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Synthesis and DNA/RNA complementation studies of peptide nucleic acids containing 5-halouracils†‡

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The monomers of peptide nucleic acids containing 5-halouracils (5-XU-PNA), incorporated into heptameric PNA in the middle position, have been synthesized. Thermodynamic analyses revealed that the heptameric PNA oligomer with DNA and RNA showed higher duplex stability compared to the unmodified PNA counterpart. NMR studies suggested that the electron withdrawing effect of the halogen atom increased the strength of the XU-A hydrogen bond.

Oligonucleotide analogs have received considerable attention during the past decade because of their present and potential therapeutic applications.¹ Both modified and artificial nucleic acids have been investigated to increase their affinity and improve their nuclease resistance. In such context, a new type of artificial oligonucleotide with a unique backbone termed peptide nucleic acid (PNA) has been designed. PNAs, first described and synthesized by Nielsen in 1991,² are completely artificial DNA analogues in which the sugar-phosphate backbone of natural nucleic acids is replaced by *N*-(2-aminoethyl)glycine units and nucleobases are attached *via* a methylene carbonyl linker. The spatial structure of PNAs is very similar to native oligonucleotides although PNAs are wholly synthetic nucleic acids. Their structure allows not only an effective recognition of complementary DNA or RNA obeying the Watson-Crick base-pairing rules, but also a remarkably high sequence specificity and affinity which are considered to be due to their uncharged backbone.³ For these reasons, PNAs are of great interest in biological medicine, with potential for development as antisense and/or antigene therapeutics. As research continues, chemical modifications of either the polyamide backbone or the nucleotide base have been performed for improved properties.⁴

Recently, introduction of halogen atoms into PNAs has attracted extensive interest because most of the halogenated modified PNAs have enhanced hybridization⁵ and cellular uptake.⁶ 5-Halouracils (5-XU), which are formed when a halogen (fluorine, chlorine or bromine) substitutes the C5 hydro-

gen in uracil or the methyl group in thymine, may be one of the most important halogenated nucleobases.⁷ Substitution of the nucleobases of DNA with 5-XU has been reported, and the base pairing configuration and stability have been investigated.⁸ The synthesis of the 5-bromouracil (5-BrU) PNA monomer using the MMT protection strategy has been described.⁹ These lines of research led us to become interested in alternative 5-halouracils [5-fluorouracil (5-FU) and 5-chlorouracil (5-ClU)], particularly 5-FU, which is the most widely used chemotherapeutic agent in oncology, as a functionalized nucleobase for conjugation to the backbone of PNAs. Moreover, a number of prodrugs of 5-FU have been synthesized¹⁰ and the release of 5-FU from the prodrugs has been studied.¹¹ Based on these studies, the combination of 5-FU and 5-FU-PNA may represent a promising new approach for cancer treatment.

In this paper, we report the synthesis of PNAs containing three kinds of 5-halouracils in order to meet the demands of urgent research. The Fmoc protection strategy has been adopted due to its mild reaction conditions and high coupling efficiency. The hybridization properties of 5-XU-PNA to

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‡ Electronic supplementary information (ESI) available: Experimental details, more spectroscopic data and UV-*T*_m profiles of PNA-DNA and PNA-RNA duplexes. See DOI: 10.1039/c6md00536e

Scheme 1 Synthesis of 5-XU-PNA monomers.

Fig. 1 Structures of synthesized PNA oligomers. **PNA1**: X = CH₃, **PNA2**: X = F, **PNA3**: X = Cl, **PNA4**: X = Br.

DNA and RNA have been described, and the effect of the substituted 5-XU has been studied.

5-XU-PNA monomers were synthesized according to a similar previously reported procedure⁹ with minor modifications, as outlined in Scheme 1. The new protocol did not involve the MMT protection strategy, which caused either high expense or acid-lability. Therefore, we synthesized 5-XU-PNA monomers using the Fmoc protection strategy. *tert*-Butyl-*N*-(2-Fmoc-aminoethyl)glycinate, which is widely used as the backbone for PNA monomer synthesis, was prepared according to a literature procedure.¹² Other key moieties are 5-halouracil acetic acids (2a–2c), which were prepared *via* direct alkylation of 5-XU with bromoacetic acid. Then using compound 2 coupled with *tert*-butyl-*N*-(2-Fmoc-aminoethyl)glycinate hydrochloride employing HATU as a condensing agent in the presence of DIEA, the Fmoc-protected PNA monomers (3a–3c) were produced. Finally, compound 3 was treated with TFA to remove the *tert*-butyl group to obtain the desired 5-XU-PNA monomers (4a–4c).

Several PNA oligomers were prepared on Wang resin using a manual device for solid-phase protocols. The sequences were: GGGTGT-Lys-NH₂ (**PNA1**), GGG-FU-GTT-Lys-NH₂ (**PNA2**), GGG-ClU-GTT-Lys-NH₂ (**PNA3**) and GGG-BrU-GTT-Lys-NH₂ (**PNA4**), where FU, ClU, and BrU indicate the 5-FU-PNA unit, 5-ClU-PNA unit, and 5-FU-PNA unit, respectively (Fig. 1). The unmodified **PNA1** was prepared for control studies, and the other embedded chimeric PNAs containing 5-XU-PNA units in the middle were designed to examine the effect of 5-XU on the stability of hybridization. The synthesis of **PNA1**–**4** was carried out using HATU–DIEA as the coupling reagent. The deprotection of the side-chain amine was performed synchronously during the final cleavage of the oligomer from the polymeric support using TFA–TIS. The lysine residue was conjugated at the C-terminus of the PNA to promote solubility

Fig. 2 UV-*T_m* profiles of PNA/DNA:DNA duplexes (top) and PNA/DNA:RNA duplexes (bottom). The *T_m* was measured in 10 mM phosphate buffer (pH 7.0); strand concentrations: 5 μM; temperature range: 10 to 80 °C; heating rate: 1 °C min^{−1}.

and prevent aggregation. All PNA oligomers were purified by reverse-phase HPLC, checked by analytical RP-HPLC, and characterized by mass spectrometry (Table 1).

The effects of halogen substitution in the base of PNA on the thermal stability *T_m* of the PNA duplexes with DNA and RNA (5'-AACYCCC-3', Y = A, T/U, G or C) were investigated by UV-melting experiments (260 nm). The UV-*T_m* profiles of the perfectly matched duplexes are shown in Fig. 2, and the other UV-*T_m* profiles are given in the ESI† (S9–S11). The *T_m* values are determined by the maxima in the first derivative curves, and the results are shown in Table 2.

Firstly, **DNA1** showed a *T_m* value of 20.9 °C (Table 2, entry 1) for the duplex with **DNA2**, whereas the *T_m* value of the **PNA1** (no substitution) duplex with **DNA2** was 36.9 °C (Table 2, entry 2) which is higher by 16.0 °C. When **RNA1** was hybridized to **DNA1** and **PNA1**, the *T_m* value of the duplex **PNA1:RNA1** was 42.0 °C (Table 2, entry 2) which is higher by 21.4 °C than the duplex **DNA1:RNA1** (*T_m* = 20.6 °C, Table 2, entry 1). The increased thermal stability of the PNA:DNA and PNA:RNA duplexes relative to their corresponding DNA:DNA or DNA:RNA duplexes is in agreement with previous studies.^{3b,9} Moreover, the thermal stability of the PNA:RNA

Table 1 ESI-MS spectral and analytical RP-HPLC data of synthesized PNA oligomers

Oligomer	Molecular formula	Mol wt calc.	Peaks observed	Purity (%)	Retention time (min)
PNA1	C ₈₃ H ₁₀₉ N ₄₃ O ₂₅	2109.0	1055.4 [M + 2H] ²⁺ ; 703.8 [M + 3H] ³⁺ ; 528.2 [M + 4H] ⁴⁺	97.24	9.212 ^a
PNA2	C ₈₂ H ₁₀₆ FN ₄₃ O ₂₅	2113.0	1057.2 [M + 2H] ²⁺ ; 705.0 [M + 3H] ³⁺ ; 529.0 [M + 4H] ⁴⁺	98.12	9.349 ^b
PNA3	C ₈₂ H ₁₀₆ ClN ₄₃ O ₂₅	2129.5	1064.7 [M + 2H] ²⁺ ; 710.3 [M + 3H] ³⁺ ; 533.1 [M + 4H] ⁴⁺	98.46	9.797 ^b
PNA4	C ₈₂ H ₁₀₆ BrN ₄₃ O ₂₅	2173.9	1087.6 [M + 2H] ²⁺ ; 725.5 [M + 3H] ³⁺ ; 544.3 [M + 4H] ⁴⁺	97.13	10.115 ^b

Conditions: ^a Venusil C-18 column (250 × 4.6 mm i.d., 5 μm); eluent A, 0.1% TFA in 100% water (v/v); eluent B, 0.1% TFA in 80% acetonitrile + 20% water (v/v); gradient, linear 12–32% B over 20 min; detection, 220 nm; flow rate, 1 mL min^{−1}. ^b Agilent Pursuit C-18 column (250 × 4.6 mm i.d., 5 μm); eluent A, 0.1% TFA in 100% water (v/v); eluent B, 0.1% TFA in 80% acetonitrile + 20% water (v/v); gradient, linear 10–30% B over 20 min; detection, 220 nm; flow rate, 1 mL min^{−1}.

Table 2 T_m data for PNA/oligonucleotide complexes^a

Oligomers	T_m (°C) ^b							
	DNA2 ^c	DNA3 ^d	DNA4 ^d	DNA5 ^d	RNA1 ^c	RNA2 ^d	RNA3 ^d	RNA4 ^d
DNA1 ^e	20.9	<10	<10	<10	20.6	<10	<10	<10
PNA1	36.9	25.1	27.8	27.3	42.0	29.2	32.4	30.2
PNA2	40.9	25.4	28.9	27.5	45.7	29.4	33.8	30.0
PNA3	40.3	25.3	29.0	26.4	45.2	29.5	33.6	29.6
PNA4	38.7	25.6	27.3	26.2	44.5	29.7	33.2	29.4

^a Solutions of PNA/oligonucleotide were prepared in 10 mM phosphate buffer (pH 7.0); strand concentrations: 5 μ M; temperature range: 10 to 80 °C; heating rate: 1 °C min⁻¹. ^b T_m was determined from the first derivative plot and the estimated error is ± 0.5 °C. ^c Fully complementary oligonucleotide (antiparallel), DNA2: 5'-AACACCC-3', RNA1: 5'-AACACCC-3'. ^d Single mismatch oligonucleotide (antiparallel), DNA3: 5'-AACTCCC-3', RNA2: 5'-AACUCCC-3', DNA4: 5'-AACGCCC-3', RNA3: 5'-AACGCC-3', DNA5: 5'-AACCCCC-3', RNA4: 5'-AACCCCC-3'. ^e Control DNA: 5'-GGGTGTT-3'.

duplex is slightly higher than the PNA:DNA duplex (42.0 *versus* 36.9 °C). This result is also consistent with a study reported previously.^{3b}

Meanwhile, the thermal denaturation studies also showed the sequence specificity of 5-XU in PNA duplex hybridizations. The standard PNA1 gave T_m values of 25.1, 27.8 and 27.3 °C (Table 2, entry 2), respectively, when hybridized to DNA3–5 (single mismatch). The standard PNA1 formed a duplex with a single mismatch sequence with T_m values lower by 9.1–11.8 °C (25.1–27.8 *versus* 36.9 °C). In the case of 5-FU-PNA (PNA2), it gave T_m values of 25.4, 28.9 and 27.5 °C, respectively, when hybridized to DNA3–5, which were lower by 12.0–15.5 °C than the duplex PNA2:DNA2 (T_m = 40.9 °C, Table 2, entry 3). Similarly, the T_m values of the duplexes PNA2:RNA2–4 are lower by 11.9–16.3 °C than the duplex PNA2:RNA1 (29.4–33.8 *versus* 45.7 °C). For other 5-XU-PNAs (PNA3 and PNA4), destabilization was also observed when the PNAs were hybridized to DNA3–5 or RNA2–4 compared with DNA2 or RNA1. These results indicated that 5-XU preferred to bind to A, while T/U, G and C were unfavorable binding partners of 5-XU.

Furthermore, PNA2–4 containing one 5-XU unit showed 1.8–4.0 °C (for DNA) or 2.5–3.7 °C (for RNA) higher T_m values

than control PNA1 (Fig. 3). The thermal stability of the XU:A base pairs varied as FU > CIU > BrU > T. Previous studies have suggested that the electron withdrawing effect of the halogen atom was a significant contributor to the higher stability of DNA duplexes.¹³ Therefore, we reason that the electron withdrawing effect of the halogen atom may be a favorable factor for the stability of PNA:DNA duplexes.

To investigate the electronic effect of the halogen atom, NMR spectroscopy was employed to characterize the changes of chemical shift and the line width of the N₃ imino proton. In this work, a series of symmetrical structure PNA heptamers have been synthesized using the method described above, and the sequences were GGGXGGG (X = T, FU, CIU and BrU). The sequences have the advantage that the ¹H NMR spectra in the imino region should exhibit one imino proton resonance for T/XU and three imino proton

Fig. 3 Comparative T_m values for 5-XU-PNAs and unmodified PNA with complementary antiparallel DNA2 and RNA1. For each 5-XU-PNA, the ΔT_m value represents the amount of stabilization over that of the control ($\Delta T_m = T_m \text{ XU-A} - T_m \text{ T-A}$).

Fig. 4 NMR spectra in the imino region of the PNA–DNA duplexes: (A) T:A, (B) FU:A, (C) CIU:A, (D) BrU:A. The spectra were recorded at 5 °C with 512 scans using a water suppression WATEGATE program with d19 of 40 μ s. The oligonucleotide was dissolved in a solution of 100 mM NaCl, 10 mM Na₂HPO₄, and 0.2 mM EDTA, pH 7.0 in 10% D₂O. Each spectrum was calibrated using TSP as an internal standard reference.

Table 3 Chemical shifts and line width of imino protons

Nucleobase	Chemical shifts of N ₃ (ppm)			Line width (half height width, Hz)
	Monomer ^a	PNA1–4:DNA2 duplexes ^b	$\Delta\delta^c$	
T	11.30	13.44	2.14	20
FU	11.88	14.20	2.32	36
ClU	11.90	14.11	2.21	25
BrU	11.87	14.06	2.19	22

^a Chemical shifts of the N₃ imino proton of PNA monomers in DMSO-d₆. ^b Chemical shifts of the N₃ imino proton of the duplexes PNA-DNA2. ¹H NMR spectra were recorded at 5 °C with 512 scans using a water suppression WATEGATE program with d19 of 40 μ s. The oligonucleotide was dissolved in a solution of 100 mM NaCl, 10 mM Na₂HPO₄, and 0.2 mM EDTA, pH 7.0 in 10% D₂O. ^c $\Delta\delta = \delta_{\text{oligo}} - \delta_{\text{monomer}}$.

resonances for G. Previous studies have shown that the chemical shift for the imino proton in T was more downfield than the chemical shift for the imino proton in G.¹⁴ Therefore, the peaks in the imino region can easily be assigned from the NMR spectra. The NMR measurements were obtained for the PNA-DNA duplexes containing intervening T/XU:A base pairs. The samples, 1 mM in single strand concentration, were dissolved in 100 mM NaCl, 10 mM Na₂HPO₄, 0.2 mM EDTA and 10% D₂O. After mixing the two strands, the oligonucleotides were heated to 80 °C and slowly cooled down to form the duplex. The NMR spectra were recorded using a previously reported method on a 500 MHz Bruker NMR system,^{13a} and the spectra of the PNA-DNA duplexes are shown in Fig. 4. The peak of the T–A duplex at 13.44 ppm is lost upon substitution with XU; however, new peaks are observed at 14.20, 14.11 and 14.06 ppm, assigned to the XU imino proton. The chemical shift and the line width of the N₃ imino proton are listed in Table 3.

In the PNA monomers, the chemical shifts of the N₃ imino proton in DMSO-*d*₆ were observed at 11.27, 11.88, 11.90 and 11.87 ppm, respectively (ESI†). The imino protons of 5-XUs are more acidic than the corresponding imino proton of T. Similarly, significant downfield shifts in the N₃ resonance of 0.62–0.76 ppm in the XU:A relative to the T:A were observed in the NMR studies of PNA-DNA duplexes (Table 3, column 3). The difference in the chemical shift of N₃ imino protons between 5-XU and T is due to the different electronic effect of the halogen atom *versus* methyl. The 5-halo substituent of XU is electron withdrawing whereas the T methyl group is electron donating. The electron withdrawing properties of all three halogens result in the low electron density of the pyrimidine ring, and increase the acidity of N₃ protons. The increased acidity of the N₃ protons of 5-XU would be expected to strengthen the hydrogen bond between N₃–H and adenine. Moreover, upon substitution of T with 5-XUs, the N₃ chemical shifts in the duplexes move further downfield (Table 3, column 4). The NMR measurements indicate that the strength order of the hydrogen bond is FU:A > ClU:A > BrU:A > T:A. This is consistent with the results of UV melting studies.

On the other hand, the N₃ imino proton line widths for duplexes containing 5-XU:A were slightly broader than those of corresponding T:A duplex (Table 3, column 5). Previous

studies have suggested that the broadening of the ClU:A imino proton peak in the DNA duplex might result from the proton exchange from within the intact base pair.^{13a} Therefore, the observed broadening of the XU:A imino proton peak is not directly related to the duplex stability.

Conclusions

In conclusion, we have synthesized the monomers of peptide nucleic acids containing 5-XU using the Fmoc protection strategy for the first time. PNA carrying 5-XU forms a duplex with complementary DNA or RNA with higher affinity than unmodified PNA. The XU:A base pair is more stable than the T:A base pair in the PNA:DNA or PNA:RNA duplex. Based on the significant roles of 5-XU in pharmaceutical applications, we can envision that 5-XU-PNA might be useful as a potent anti-tumor agent. Further work that includes the cellular delivery of 5-XU-PNA and the antisense effect of the functionalized nucleobase of 5-XU-PNA oligomers, in combination with the release of 5-XU from 5-XU-PNA, is in progress.

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