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PPG Peptide Nucleic Acids that Promote DNA Guanine Quadruplexes**

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Abstract

Properly designed peptide nucleic acids (PNAs) can invade G-rich DNA duplexes and induce the formation of a G-quadruplex in the free DNA strand. Replaceing guanines in the PNA sequence with PPG nucleobases eliminates G-quadruplex formation with PNA and promotes invasion of the target DNA.

Keywords

peptide nucleic acids; G-Quadruplexes; DNA recognition; Nucleic acids; Nucleobases

DNA oligomers form a wide variety of sequence-dependent secondary structures distinct from the canonical B-form duplex. For example, guanine-quadruplexes are a well-known type of DNA secondary structure that can form when guanine-rich oligonucleotides are in the presence of appropriately sized cations such as sodium, potassium, or ammonium (Figure 1a).[1] Sequences containing guanine quadruplexesarecommonly found in telomeres that are located at the tails of chromosomes.[2] Recent studies have also shown that guanine-rich sequences with the potential to form quadruplexes are over represented and conserved in the regulatory regions of many genes and could play a role in normal transcription as well as overexpression of oncogenes.[3] Although many of these guanine-rich sequences readily form guanine-tetrads *in vitro*, researchers continue to examine the specific roles of quadruplex formation *in vivo* and their association with gene regulation.[4] Chemical tools that allow guanine quadruplexes to be probed will be essential to understanding their functions and could lead to beneficial therapies.

Peptide Nucleic Acids (PNAs) are non-natural nucleic acids in which the natural nucleobases are preserved while the sugar phosphate backbone is replaced with pseudo-

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Supporting information for this article is available

peptide residues. PNA oligomersform stable complexes with natural nucleic acids through typical Watson-Crick hydrogen bond pairing rules between nucleobases.[5] PNA oligomers are also chemically stable, resistant to enzymatic degradation,[6] and can be readily derivatized to alter their physical and chemical characteristics.[7] Because of these properties, PNA can be employed in medicinal and diagnostic applications.[8]

Despite their high affinity and selectivity for natural nucleic acids, unmodified PNA oligomers do not readily invade duplex DNA unless specifically modified using one of several strategies.For instance, DNA invasion and binding can be improved using PNA modifications that preform the oligomer in a right-handed helix, or by appending ligands that non-selectively intercalate into duplex DNA, or with specific PNA-peptide chimeras.[9] Bis-PNA oligomers, covalently linked PNA strands that contain both Watson-Crick and Hoogsteen complements, can invade and tightly bind duplex DNA that has a poly-purine sequence, forming a bis-PNA-DNA triplex.[10]

PNA invasion into duplex DNA can also be facilitated if the strand complementary to the target sequence can be involved in further secondary structure formation. This can be achieved if the complementary sequence complexes with another PNA probe (the strategy of using pseudo-complimentary PNA oligomers),[11] or forms an intramolecular complex (e.g. PD-loops).[12] In our previous work, we discovered that short, guanine-rich PNA oligomers could invade and bind to plasmid DNA (Figure 1b).[13] We showed that invasion of duplex DNAdepends on PNA binding to its target sequence by Watson-Crick hydrogen bonding *and* on quadruplex formation of the displaced DNA strand.[13] Using PNA to promote quadruplex formation in this manner could work in conjunction with other strategies to target quadruplexes, such as small molecules[14] and anti-bodies for specific G-rich sequences.[15] Since there is a large structural diversity in DNA quadruplexes, combining strategies may also help improve specificity of targeting one type of G-quadruplex over others.[16]

Although promising, the use of unmodified PNA probes still presents several limitations. Guanine-rich PNA oligomers aggregate, reducing the availability of these PNA probes to bind their intended target. Guanine-rich PNA oligomers readily form quadruplexes with DNA,[17] raising the possibility that the PNA probe could bind directly to the guanine rich strand, or other off-target G-rich sequences. Furthermore, PNA forms homogeneous quadruplexes,[18] reducing the availability of free PNA to bind target DNA.

To address these concerns, we synthesized a PNA monomer containing a non-natural guanine analogue. Pyrazolo[3,4-d]pyrimidine guanine (PPG) is a guanine analogue that lacks a nitrogen atom at the N7 position (Figure 2).[19] Because of this modification, PPG lacks the electron lone pair necessary to coordinate metal ions and form tetrad structures. However, PPG maintains the hydrogen-bonding motif on the Watson-Crick face to facilitate cytosine recognition. DNA oligomers containing PPG showed higher thermal stability and higher mismatch discrimination than guanine-rich oligomers.[20] Such oligomers also aggregate less than the corresponding guanine-rich oligomers.[21]

In this article, we describe the synthesis and physical properties of PPG-containing PNA oligomers. We synthesized both amino ethyl glycine (*aeg*) PNA, as well as (S,S)-*trans*-cyclopentane PPG monomers, a PNA residue that increases PNA oligomer binding and base-mismatch discrimination.[22] We investigated the abilities of PPG-rich oligomers to invade the duplex plasmid DNA of the BCL2 gene promoter region sequence, freeing the complimentary strand to undergo guanine quadruplex formation. BCL2 is of particular interest because of its role in cell death regulation and over expression in many types of cancers.[23] We used multiple PNA oligomers differing in charge, polarity, length and PPG content to better understand the role duplex stability and kinetics play in DNA duplex invasion. We accomplished this by using chemical probes and protection assays to assess binding events within the plasmid DNA, as well as restriction enzyme assays to quantify the efficacy and efficiency of such PNA binding.

The synthesis of PPG-PNA was carried out by first developing a method to make PPG-acetic acid with a protecting group on the exocyclic nitrogen (Supporting Information Scheme S1). Briefly, 2-amino-4,6-dichloropyrimidine-5-carboxyaldehyde was condensed with ethyl hydrazinoacetate followed by protection of the exocyclic amine as a Cbz carbamate.[24] A 2-step hydrolysis was then performed to afford the PPG-acetic acid. This nucleobase acetic acid was then coupled to aminoethyl glycine PNA backbone or (S,S)-*trans*-cyclopentane PNA backbone, to make aegPNA PPG monomer (**1**) and cypPNA PPG monomer (**2**), respectively.

All the PNAs were synthesized using a standard PNA procedure on Applied BioSystems 433A automated peptide synthesizer, purified by HPLC, and characterized by mass spectrometry as previously reported (Supporting information, Table S1).[13] The use of zwitterionic oligomers greatly increased the specificity of the PNA oligomers and reduced aggregation without sacrificing solubility. PPG monomers were incorporated into the PNA oligomers without issue, and the resulting product was generally superior in yield and purity to that of guanine analogues.

Variable temperature UV was used to determine the effect PPG residues have on the thermal stabilities of PNA oligomers, as well as determine their propensity for self quadruplex formation (Table 1).

Annealing experiments showed that the incorporation of two aegPNA PPG residues in PNA 8 reduced the melting temperature of the oligomer with its complementary DNA by ∼5.5 °C compared to the PNA oligomer containing only guanine (PNA 4). This result was somewhat unexpected based on the fact that PPG has been shown to maintain and even slightly increase the thermal stability of DNA oligomers in which PPG has been substituted for guanine.[20] However, this discrepancy could simply be further evidence that the factors that control double helix formation in DNA duplexes (hydrogen bonding, base stacking, solvent effects, preorganization, etc.) differ in importance when considering the stability of PNA/DNA duplexes. When (S,S) *trans*-cyclopentane PNA PPG residues were incorporated (PNA 9), the thermal stability increased compared to the aegPNA PPG analogue, consistent with previous results. The increase in thermal stability from cyclopentane residues successfully recovered (and further increased) the stability lost from substituting guanine

with PPG. Similar to natural nucleic acids, PNA can form stable guanine tetrads in G-rich sequences in the presence appropriate cations.[18] When the aforementioned oligomers were examined for evidence of quaduplex formation, PNA 4 showed evidence of stable quadruplex formation. However, there was no evidence for quadruplex formation in PNA 8. Despite (S,S) *trans*-cyclopentane PNA having the ability to strengthen PNA quadruplexes,[22b] no evidence for any quaduplex was observed in PNA 9.

As described previously, we established through the use of chemical probes that short, single-stranded PNA oligomers could invade plasmid DNA when the potential for guanine quadruplex formation existed in the sequence complementary to the target sequence[13]. When DEPC analysis was performed to quantify the efficacy of DNA-duplex invasion, PNA 5 showed greater propensity for exposing adenines in the quadruplex- forming strand to chemical modification compared to PNA 1 containing only guanine (Supporting information, Figure S1). Similar improvements in the invasion of DNA were observed when the overall charge of the PNA is zwitterionic instead of cationic, and when the PNA sequence is antiparallel instead of parallel. Probing the PNA oligomers for their ability to invade plasmid DNA with OsO_4 showed a similar trend(data not shown). Since PPG lacks the capability of forming guanine-quadruplexes, positive results in these assays with the use of PPG PNA means that the PNA probes are binding only to the target sequence.

To further assess the binding properties of different PNAs to plasmid DNA, we established two new assays: an S1 nuclease assay and an HpyF10VI restriction endonuclease protection assay. Nuclease S1 attacks single-stranded DNA, and is a highly sensitive probe for non-B form duplex DNA.[25] PNAs bound to plasmid pCRBcl2 with the human BCL2 gene promoter target were incubated with S1 nuclease on ice as described in the Supporting Information. The samples were then digested with Dra1 restriction endonuclease to reveal the sites for S1 cleavage. Dra1 has a single restriction site in the pCRBcl2 plasmid located 2683 base pairs (bp) from the Bcl2 quadruplex-forming sequence. Therefore, S1 cleavage in the quadruplex-forming sequence would result in the appearance of a 2683 bp fragment in addition to the linear DNA. In a control sample with no PNA, there was no S1 cleavage observed at the PNA target sequence.

The intensity of this fragment would reflect the efficiency of S1 cleavage and therefore PNA invasion at the target sequence. Figure S2 (Supporting Information) shows percentage of the S1 cleavage measured for the panel of PNAs. The trends observed are in broad agreement with what was seen in with the DEPC probe.For example, anti-parallel PNA probes (PNAs 3, 6, and 9) are uniformly better at invading and binding to the target sequence compared to their parallel analogues (PNAs 1, 2, 5 and 7).

In the HpyF10VI restriction endonuclease protection assay, the PNA binding sequence partially covers an hpyF10VI restriction site. Although HpyF10VI has numeroussites within pCRBcl2 plasmid, the PNA-covered HpyF10VI restriction site is located between two EcoR1 restriction sites. We cut PNA-plasmid complexes with both HpyF10VI and EcoR1 and labeled EcoR1 ends with [α-^{32}P]-dATP and the Klenow Fragment of DNA polymerase. If PNA is bound to its recognition sequence at the time of the digestion with restriction enzymes, we should see a single 60 nucleotide (nt) radiolabeled fragment instead of two (26

nt and 34 nt) fragments. The intensity of this 60 nt fragment is proportional to the extent of PNA binding. The intensities of the 60 nt and 34 nt fragments were measured and the percentages of the intensity of the 60 nt fragment for different PNAs are shown in Figure 3. This assay further reinforces the results from the previous assays. Once again, PPG containing PNAs (PNAs 6, 9) show better binding to the target in the plasmid compared to their guanine-rich counterparts (PNAs 1, 2).

In summary, we have detailed the synthesis and properties of using PPG attached to two types of peptide nucleic acid backbones. As the number of guanine-rich sequences identified in the regulatory region of genes continues to grow, PNAs containing PPG residues present a means of targeting such regions in a sequence specific manner. Incorporation of PPG residues in PNA oligomers increases the potency of such probes by elimination of quadruplex formation. This improvement in properties of PPG PNA may be due to the reduction of aggregation associated with guanine-rich PNAs that may form intra- and intermolecular guanine quadruplexes with themselves. In theory, the incorporation of PPG residues should allow for the synthesis of longer, more guanine-rich PNA probes than is currently feasible using only guanine. Finally, the use of the (S,S)-*trans*-cyclopentane PNA backbone recovers the loss in thermal stability of binding DNA seen when PPG is introduced into aegPNA oligomers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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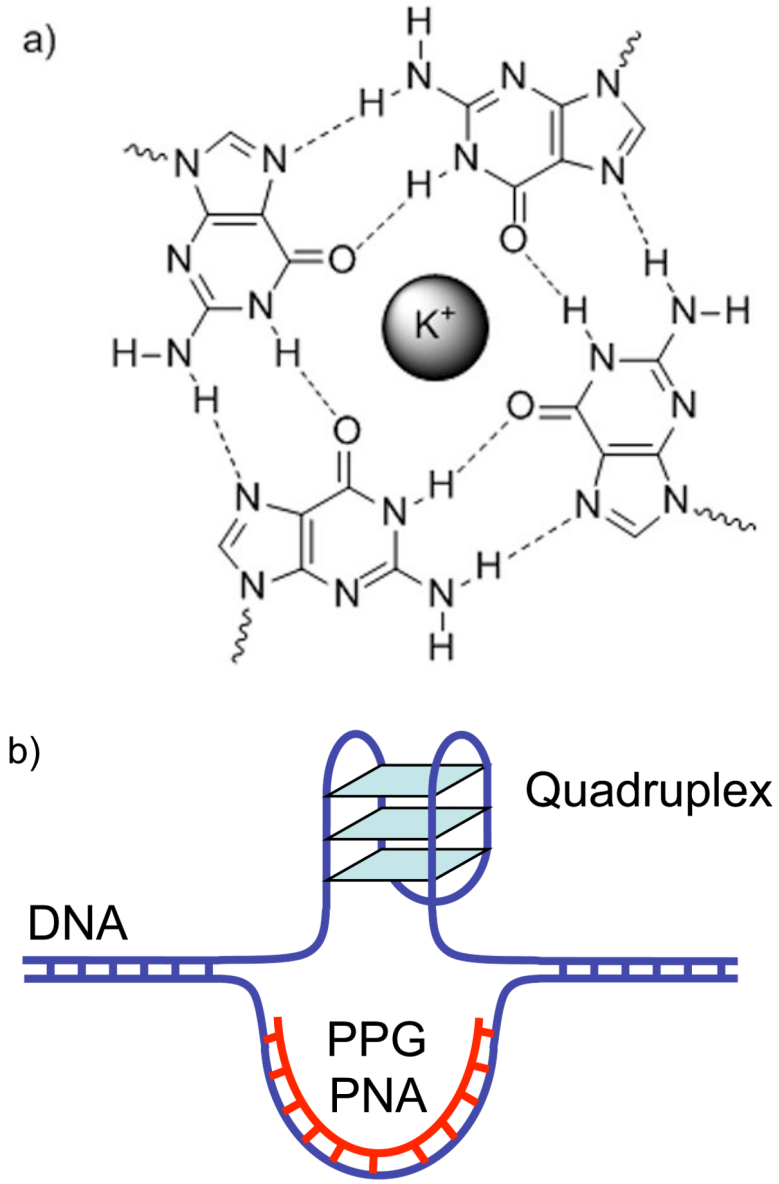

Figure 1.

a) Arrangement of guanines around a central cation resulting in quadruplex formation. Guanine-tetrads have been observed in G-rich sequences in both natural and non-natural nucleic acids. b) Basic strategy for invasion of PPG PNA into DNA to promote quadruplex formation.

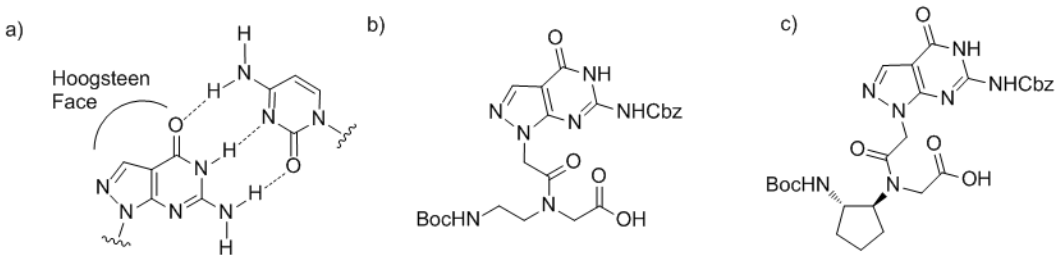

Figure 2.

a) Pyrazolo[3,4-d]pyrimidine (PPG) hydrogen bonding to cytosine in the typical Watson-Crick motif. Compared to guanine, the lack of the N7 nitrogen in PPG greatly reduces the potential for coordination on the Hoogsteen face. b) PPG aeg-PNA monomer (X). c) PPG cyp-PNA monomer (X).

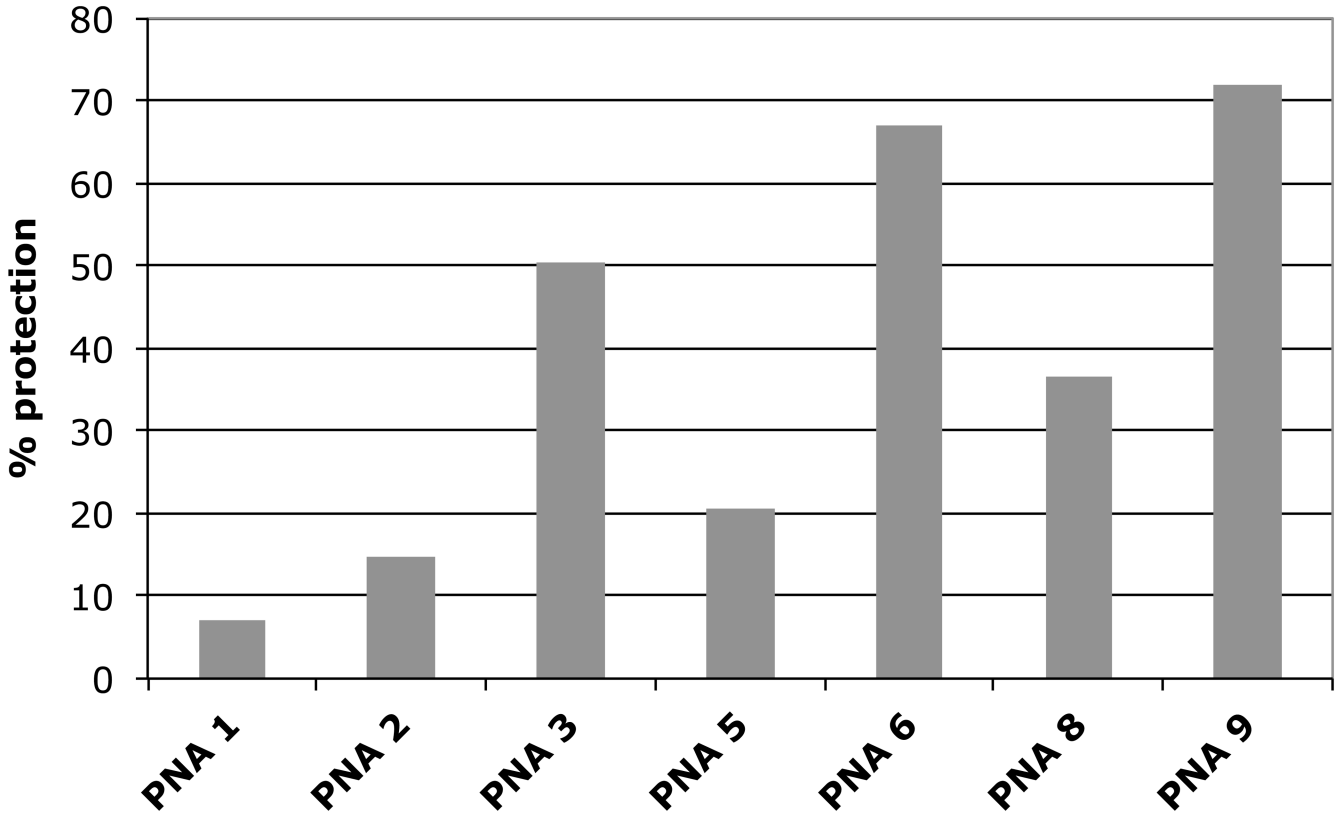

Figure 3.

PNA invasion measured by HpyF10VI protection assay. Percent of protection was calculated from the ratio of intensities of the 60 nt fragment that appeared as a result of PNA invasion and the intensity of the 34 nt fragment resulted from the normal cleavage by the restriction enzyme of the PNA binding site in the same lane of the gel.

Table 1

Thermal melting data[a] for PNA oligomers with complementary DNA and alone.

PNA	Sequence[b]	T_m[°C] DNA[c]	T_m[°C] Quadruplex[d]
4	H_2N-Glu-G-G-G-C-G-G-A-G-G-Glu-Lys	82.4	54.0
8	H_2N-Glu-G-**X**-G-C-G-**X**-A-G-G-Glu-Lys	77.0	-
9	H_2N-Glu-G-**X**-G-C-G-**X**-A-G-G-Glu-Lys	88.0	-

[a] Solutions of PNA/DNA (1:1) were prepared in pH 7.0 PBS buffer containing 10 mM sodium phosphate, 0.1 mM EDTA, and 150 mM NaCl.

[b] PNA oligomers are written from N terminus to C terminus. Lys = lysine, Glu = glutamic acid.

[c] Complementary antiparallel DNA 5′-CCT CCG CCC-3′. Strand concentration is 5 microM for both PNA and DNA. T_m is estimated from the inflection point in the melting curve monitored at 260 nm. Estimated error is +/- 0.5 °C.

[d] PNA concentration is 10 microM. T_m is estimated from the inflection point in the melting curve (reverse sigmoidal) monitored at 295 nm. Estimated error is +/- 0.5 °C.

Table 2

Charge state and polarity of PNA oligomers.

PNA	Sequence[a]	Charge State[b]	Polarity[c]
1	H_2N-G-G-A-G-G-C-G-G-G-Lys	positive	parallel
2	H_2N-Glu-G-G-A-G-G-C-G-G-G-Glu-Lys	zwitterionic	parallel
3	H_2N-G-G-G-C-G-G-A-G-G-Lys	positive	anti-parallel
4	H_2N-Glu-G-G-G-C-G-G-A-G-G-Glu-Lys	zwitterionic	anti-parallel
5	H_2N-G-G-A-**X**-G-C-G-**X**-G-Lys	positive	parallel
6	H_2N-G- **X**-G-C-G-**X**-A-G-G-Lys	positive	anti-parallel
7	H_2N-Glu-G-G-A-**X**-G-C-G-**X**-G-Glu-Lys	zwitterionic	parallel
8	H_2N Glu-G-**X**-G-C-G-**X**-A-G-G-Glu-Lys	zwitterionic	anti-parallel
9	H_2N Glu-G-**X**-G-C-G-**X**-A-G-G-Glu-Lys	zwitterionic	anti-parallel

[a] PNA oligomers are written from N terminus to C terminus. Lys = lysine, Glu = glutamic acid, X = aeg PPG residues, X = cyclopentane PPG residues.

[b] The charge state is the most likely overall charge state for each oligomer at physiological pH. Positively charged sequences have primary amines (lysine side chain and N terminus) while zwitterionic oligomers also contain two acid moieties (glutamic acid side chains).

[c] The polarity is defined in relation to the target sequence (5′-CCT CCG CCC-3′) where the N terminus of PNA oligomers is analogous to the 5′ end of natural nucleic acids.