer was washed with brine and dried over MgSO₄. After drying under vacuum, the product **2**(4.18g, 60% yield) was obtained as clear oil liquid.

Ethyl 2-((2-((tert-butoxycarbonyl)amino)ethyl)amino)acetate (3): compound **2** (3.3g, 0.0206 mol) was dissolved in acetonitrile (100mL) and the solution was cooled in ice bath. Triethylamine (4.32mL 0.0309 mol) was added into the cool solution and the mixture was allowed to stir for 2 minutes. Ethylbromoacetate (2.28mL, 0.0206mol) was diluted with acetonitrile (25mL), and the solution was added drop-wise at 0°C. After addition, the reaction mixture was stirred for another 8 hours at room temperature. The solvent was removed via rotavap. After extraction and flush column chromatography with ethyl acetate as eluent, the product **3** (3.01g, 60% yield) was obtained as clear oil liquid.

4-(3-nitrophenyl)thiazol-2-amine (6): 2-bromo-3-nitroacetophenone **4** (10g, 40.9mol) and thiourea **5** (3.03g, 39.8mol) were added into a mixture of H₂O (17mL) and EtOH (28mL). The mixture was heated to reflux for 2 hours. After the mixture was cooled to room temperature, the solution was set to very basic conditions by adding 2M solution of NaOH (80mL). The solution was stirred for another 1 hour. Yellow colour precipitate was formed and filtered off, washed with diethyl ether. After drying under vacuum overnight, the product (7.4g, 81% yield) was used for next step without further purification.

N-(4-(3-nitrophenyl)thiazol-2-yl)acetamide (7): compound **6** (3g, 0.0136 mol) was dissolved in acetic anhydride (60mL). The solution was heated to reflux for 5 hours. After the reaction mixture was cooled to room temperature, the formed yellow colour precipitate was filtered off and emulsified in 0.1M NaOH solution (37mL). The mixture was stirred for another 30 minutes before filtration. The obtained yellow colour product (3.3g, 92% yield) was dried under high vacuum without further purification.

N-(4-(3-aminophenyl)thiazol-2-yl)acetamide (8): compound **7** (0.5g, 1.9 mmol) was dissolved in a mixture of EtOH/MeOH (12mL, 1:1 v/v). Pd/C (10%, 0.2022g, 0.19 mmol) was added in fraction to the solution. The mixture was degassed and filled with hydrogen. The reaction mixture was stirred for 20 hours at room temperature under hydrogen pressure. Upon the completion of the reaction, the mixture was filtered off and washed with MeOH. The solvent was removed via rotavap and high vacuum. The obtained product (0.33g, 66% yield) was obtained as light beige solid.

N-(3-(2-acetamidothiazol-4-yl)phenyl)-2-chloroacetamide (9): compound **8** (2 g, 8.58 mmol) was dissolved in minimal of dimethylfuran. DCM (60mL) was added into the solution for dilution, and the overall solution was cooled in ice bath. Triethylamine (1.795mL, 12.9 mmol) was added to the cool solution. Chloroacetyl chloride (0.684mL, 8.58 mmol) was diluted with DCM (15mL), and the solution was added drop-wise over 2 hours. After extraction, the residue was purified by flash column chromatography. The product (1.7 g, 64% yield).

Ethyl 2-(((3-(2-acetamidothiazol-4-yl)phenyl)amino)-2-oxoethyl)(2-((tert-butoxycarbonyl)amino)ethyl)amino)acetate (10): compound **9** (2.4 g, 7.77 mmol) was dissolved in minimal of DMF. Compound **3** (1.8g, 7.77 mmol) and anhydrous potassium carbonate (1.6 g, 11.65 mmol) were added into the solution. The reaction mixture was stirred at room temperature for 8 hours. After extraction and flash column chromatography purification, the product (0.37 g, 8.6% yield) was obtained as pale yellow solid.

2-(((3-(2-acetamidothiazol-4-yl)phenyl)amino)-2-oxoethyl)(2-((tert-butoxycarbonyl)amino)ethyl)amino)acetic acid (11): Compound **10** (0.5g, 0.963 mmol) was dissolved in THF (10 mL), and the solution was cooled in ice bath. 0.5M of LiOH (4 mL) solution was added in intervals over 4 hours. After addition, the solution was allowed to stirred another 1 hour at room temperature. The THF was removed via rotavap. The pH of the aqueous solution was adjusted below 2.0 with 2M HCl (aqueous). The slightly grey solid that precipitated was filtered off and washed with cold MeOH to yield the product (0.31g, 65% yield).

Appendix v Fluorescence Measurement for PNA S-2-tau