Extended Target Sequence Specificity of PNA–Minor-Groove Binder Conjugates

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Peptide nucleic acid (PNA), the pseudopeptide DNA mimic, has been used extensively for sequence-selective targeting of double-stranded DNA (see, for example, refs. [1–4]). Most studies have focused on triplex invasion by using homopyrimidine *bis*-PNAs,[5–7] and more recently on double duplex invasion by exploiting pseudocomplementary PNA oligomers.[8–10]

Even when using relatively short PNAs and DNA targets (8–10 bases), the helix invasion binding is kinetically controlled due to the very high stability and consequently slow dissociation rates of the complexes.[11–12] Despite these binding characteristics, 10-mer *bis*-PNAs very effectively (in kinetic terms) discriminate between a full-sequence complementary target and targets containing internal mismatches.[11–12] However, not surprisingly, sequence-specific preference for longer targets (12–15 bases) over shorter targets that are contained within a longer sequence is not efficient (unpublished data), and no evident solution to this problem presents itself when using simple PNAs.

We have recently shown that binding kinetics are dramatically enhanced when using PNA–acridine conjugates.[13] This effect of the DNA intercalator is ascribed to its DNA-binding properties which are thought to ensure a high local concentration of PNA close to the DNA helix and thus bring about an increased probability for the PNA to "catch" a "breathing" target.[13] Analogous effects are observed with DNA oligomers that are conjugated to catioinc peptides.[14,15] We speculated that this principle could be refined by employing other DNA-binding ligands with sequence specificity, such as minor-groove binders.[16–17] We have previously described an amino acid–Hoechst analogue that is readily incorporated into peptides,[18] and, using this ligand, we have now prepared a PNA–Hoechst conjugate and examined whether this conjugate would kinetically and sequence preferentially guide the PNA moiety to target a binding site proximal to an A–T region with an affinity for the minor-groove binder.

In deciding the binding site and the PNA sequence, we considered that binding of the Hoechst moiety within the PNA target would inhibit PNA binding,[19] and therefore designed the PNA target without A_n stretches ($n = 3$). Furthermore, we chose an alternating A–T target for the Hoechst moiety, as this would not create a partial binding site for the PNA and separated the two targets by four G–C base pairs. Both chemical and enzymatic probing indicate that the helical distortion proximal to a PNA triplex invasion complex is no more than 2–3 base pairs,[20,21] and therefore no interference between the two types of targets should occur. Finally, the conjugate was constructed with a long (30-atom) hydrophilic linker (that also included an amine to provide an extra charge) between the PNA and the Hoechst moiety (Figure 1), and pseudoisocytosine (J) was used in the Hoogsteen binding domain of the *bis*-PNA to eliminate pH dependence of the binding.[22]

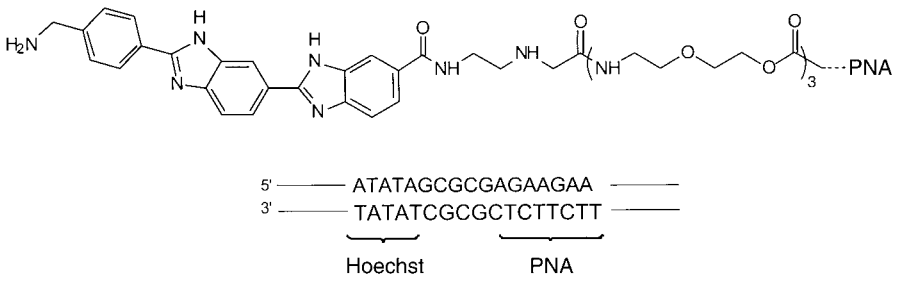

Figure 1. *Chemical structure of the PNA–Hoechst conjugate (Hoe-aeg-(eg1)$_3$-JTJTTJTT-(eg1)$_3$-TTCTTCTC-Lys-NH$_2$) where J denotes pseudoisocytosine, the control PNA (H-aeg-(eg1)$_3$-JTJTTJTT-(eg1)$_3$-TTCTTCTC-Lys-NH$_2$), and base sequence of the DNA target and the PNA. The PNA conjugate was prepared by continuous, solid-phase Boc-synthesis, purified by reverse-phase HPLC, and characterized by MALDI-TOF mass spectrometry, as described.*[18,29]

In order to study whether the PNA moiety of this conjugate was able to discriminate between an "isolated" PNA target and one proximal to an A–T region, a binding experiment was performed with a mixture of two DNA fragments, a longer one with only the PNA target (−) and a shorter one containing the combined target (+). Free and bound forms of these DNA fragments are readily separated by using gel mobility shift analysis (Figure 2). The results clearly show that the PNA–Hoechst conjugate preferably bound the + target than the − target with approximately tenfold more binding (Figure 2A); this is not the case for the corresponding control PNA (Figure 2B). Additionally, the conjugate binds the target 40-fold more efficiently than the simple *bis*-PNA; this indicates that the minor-groove binder, in addition to a specific-sequence directing effect, also provides general DNA affinity, analogous to the effect of 9-aminoacridine,[13] or of cationic peptides.[14,15] This general affinity effect might to a certain extent be of electrostatic origin. We therefore decided to perform an experiment at higher ionic strength with the expectation that the sequence-directing effect of the Hoechst moiety would be more pronounced.

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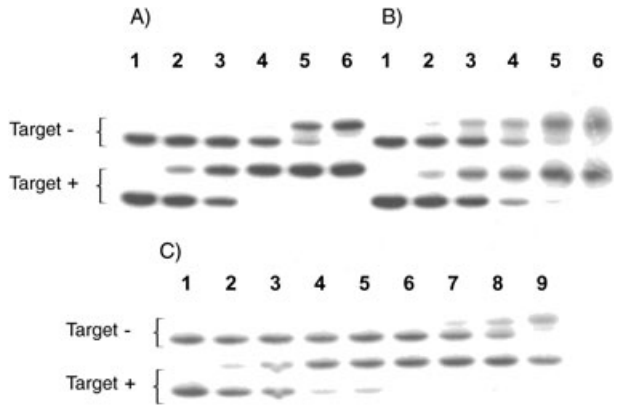

Figure 2. *Binding of PNA to DNA fragments containing only the PNA target (−)(5′-GAGAAGAA) or the PNA target as well as a Hoechst target (+)(5′-TATAT). A mixture of the two ^{32}P-end-labeled DNA fragments was incubated with the PNA in Na-phosphate (10 mM) and EDTA (1 mM) at pH 6.3 and 37 °C for 20 h and analyzed by electrophoresis on a polyacrylamide gel (10%) in TBE buffer, followed by autoradiography. A) bis-PNA–Hoechst conjugate at 0, 5, 10, 20, 40, or 80 nM (lanes 1–6). B)* bis-*PNA at 0, 0.6, 1, 2, 2.5, 5, or 10 μM (lanes 1–6). "Target+" is the* HindIII/SstI *fragment of a pBluescript KS+ clone: 5′-GATCTTCTTCTCGCGCTATAT/5′-GATCATATAGCGCGAGAAGAA in the* BamHI *site; and "Target−" is the* HindIII/PvuII *fragment of a pBluescript KS$^+$ clone: 5′-GATCTTCTTCTCGCGC/5′-GATCGCGCGAGAAGAA). C) Experiment same as in (A) except that the incubation was performed in the presence of KCl (50 mM). The PNA–Hoechst concentrations were: 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.3, 2.5, or 5 μM (lanes 1–9).*

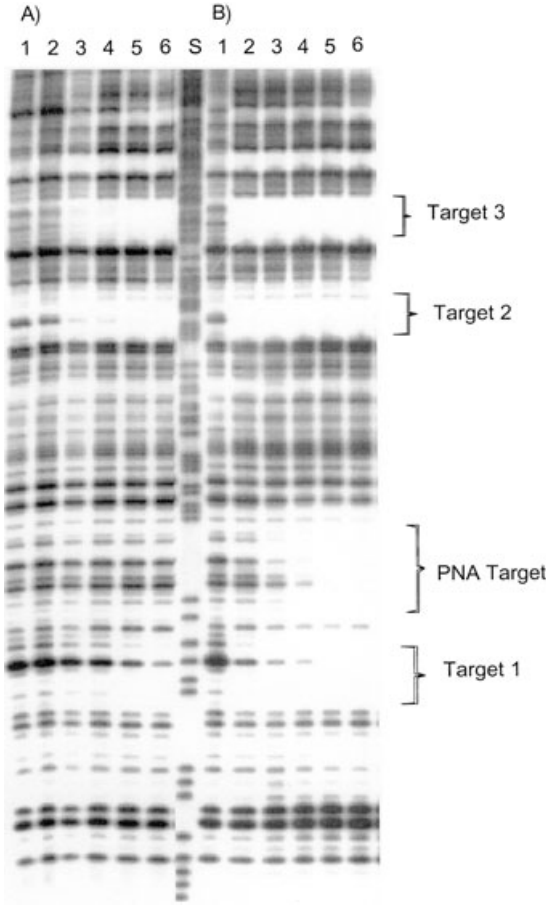

Figure 3. *DNA sequence specificity of the PNA–Hoechst conjugate analyzed by DNaseI footprinting. Incubation was carried out for A) 30 min or B) 20 h in Tris-HCl (10 mM) and EDTA (1 mM) at pH 7.4 and 37 °C by using 0, 0.12, 0.4, 1.2, 4, or 12 μM PNA (lanes 1–6). The* HindIII/PvuII *fragment of the "Target+" plasmid (Figure 2) 3′-labeled with ^{32}P at the* HindIII *site was used, and following incubation the samples were treated with DNaseI after adjusting the buffer to Tris-HCl (50 mM), Mg^{2+} (3 mM). The samples were analyzed by electrophoresis on a polyacrylamide (10%) gel with urea (7 M) in TBE buffer followed by autroradiography. S is an A/G sequence marker. The targets are: PNA: TTCTTCT, 1: TATAT, 2: TTAA, and 3: TTAAGATAAT.*

Indeed, the results clearly show that upon addition of 50 mM K^+, the preference of the conjugate for the + target over the − target increases to 30-fold (Figure 2C). Finally, we asked whether the conjugate was actually binding to the correct target by using DNaseI footprinting (Figure 3). These results allow several conclusions. At the shorter incubation time (30 min, Figure 3A), the PNA target is not occupied, but protection is seen at the A–T target proximal to the PNA target and at two other A–T-rich regions (targets 2 and 3). In fact, the Hoechst moiety of the conjugate binds with higher affinity to targets 2 and 3 than to the designated target 1. At the longer incubation time (20 h, Figure 3B), the PNA target is fully occupied at 4 μM PNA conjugate, and the A–T-rich regions are still also protected. Clearly and not surprisingly, binding to the minor-groove sites is fast whereas PNA (triplex invasion) binding is slow. Therefore in conclusion, these results demonstrate a new strategy for constructing DNA-recognition ligands composed of two domains: a sequence-guiding domain with fast binding kinetics (fast on and off rates), which increases the target specificity, and a DNA-modification domain characterized by a slow on rate and a very slow off rate, which determines the biological activity of the conjugate. This type of targeting should not be confused with equilibrium affinity, but should rather be compared to sequence-directed modification of DNA, for example, with alkylating agents.[23] In the present case, the modification consists of a PNA P-loop that might arrest transcription elongation,[24,25] induce transcription initiation,[26,27] or provoke site-specific repair.[28]

Moreover, the specific results clearly show that the present conjugate is far from optimal. The Hoechst moiety has higher affinity for other sites than the one directing the PNA to its target. The affinity for the latter is also smaller than desired—full occupancy is not observed at PNA concentrations that eventually lead to full PNA binding (Figure 3, lanes 5 and 6). In an attempt to improve targeting, we constructed a DNA fragment in which minor-groove target 2 (5′-TTAA) was positioned adjacent to the PNA site (5′-TTAAGCGCGAGAAGAA). However, the behavior of this target was virtually identical to that described above. In view of this unexpected result, we are now performing in vitro selection experiments in order to identify optimal targets for the PNA–Hoechst conjugate. Furthermore, the system could be significantly improved, both in terms of affinity/efficacy and, most importantly, in terms of targetable sequences, by employing minor-groove binding hairpin polyamides.[16–17] Experiments exploring these aspects are now in progress.

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Keywords: DNA recognition · DNA targeting · minor-groove binder · nucleic acids · peptide nucleic acids

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