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Gene Targeting Using Peptide Nucleic Acid

Peter E. Nielsen

Summary

A brief overview of the properties of the deoxyribonucleic acid mimic peptide nucleic acid (PNA) is given, and the recent progress in cellular delivery of PNA and of using PNA oligomers for antisense and antigene gene targeting is presented.

Key Words: Gene targeting; antisense; antigene; DNA recognition; peptide nucleic acid; PNA.

1. Introduction

Peptide nucleic acids (PNA) (**Fig. 1**) were originally conceived and developed as agents that sequence specifically target duplex deoxyribonucleic acid (DNA) by triple helix formation ***(1,2)***. However, reality showed that although (homopyrimidine) PNAs bind very tightly and with excellent sequence discrimination to duplex DNA targets, they preferably do so via a helix invasion mechanism and not through conventional triple helix formation ***(1–4)***. Nonetheless, the PNA structure does to a surprising extent mimic the structure of nucleic acids despite the fact that the chemical resemblance is minimal. In fact PNAs are essentially pseudopeptides (polyamides) with pendant nucleobases.

Over the past decade the properties of PNA have intrigued both chemists and biologists. Chemists to study, elaborate and modify the rather simple structure of PNA ***(5,6)*** and biologists to exploit the DNA and ribonucleic acid (RNA)-recognizing properties of PNA within antisense and antigene drug discovery, genetic diagnostics, and molecular biology ***(7–11)***.

2. Backbone Modifications

The very straightforward chemistry of the PNA backbone is an open invitation to modification, and numerous analogs of the original aminoethylglycin

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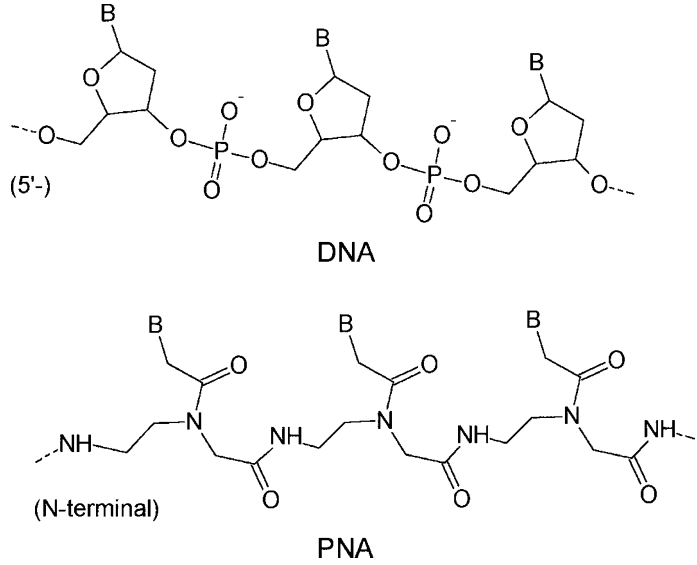

Fig. 1. Chemical structure of PNA compared to DNA.

(aeg) backbone have been designed, synthesized, and studied to understand the DNA recognition properties of peptide nucleic acidsEfforts have also been made to discover molecules with improved DNA/RNA hybridization properties. PNA is unique among high-affinity DNA analogs and mimics in having an acyclic backbone. Acyclic DNA derivatives show very poor hybridization efficacy ***(12)***, which is explained by the high flexibility of such backbones leading to a greatly increased loss of entropy on duplex formation as compared to the much more conformationally constrained cyclic backbones, such as the deoxyribose phosphodiester of DNA. Clearly, the two amide bonds in the PNA backbone restrict the conformational flexibility of this backbone considerably. Accordingly, it was found that reduction of the nucleobase side-chain amide to the ethylamino backbone (**Fig. 2**) results in PNA oligomers with significantly inferior hybridization properties (ΔTm – 20°C per modification) ***(13)***. Along these lines, Leumann et al. have attempted to replace the amide function with an isostructural olefin function (O-PNA). However, such O-PNAs are inferior to aegPNA in terms of DNA/RNA hybridization ***(14–16)***. This observation has not been explained, but it may well be connected to differences in hydration of the two backbones ***(17)***.

Many attempts have been made to improve on the aegPNA backbone through introduction of a cyclic structure to impose restricted flexibility. The efforts have, however, so far only been met with limited success ***(18)***. One

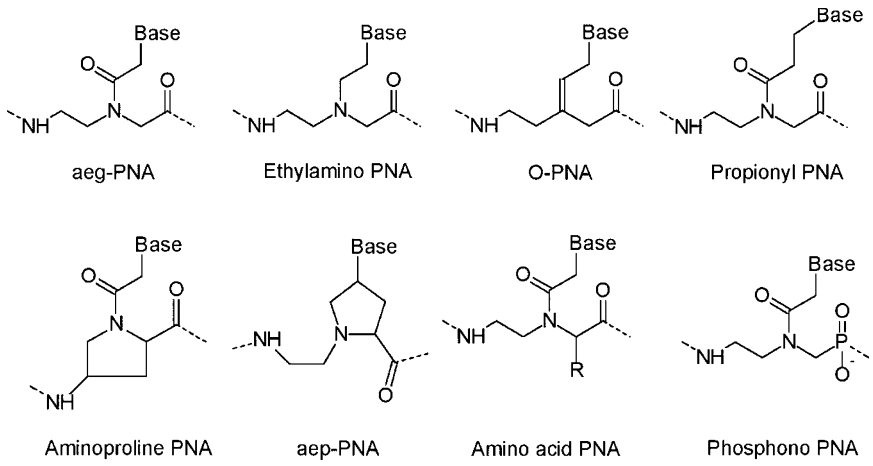

Fig. 2. Modified PNA backbones.

derivative, the aminoethylprolyl (aep) backbone ***(19,20)*** appears to stabilize the PNA–DNA triplex and at least some duplexes, but the limited data available so far indicate a significant sequence context variation, and more data are required to fully evaluate this backbone modification.

Finally, more nucleotide-like phosphono-PNA has been synthesized, and in particular in combination with prolyl backbone, this alternating structure confers favorable hybridization properties.

3. Non-Natural Nucleobases

A number of non-natural nucleobases have been exploited in a PNA backbone context (**Fig. 3**). Of these the pseudoisocytosine is very useful for pH-independent Hoogsteen recognition of guanine in triplex forming *bis*-PNA clamps ***(21)***. The diaminopurine–thiouracil basepair has been exploited in the development of pcPNAs for efficient recognition of duplex DNA by double duplex invasion (*vide supra*) ***(22)***. Finally, a range of bi- and tricyclic nucleobase analogs have been tested in efforts to exploit increased stacking interaction to stabilize PNA–DNA duplexes. A significant stabilization was observed with the bicyclic thymine analog (7-chloro-naphthyridinone) ***(23)***, although a very dramatic (ΔT_m up to 15°C per modification) stabilization was obtained using the G-clamp ***(24,25)***, which is a cytosine analog previously developed in a DNA context, and with similar effects on hybridization potency. It is, however, noteworthy that antisense oligonucleotides containing G-clamps do not appear more potent in cellular antisense assays than unmodified oligonucleotides ***(26)***.

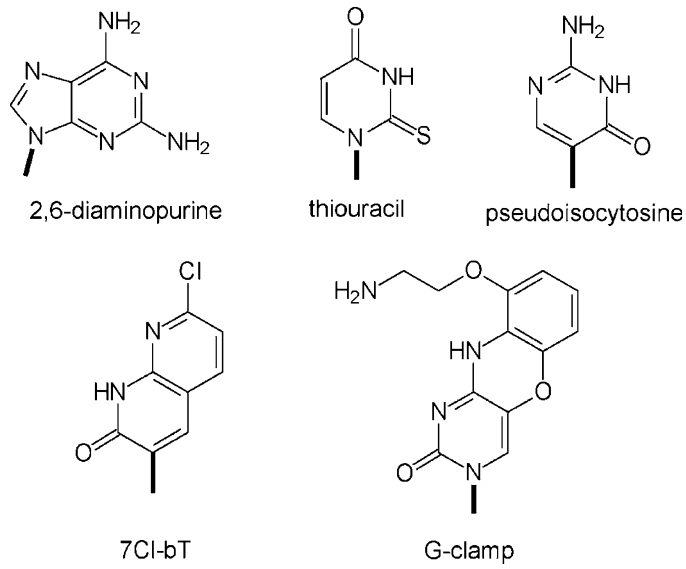

Fig. 3. Examples of nonstandard nucleobases that have been in used PNA oligomers.

4. Genetic Detection

PNA oligomers have been used to improve the performance of a number of "diagnostic" techniques for genetic detection. Two methods in particular are finding widespread applications. PNA clamping of polymerase chain reactions (PCRs) by which a PNA is blocking a PCR primer very effectively suppresses amplification of undesired templates. This is especially useful for point mutation analysis in which two targets differing by only one nucleobase can be selectively amplified ***(27–29)***. For instance, it has been shown that single mutant oncogenes in cancer cells from human tissues can be detected in a background of 10^{+}-fold normal cells ***(29)***. Fluorescent *in situ* hybridization(FISH) is another area in which PNA probes perform extremely well ***(30–32)***. Finally, PNA beacons ***(33)*** and other "light-up" probes ***(34)*** are attracting increasing interest.

5. Antisense mRNA Targeting

PNA–RNA duplexes are not substrates for ribonuclease H. Cellular antisense inhibition of gene expression by PNA must therefore rely on other mechanisms, in particular steric blocking of the translational machinery (ribosomes) or of messenger RNA (mRNA) processing enzymes (e.g., the spliceosome). Both principles appear as viable routes to PNA antisense targeting. Although ribosomal translation elongation is not readily arrested by PNAs (or

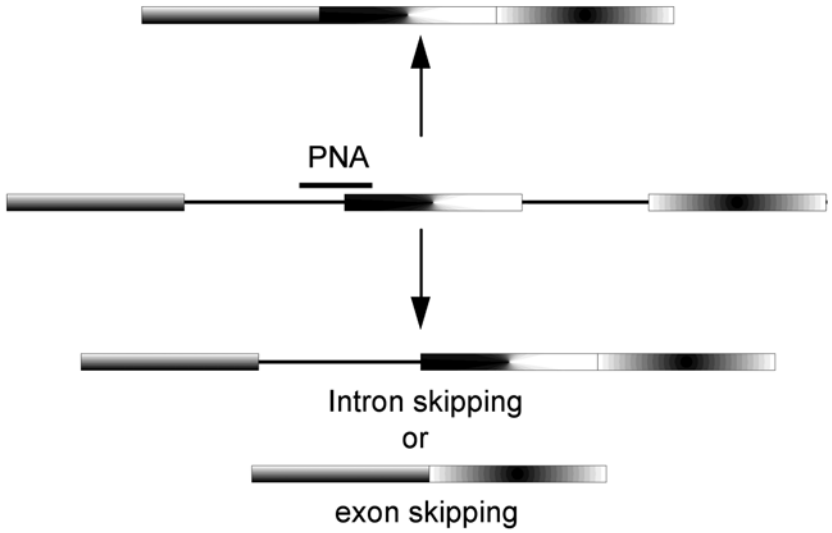

Fig. 4. Effect of PNA targeting of a pre-mRNA intron–exon splice junction. Thick lines signify exons, and thin lines signify introns.

other oligonucleotide analogs or mimics) bound to the coding part of the mRNA ***(35–37)***, PNA oligomers targeted to the translation initiation (AUG) region or to the 5-UTR (untranslated region) of the mRNA effectively cause inhibition of protein synthesis, presumably by interfering with ribosome scanning and assembly prior to translation initiation ***(38)***.

Likewise, recent results have demonstrated that PNA oligomers targeted to exon–intron splice junctions are potent inhibitors of correct mRNA splicing presumably by steric interference with the spliceosome ***(39,40)***. Although no systematic studies have yet been published, at least two outcomes of targeting a 5'-junction (**Fig. 4**) have been found. The spliceosome may either skip the entire exon and thus produce a truncated mRNA missing an exon, or it may skip the intron in the processing and thereby produce a larger mRNA still containing the intron (**Fig. 5**). Therefore, in a drug discovery or molecular biology context, targeting of splice junctions may (in principle) be exploited to correct the splicing of mRNA containing aberrant splice sites (because of mutation), to simply inhibit the synthesis of the correct mRNA (and thus protein product), or perhaps to shift the ratio between biologically functional splice variants. One may also imagine inducing non-natural splice variants with altered (novel) biological function of the resulting protein product. Therefore, splice interference technology may open a range of novel opportunities for gene targeting and drug discovery.

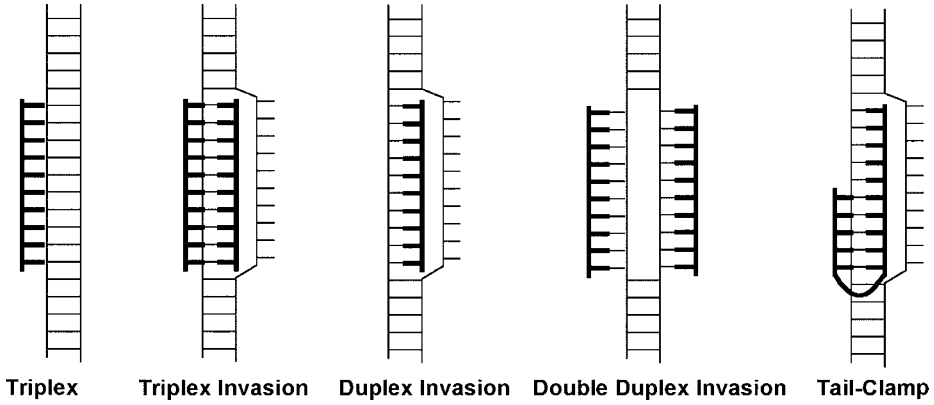

Fig. 5. Five different types of PNA–double-stranded DNA complexes. DNA is schematically drawn as a ladder, and the PNA oligomers are in bold.

6. Duplex DNA Targeting

The concept of PNA was originally aimed at triplex recognition of double-stranded DNA *(1,2)*. However, in contrast to triplex-forming oligonucleotides, homopyrimidine PNAs invade the double helix through the formation of triplex invasion complexes (**Figs. 5** and **6**). Conventional triplexes can be observed with cytosine-rich homopyrimidine PNAs, especially at elevated ionic strength, where triplex invasion is very slow, or as kinetic intermediates (unpublished results and *41,42*). Duplex invasion is seen with mixed purine–pyrimidine PNAs binding to negatively supercoiled DNA, in particular using positively charged PNA–peptide conjugates *(43,44)* or with high-affinity very purine-rich PNAs *(45)*. Mixed purine–pyrimidine sequences can be targeted using sets of pcPNAs, for example, those containing diaminopurine–thiouracil basepairs. Such pcPNAs have greatly reduced affinity for each other owing to steric clashes in the diaminopurine–thiouracil basepair, but bind sequence-complementary DNA with essentially unchanged affinity *(22)*. Finally, triplex and duplex invasion can be combined in two-domain tail-clamp PNAs.

It is well established from in vitro cell-free experiments that triplex invasion complexes are effective inhibitors of transcription initiation as well as of transcription elongation *(35,46)*. Most intriguingly, it was also demonstrated several years ago that these invasion complexes are recognized by RNA polymerase as transcription initiation sites *(47)*. Thus PNA may "mimic" both a transcriptional repressor as well as an activator (transcription factor). Although these effects have also been reported in cell culture experiments *(48,49)*, the results are less clear-cut, and other laboratories are having difficulties reproducing PNA gene activation in cell culture (unpublished results and

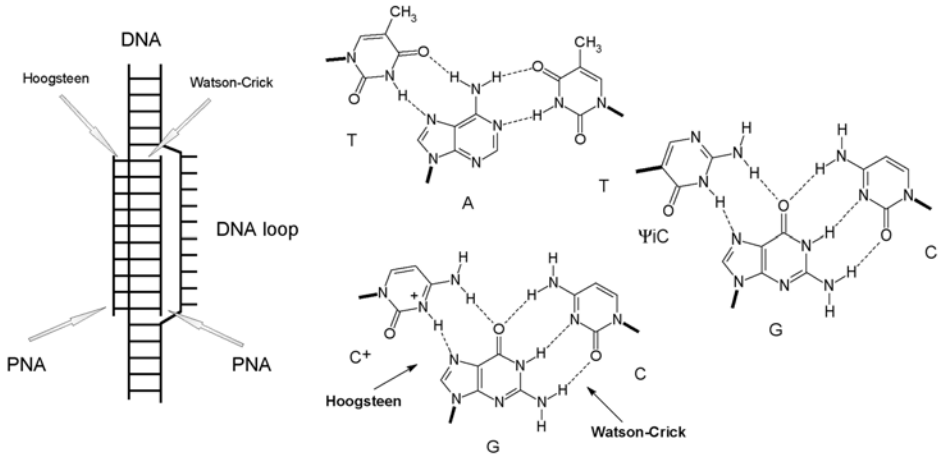

Fig. 6. Triplex invasion by homopyrimidine PNA oligomers. One PNA strand binds via Watson–Crick basepairing (preferably in the antiparallel orientation), whereas the other binds via Hoogsteen basepairing (preferably in the parallel orientation). It is usually advantageous to connect the two PNA strands covalently via a flexible linker into a *bis*-PNA, and to substitute all cytosines in the Hoogsteen strand with pseudoisocytosines (ψiC), which does not require low pH for N3 protonation.

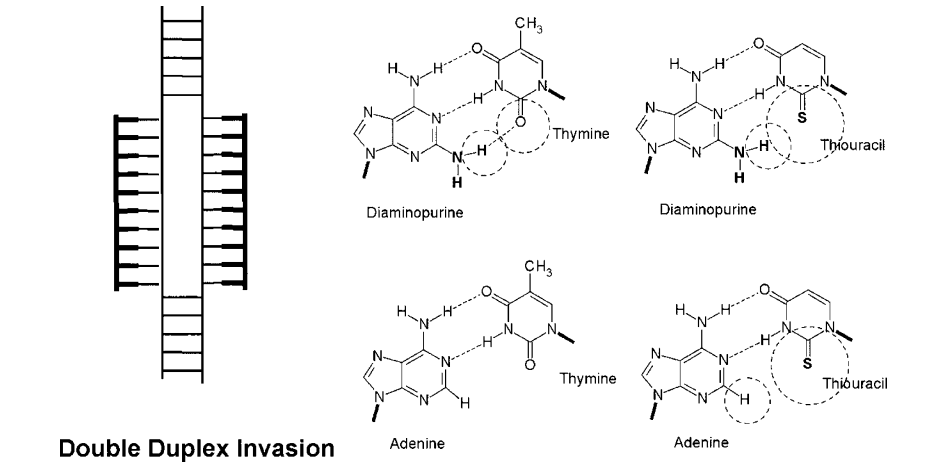

Fig. 7. Double duplex invasion of pseudocomplementary PNAs. To obtain efficient binding the target (and thus the PNAs) should contain at least 50% AT (no other sequence constraints), and in the PNA oligomers all A/T basepairs are substituted with 2,6-diaminopurine/2-thiouracil basepairs. This basepair is very unstable owing to steric hindrance. Therefore the two sequence-complementary PNAs will not be able to bind each other, but they bind their DNA complement very well.

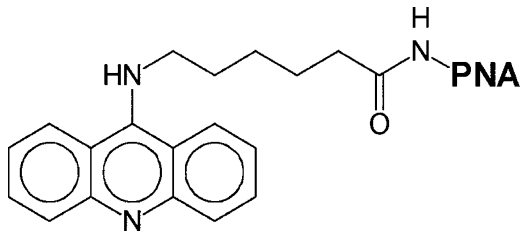

Fig. 8. Acridine–PNA conjugate.

personal communications). Targeting duplex DNA in vivo may also be a challenge because it is known from in vitro studies that physiological ionic conditions (e.g., 140 m*M* K^+, 2 m*M* Mg^{2+}) are inhibitory to effective helix invasion ***(50–52)***. Although natural negative DNA supercoiling may to a large extent alleviate this obstacle ***(53)***, and it is possible to construct PNA–peptide in PNA–acridine conjugates (**Fig. 8**) that are able to invade duplex DNA at physiologically relevant ionic conditions ***(43,52)***, targeting DNA in a nuclear context is an area where quantitative and well-controlled experiments are highly warranted.

7. Cellular Delivery

Any gene targeting approach requires the PNA oligomer to reach the target in the cell cytoplasm or nucleus. Unfortunately, the largely hydrophilic PNA molecules do not spontaneously cross lipid membranes ***(54)***, and they are therefore in general very poorly taken up by living cells ***(55,56)***. Within the past few years a number of protocols have been developed that give greatly improved intracellular bioavailability of PNA. Two protocols exploit cationic liposomes, analogous to the standard method for oligonucleotide delivery. However, because PNAs are not inherently anionic and therefore do not spontaneously assemble into PNA–liposome complexes, an assembly auxiliary is required. This may be a fatty acid conjugated to the PNA ***(57)*** preferably through a cleavable inker ***(58)***, but the more general and effective approach is to use a partly complementary oligonucleotide to hybridize to the PNA and thereby "piggyback" the PNA into the cationic liposomes and further into the cell ***(59)***.

Alternatively a variety of cell-penetrating peptides have been used via chemical conjugation to PNA oligomers (**Table 1**). It was originally reported that several of these peptides (e.g., pAntp) transverse the membrane by a carrier- and energy-independent mechanism ***(60)***, but recent careful reevaluations clearly indicate that the main cellular internalization route is via an endosomal pathway ***(56,61,62)***. Nonetheless, PNA–peptide conjugates in particular using transportan ***(62,63)*** or oligo–arginine ***(64)*** are quite potent cellular antisense

Table 1
Cell Penetrating Pepides

Peptide	Sequence	Cell type[a]	Reference
penetratin (pAntp)	RQIKIWFQNRRMKWKK	JR8/M14, human melanoma	***72***
Transportan	GWTLNSAGYLLG**K**INLAALAKKIL[b]	Bowes cells	***63***
Retro–inverso penetratin	D-(KKWKMRRNQFWVKVQR)	rat neurones	***73***
pTat	GRKKRRQRRRPPQ	HeLa	***62***
SV40 Nuclear Localization Signal (NLS)	PKKKRKV	Burkitt's lymphoma	***74***
Somatostatin	DKDYKDYDK	IMR32	***75***
	MSVLTPLLLRGLTGSARRLPVPRAKIHSL	Mitochondria in IMR32, HeLa, a.o	***76***
KFF	KFFKFFKFFK	*Escherichia coli*	***77***
Polyarginine	R9		***78***

[a]Demonstrated cell types.
[b]Conjugated to the PNA via a disulfide bridge at the central lysine.

agents. However, it is also clear that better delivery systems for PNA are still very much desired.

8. In Vivo Experiments

Only a handful of in vivo animal studies have so far been published using PNA *(65–68)*. Of these a major part concerns neurological targets in the brain *(65–67)*. Unfortunately, these studies do not comply with good standards for antisense studies in terms of proper mismatch controls and/or molecular biology end points (mRNA and/or protein levels including controls), and independent confirmation of the conclusion should be awaited. In one study effective passage of PNA oligomers over the blood–brain barrier was also reported *(67)*, but this could not be independently confirmed as a general feature of PNA *(69)*.

However, a very convincing study showing systemic antisense effects of PNA in a mouse model was recently published *(68)*. In this study Kole and coworkers used a transgenic mouse with an aberrant splice variant of GFP. On treatment with PNA (or comparable antisense agents such as morpholino) targeted to the exon–intron junction of the aberrant splice site, the pre-mRNA is correctly processed, and the cells and tissues thus express functional GFP. Using this model it was shown that intraperitoneal administration of PNA results in significant antisense activation of green fluorescent protein (GFP) in liver, intestine, heart, and kidneys *(68)*.

In this context it is also worth noting that the pharmacokinetic profile of simple PNAs is not very favorable for in vivo activity as the compound exhibits a very small volume of distribution, low tissue availability, and is excreted very quickly through the kidneys *(70,71)*. Thus it should be possible to obtain much higher in vivo efficacy with PNA derivatives having improved bioavailability.

9. Prospects

The PNA field is steadily moving forward in all areas: chemistry, molecular biology, diagnostics, and cell biology, and also very slowly into drug discovery. The recent results in mouse models as well as the emerging information on the bioavailability and pharmacokinetic behavior of PNA oligomers in animals are extremely encouraging. Thus scientist in all areas are still being inspired by the very simple PNA structure and are still able to bring new and exiting ideas into the field, which may eventually also lead this molecule onto new avenues, such as nanotechnology or artificial life.

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