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Review

Antisense properties of peptide nucleic acid

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Abstract

Peptide nucleic acid (PNA) is a nucleic acid mimic in which the deoxyribose phosphate backbone has been replaced by a pseudo-peptide polymer to which the nucleobases are linked. PNA-oligomers can be synthesized in relatively large amounts, are highly stable in biological environments, and bind complementary DNA and RNA targets with remarkably high affinity and specificity. Thus PNA possesses many of the properties desired for a good antisense agent. Until recently, limited uptake of PNA into cells has been the major obstacle for applying PNA as an antisense agent in cell cultures and in vivo. Here, the antisense properties of PNA in vitro and in vivo will be reviewed. In particular, we will focus on recent observations indicating that PNA equipped with or without various uptake moieties may function as an efficient and gene-specific inhibitor of translation in *Escherichia coli* and in certain mammalian cell types. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Peptide nucleic acid; Cell permeation

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1. Introduction

Antisense – the regulation of gene expression by short oligonucleotides or oligonucleotide mimics – relies on the formation of Watson–Crick hydrogen bonds between the antisense oligomer and the complementary mRNA strand, thereby providing target specificity of the agent. Ideally this renders the mRNA non-functional and therefore results in the inhibition of synthesis of a specific protein. Although simple in concept, antisense is faced with numerous challenges, which must be met in order to achieve efficient gene repression. Most of the oligonucleotide analogues that have been investigated are close chemical relatives of DNA [1,2]. In contrast, peptide nucleic acid (PNA) is a DNA mimic that is only remotely chemically related to DNA [3–5].

In PNA the sugar phosphate backbone of DNA is replaced by a homomorphous, achiral, uncharged and relatively flexible backbone composed of 2-aminoethyl glycine units to which the nucleobases are attached by methylenecarbonyl linkers (Fig. 1). PNA can be synthesized in relatively large quantities employing conventional solid phase peptide technology using either Boc or Fmoc protected monomers [6–9]. Furthermore, PNA is resistant to biological degradation [10] and can bind complementary RNA or DNA sequences with extraordinary high affinity and specificity [11].

1.1. Duplex formation

PNA can bind complementary polynucleotide targets in at least two modes (Fig. 2). PNAs containing mixed purine/pyrimidine nucleobases recognize complementary nucleic acid targets by forming duplexes based on Watson–Crick hydrogen bonding (Fig. 2, upper), and are termed duplex-forming PNAs [11,13,14]. PNAs only containing pyrimidines form PNA_2/nucleic acid triplexes via Watson–Crick- and Hoogsteen hydrogen bonds, and will consequently be referred to as triplex-forming PNAs [15,16].

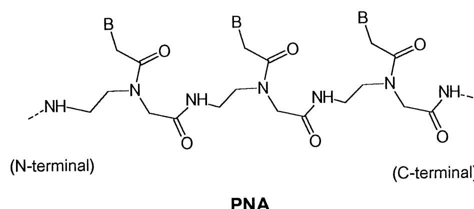

Fig. 1. Chemical structure of a PNA oligomer. B indicates a nucleobase.

The thermal stability of PNA/nucleic acid duplexes is virtually independent of ionic strength and usually exceeds those of the corresponding RNA/DNA or DNA/DNA duplexes [11,17,18]. Binding in the antiparallel orientation (the amino terminus of the PNA facing the 3′-end of the nucleic acid target strand) is the more stable. However, in contrast to conventional nucleic acids, binding in the parallel orientation is also possible with appreciable efficiency [11].

The sequence specificity of PNA/DNA duplex formation has been studied systematically by determining the effect of all possible mismatches in the four central positions of a 15-mer duplex. Introduction of single mismatches resulted in significant decreases in T_m (8–20°C depending on the actual mismatch). Notably, in almost all cases the mismatch dependent decreases in T_m for the duplexes involving PNA were higher than those found for the analogous DNA/DNA duplexes. Thus the sequence discrimination of PNA binding is as high or even higher than that of DNA [11,12].

1.2. Triplex formation

A triplex consisting of two 10-mer homopyrimidine PNA strands bound to a complementary nucleic acid target (Fig. 2, lower) is very stable (a T_m of 70°C is typical [5,15]) and has a lifetime at 37°C that is measured in days or weeks [19]. A $(PNA)_2$/nucleic acid triplex is most stable when the "Watson Crick PNA strand" is anti-parallel relative to the target, whereas the Hoogsteen strand should be in the parallel orientation [15]. In order to ensure opti-

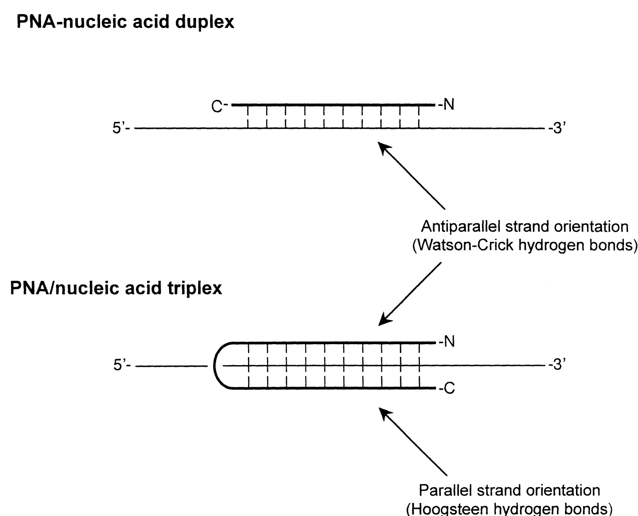

Fig. 2. Schematic representation of PNA binding to complementary single-stranded nucleic acids. PNA carrying mixed purine/pyrimidine bases forms a duplex, preferentially in the antiparallel orientation (upper). A bis-PNA composed of two homopyrimidine strands connected by a flexible linker forms a triplex upon binding a complementary PNA (lower).

mal orientation for both strands it is advantageous to connect the two homopyrimidine PNA strands with a flexible linker, and such molecules are termed bis-PNAs [15]. In addition, this facilitates correct positioning of cytosine analogues especially designed for making Hoogsteen hydrogen bonds at neutral pH as described below.

Because cytosines require protonation in the N^3 position in order to make Hoogsteen interactions with N^7 of guanine residues, homopyrimidine PNA containing cytosines has a reduced ability to form $(PNA)_2$/nucleic acid triplexes at neutral and alkaline pH values. This pH effect can be avoided by replacing the cytosines with the nucleobase analogue pseudoisocytosine (typically denoted 'J') in the 'Hoogsteen strand' of bis-PNA [15] Thus, pseudoisocytosine mimics a cytosine that is permanently protonated.

2. Biostability and cell permeation

2.1. Stability in biological fluids

Considering pharmacokinetic issues, an antisense agent should ideally be resistant towards enzymatic degradation. It is reasonable to assume that PNA has sufficient biostability to be used as an antisense drug. For instance, incubation of PNA with human serum, with bacterial cell extracts from *Micrococcus luteus* or *Escherichia coli*, with nuclear extracts from mouse Erhlich ascites tumor cells, or even with high concentrations of isolated proteinase or peptidase does not result in significant degradation [10].

2.2. Membrane penetrability

Poor cellular uptake is the main reason why the antisense properties of PNA only to a limited degree have been studied in cell cultures and in vivo. Phospholipid vesicles used as a model system for biological membranes have been used to examine the uptake properties of PNA. The permeability of such membranes for PNA is relatively poor, i.e., of the same order of magnitude as that for DNA oligonucleotides [20]. The uptake issue has also been examined in cell cultures. CV-1 cells or cloned rat embryo fibroblast cells incubated with fluorescein conjugated PNA, resulted in fluorescence in cytoplasmic vesicles but not in the cytosol. Neither increased PNA concentrations nor prolonged incubation time changed this pattern [21,22]. However, the uptake properties may vary considerably among cell types as illustrated by recent work indicating that PNA may enter certain cell types much more efficiently than others. Notably, as described below neuronal cells in culture appear to internalize PNA relatively efficiently. In general, however, passive diffusion of simple PNAs into cells is presumably not the mechanism to rely on for intracellular delivery.

2.3. Carrier moieties

Recent work has demonstrated that the uptake of PNA into cells can be increased by the conjugation of various carrier groups. Because PNA is synthesized by conventional peptide chemistry it is relatively easy to equip PNA oligomers with a wide variety of peptides [23,24]. For instance, 'transportan' and the third helix of the Antennapedia homeo domain peptide (16 amino acids) has been shown to increase the cellular uptake of attached PNA oligomers [24,25]. Transportan preferentially ends up in membranous structure of the cell [25] whereas the Antennapedia peptide has a preference for the nucleus [26].

The uptake of these carriers are believed to be ATP and receptor independent and thus rely on their physical properties [27–29]. The retro-inverso counterpart of the Antennapedia peptide is also active and presumably much more resistant to proteolytic degradation. For instance, the uptake of a PNA into cerebral cortical neurones were shown to be increased by a factor of five by the attachment of a retro-inverso form of a 16-residue peptide from the Antennapedia homeodomain [29].

Receptor-mediated endocytosis has also been exploited by the attachment of a D-amino acid analog of insulin-like growth factor to a PNA. This conjugate displayed significantly higher uptake than a similar PNA lacking the carrier peptide but only in cells that expressed the receptor for insulin-like growth factor [30]. Similarly, by conjugating PNA to an antibody recognizing the rat transferrin receptor it has been possible to increase its transport over the rat blood–brain barrier [31]. Such approaches, could facilitate cell- or tissue-specific administration of PNA.

Also, lipophilic groups such as adamantyl have been attached to PNA. Although these carriers resulted in increased cellular uptake, most of the PNA apparently ended up in endosomes [32]. At least in some cell types, however, homogenous cytoplasmic uptake was obtained by liposome-mediated adamantyl–PNA delivery.

3. Antisense activity of PNA

3.1. Mode of action

The antisense effect of conventional oligonucleotides is generally believed to rely on the activation of RNase H which cleaves the RNA portion of the heteroduplex. Notably, PNA/RNA hybrids are not substrates for RNase H [33–35]. Thus, antisense effects mediated by PNA are thought to depend on steric interference with the translation machinery and the efficiency of PNA should therefore rely on the stability of the resulting RNA/PNA hybrid. The observation that PNA/RNA hybrids are not a substrate for RNase H in vitro does not, however, exclude the possibility that PNA may cause degradation of the affected mRNA by alternative catabolic pathways in vivo and thus, indirectly, may function in a catalytic manner.

Alternative strategies facilitating catalytic action of PNA, include chimeras between PNA and DNA [36–41]. The PNA part of such chimeras may provide the necessary target affinity and sequence discrimination and the DNA part may trigger sequence-specific RNase-mediated hydrolysis of the relevant RNA. Finally, an option may be to attach nucleic acid cleavers to the PNA and in this way make its action catalytic [42,43].

3.2. Antisense properties in vitro

Several investigators have examined the antisense properties of PNA in vitro using a rabbit reticulocyte cell free translation system [33,34,44,45]. The presence of a triplex-forming PNA complementary to the mRNA in the translation reaction indeed results in translational arrest and in many cases results in a truncated protein product corresponding to the position of the PNA target [34,44,46]. It appears that translation arrest may be obtained using homopyrimidine PNAs as short as 6-mers [34].

When using duplex-forming PNAs, the situation is quite different. Hybridization of up to 20-mer duplex-forming PNAs were not sufficient to arrest translation elongation in vitro [34,44]. In contrast, when targeting a duplex-forming PNA to the region spanning the AUG initiation codon, effective inhibition of translation initiation was observed [34,44,45]. The fact that this site of the mRNA, in contrast to sites in the coding region, is sensitive to in vitro antisense inhibition PNA is presumably because it requires lower complex stability to prevent ribosome assembly than to prevent ribosome elongation. Thus in general duplex-forming PNAs can inhibit translation in vitro if they are targeted towards the ribosome binding site whereas triplex-forming PNAs are effective when targeted against polypurine targets downstream of the translation initiation site.

Recently it was shown that PNA can act as a potent antisense agent in bacteria [47]. PNAs were designed to recognize regions spanning the AUG start codon of mRNAs encoding β-lactamase or β-galactosidase. Using an *E. coli* cell lysate for coupled transcription and translation, it was shown that these PNAs efficiently inhibited in vitro translation of the

relevant mRNAs in a highly sequence-specific manner.

3.3. Antisense properties in prokaryotes

The ability of PNA to inhibit translation in a sequence-specific manner has been tested in bacteria in culture [47]. Cultures of wild-type *E. coli* K12 or the hyperpermeable strain AS19 were supplied with PNA recognizing the translation initiation regions of mRNAs encoding β-lactamase or β-galactosidase. The cells were grown to the mid-log phase and their content of β-galactosidase and β-lactamase activity were determined and compared with those of untreated cultures or cultures treated with irrelevant PNAs. PNA dose-dependent and -specific antisense inhibition was observed for both reporter genes in both strains. However, antisense effects were obtained by much lower PNA concentrations when employing the AS19 strain ($EC_{50} \sim 2$ μM). This is compatible with the notion that uptake is a major limiting factor. Using the AS19 strain it was also possible to demonstrate a phenotypic effect of the anti β-lactamase PNA because it turned otherwise resistant cells sensitive to ampicillin. Notably, the elongated cell morphology typical for cells lacking the β-lactamase gene treated with ampicillin was closely mimicked in cells containing the PNA inactivated β-lactamase gene.

To substantiate that the PNA acted by an antisense mechanism, silent mutations were introduced in the regions complementary to the relevant PNA β-lactamase mRNA. The presence of two or six mismatches reduced or eliminated the inhibitory effect of the PNA, and most importantly, the inhibition of the β-lactamase mRNA carrying six point mutations could be restored by employing a PNA with compensating base changes. This is convincing evidence that the observed repression of gene expression takes place through specific Watson–Crick hydrogen bonding and thus by a true antisense mechanism.

3.4. Antisense properties in eukaryotes

The first indications that PNA could act as an antisense agent in eukaryotic cells came from experiments in which a pyrimidine-rich (90% pyrimidines) 20-mer PNA targeted against the coding region of an SV40 T antigen-reporter mRNA was microinjected into cultured cells [33]. The PNA caused a significant reduction in the SV40 T-antigen product, indicating that PNA can indeed arrest translation under intracellular conditions provided that it can get there.

Recently a few papers have reported on efficient antisense effects of PNA in living cells and whole animals. Pooga et al. [25] used a 21-mer PNA conjugated to one of the cell penetrating peptides transportan or the third helix of the Antennapedia homeodomain by a disulfide linker that is believed to be cleaved when subjected to the intracellular reducing environment. Two duplex-forming PNAs were designed to recognize and downregulate the mRNA of the human galanin receptor which mediates the effect(s) of the neuropeptide galanin. One of the PNAs was targeted to the translation start codon whereas the other recognized the mRNA further downstream in the coding region.

First, the PNA conjugates were tested in Bowes melanoma cells and were shown capable of downregulating the level of [^{125}I]galanin binding capacity in Bowes cellular membranes. Curiously, the PNA recognizing the coding region appeared to be slightly but significantly more potent than that recognizing the AUG start codon. The PNA conjugates were much more potent ($EC_{50} \sim 0.2$–2 μM) than the analogous phosporothioates ($EC_{50} \sim 21.5$ μM) or phosphordiester oligomers ($EC_{50} > 100$ μM). Neither the carrier peptides nor the native PNA alone could produce such an effect and the effect appeared to be reasonably specific because no antisense effect was observed using high concentrations of PNAs with scrambled sequences and only a low effect using similar sequences (5 or 6 mismatches) specific for the rat galanin receptor.

The analogous PNAs designed to target the rat galanin receptor were also tested in living rats by intrathecally administration into the spinal cord. Rats receiving a duplex-forming PNA targeted to the coding region of the galanin receptor PNA, showed significantly reduced response to galanin injections, whereas rats receiving a PNA with scrambled sequence responded similar to those not receiving PNA.

In an analogous study, 'simple' PNAs (without peptide conjugations) were targeted to a mixed pu-

rine/pyrimidine region in the coding sequence of the mRNA for the neurotensin receptor 1 (NTR-1) and a pyrimidine-rich region in the opioid μ-receptor mRNA. The PNAs were injected into the periaqueductal gray region of the rat brain [48]. Neurotensin mediates hypothermia and antinociception. The rats receiving the PNAs against NTR-1 and the opioid μ receptor had a dramatically reduced physiological response to neurotensin and morphine, respectively. In contrast, the anti-NTR-1 PNA had no effect on the response to morphine and the anti opioid μ-receptor PNA did not affect the response to neurotensin. Consistent with an antisense-based mechanism, rats treated with anti-NTR-1 had a significant reduced number of receptor binding sites for NT, and the K_d of the remaining receptors remained unaffected. Rats receiving anti opioid μ-receptor PNA or a scrambled PNA had a normal number of receptor NT binding sites indicating that the PNA acts in a specific manner.

In addition to the neurophysiological aspects that these works may imply, they suggest that PNA administered directly into the brain may function as an effective and specific antisense agent.

A third study substantiating that PNA may enter and function as an antisense drug in neuronal cells involved PNA targeted to the AUG region of the mRNA encoding the neuropeptide oxytocin [29] in cultured magnocellular oxytocin neurons. The presence of the anti-oxytocin PNAs reduced the amount of oxytocin mRNA as assayed by RT–PCR and it reduced the immunocytochemical signal of an antibody specific for prepro-oxytocin. The observation that the PNA apparently leads to reduced levels of the targeted oxytocin mRNA is not readily explained, but implies that PNA does not necessarily inhibit translation in vivo just by mechanical disruption of the translation process but may also induce RNA degradation by an RNase H-independent pathway.

4. Concluding remarks

If the findings described above turn out to be general they may represent a breakthrough in PNA antisense technology. However, important issues have to be addressed. For instance, it is not clear by which mechanism duplex-forming PNAs exert their inhibitory effect on the mRNA in vivo: are the selected system (rat brain), the genes or the targets in the mRNAs especially hypersensitive to antisense inhibition so these regions represent relatively unique 'lucky punches' or do these results infer generally efficient in vivo antisense activity of PNAs?

Furthermore, very little information is available regarding the pharmacokinetic and dynamic properties of PNAs as well as their long-term systemic effects. These issues are naturally of the utmost importance for evaluating the therapeutic potential of PNA. Nonetheless, the extremely exciting results obtained so far with PNA–peptide conjugates in cell cultures and with just 'naked' PNAs in bacteria holds promise that PNA may become a valuable tool in medical target validation and functional genomics studies both in pro- and eukaryotic systems, and also suggests PNA as a 'lead' for development of 'genetic antibiotics'.

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