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ANTISENSE PROPERTIES OF PEPTIDE NUCLEIC ACIDS

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1. ABSTRACT

In this paper, a summary of recent antisense PNA (Peptide Nucleic Acid) applications is presented. We discuss in detail reports that demonstrate that the effects of PNA may be considerably improved by enhancing its cellular uptake.

2. INTRODUCTION

PNA is a nucleic acid analog with an achiral polyamide backbone consisting of N-(2-aminoethyl)glycine units (figure 1). The purine or pyrimidine bases are linked to the each unit via a methylene carbonyl linker (1-3) to target the complementary nucleic acid (4). PNA binds to complementary RNA or DNA in a parallel or antiparallel orientation following the Watson-Crick base-pairing rules (5-7). The uncharged nature of the PNA oligomers enhances the stability of the hybrid PNA/DNA(RNA) duplexes as compared to the natural homoduplexes. The non-natural character of the PNA makes PNA oligomers highly resistant to protease and nuclease attacks (8). These properties of PNA oligomers suggest that they could potentially serve as efficient antisense or antigene reagents. Indeed, peptide nucleic acids have been applied to block protein expression on the transcriptional (9) and translational level (10,11), and microinjected PNA oligomers demonstrate a strong antisense effect in intact cells (12). However, contrary to the "normal" nucleic acid analogs, PNA oligomers are not efficiently delivered into the cytoplasm of the cell, and until recently this has hindered the application of PNA oligomers as antisense reagents. In this work we summarize some recent achievements on PNA antisense application, especially these concerned with whole cell or tissue delivery of the PNA.

3. PNAs AS ANTISENSE REAGENTS

This summary deals with two issues of PNA applications, the use of unmodified PNA as antisense reagent, and, the improvement of cellular uptake of PNA.

Although PNAs have several characteristics required for a good antisense molecule, they suffer from poor membrane penetrability. Therefore, the initial antisense experiments using PNA relayed on microinjection and cell permeabilization techniques.

Gray *et al* (13) have studied the uptake of different oligonucleotide analogues in transformed and non-transformed fibroblasts. In this study, PNA was shown to be poorly taken up by the cells, whereas transformed cells internalized all analogues better than non-transformed cells.The authors suggest that PNA and other uncharged oligonucleotide analogues are taken up through fluid-phase endocytosis, while charged analogues are internalized through receptor mediated endocytosis

In 1992, the first study about the potential application of PNA was published (10). The microinjection into the nuclei of cultured cells of PNA complementary to the coding region of SV40 T antigen (T Ag) mRNA, caused down-regulation of translation of the respective protein. The extent of the inhibition was dose-dependent and specific when the intracellular concentration of PNA was below 5 μM. Moreover, the effect of the length of the PNA was investigated, using 10-, 15-, and 20-mer PNAs. The antisense effect was correlated to PNA length: at 1 μM concentration, the 20-, 15- and 10-mers gave 50%, 40% and 0% inhibition, respectively.

Bonham *et al* (12) published a study in which CV-1 cells, transiently transfected with T Ag expression vector plasmid, were microinjected with FITC-labeled PNA targeted against the T Ag. After injection they observed a quick redistribution of the fluorescent label into the nuclear compartment, whereas nucleoli were completely excluded. 6 h after injection, the expression of T Ag was inhibited by 57%. As a comparison, 10 times less of C-5 propynyl pyrimidine phosphorothioate oligonucleotides (propyne-S-ON), was needed for 70% inhibition. The authors suggest that the differences in inhibition are due to the capability of the propyne-S-ONs to activate RNAse H and due the lower rate of PNA association with the RNA than of the propyne-S-ON.

Several groups have used a rabbit reticulocyte cell-free translation system (10,11,14-17) or rat hepatocyte nuclear extract (10) to study antisense/antigene effects of

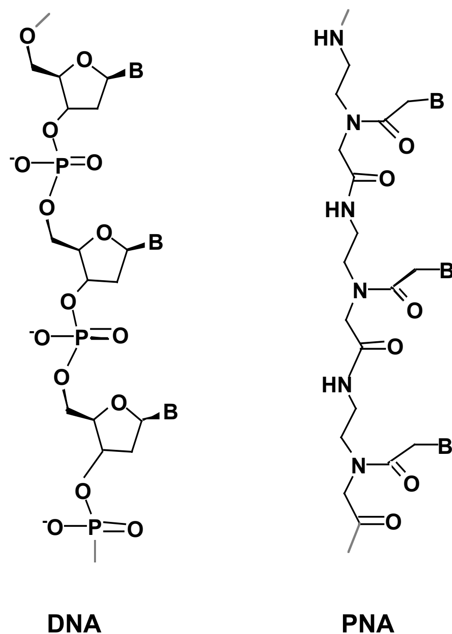

Figure 1. General structures of DNA and PNA.

PNAs *in vitro*. Using this technique, the inhibition of translation of the interleukin-2 receptor α subunit mRNA (10), chloramphenicol acetyltransferase mRNA (11), promyelocytic leukemia/retinoic acid receptor α mRNA (15,16) and inducible nitric oxide synthase mRNA (14) have been shown. In addition, the selective inhibition of mitochondrial DNA replication (18) and reverse transcription of the HIV-1 *gag* gene (17) by PNAs have been demonstrated. These results showed that the homopyrimidine or pyrimidine rich PNA sequences (as short as 6-mers) inhibited translation, whereas PNAs with mixed sequence had effect only when they were complementary to the AUG initiation codon region.

3.1. UNMODIFIED PNA

In 1998, the first *in vivo* results describing antisense effects of unmodified PNA were published by Tyler *et al* (19). In this study, 14-mer PNAs, directed against the neurotensin, NTR1, (position +103) and mu opioid (position -70) receptors mRNAs, were injected into the periaqueductal gray (PAG) of rats. Neurotensin as well as opioids are well known to exert an antinociceptive effect. In addition, neurotensin induces hypothermia. Behavioral studies of anti-NTR1 or anti-opioid mu receptor PNA treated animals showed dramatically reduced responses to neurotensin and morphine, respectively. Furthermore, hypothermic effect of neurotensin was substantially reduced. These effects where reversible and responses returned to normal 5-14 days after final injection. The effects were specific since PNA targeted against the NTR-1 mRNA had no effect on the morphine response and the antisense PNA targeted to mu receptor mRNA did not decrease the effect of neurotensin. These changes were accompanied by a specific decrease of the number of neurotensin or morphine binding sites.

Continuing their work, Tyler *et al* (20) studied the effects of the antisense NTR1 PNA (injected intraperitoneally, i.p.) or sense NTR1 PNA (injected directly into the PAG of rats). Surprisingly, 24 h post injection, both sense and antisense PNAs decreased the antinociceptive effect of neurotensin injected into the PAG. However, mismatched anti NTR1 and mu opioid receptor PNAs showed no significant effect. A similar profile was found for the hypothermic responses of neurotensin. These effects were reversible and responses returned to normal 48 h post injection. The NTR1 mRNA level in brain was determined using quantitative PCR. The antisense PNA treated animals showed no change in NTR1 mRNA levels while the sense PNA treated animals showed a 50% decrease in mRNA levels, suggesting that the sense PNA acted by an antigene mechanism. In order to detect the uptake of PNA into the brain, a gel shift assay with brain homogenate was used. The data in (20) enable to evaluate the approximate concentration of the PNA in brain at 0.1-1 nM range. Such low and efficient concentration of the antisense PNA is at least remarkable.

The brain is protected from the surrounding blood vessels by a tight layer of non permeable cells, the blood-brain barrier (BBB), that restricts the access of many molecules, like glucose and amino acids into the brain. BBB protects the brain from infections and toxins, but also prevents the delivery of potential pharmacologically active molecules into the brain. Therefore, the results of Tyler *et al* (20) are surprising, especially since Pardridge *et al* (21) have shown that significant levels of unmodified PNA do not cross the BBB, and only the conjugation of the PNA to an anti-transferrin antibody allowed it to be transported through BBB. However, the concentration of PNA used by Tyler *et al* (10 mg/kg) was significantly higher than that was used in the other studies and the authors suggest that such a high amount of PNA may disrupt the BBB.

Aldrian-Herrada *et al* (22) have shown that PNAs can enter neurons in culture, probably through an endocytotic mechanism. Also, Taylor *et al* (18) have shown the uptake of PNAs by cultured human myoblasts and specific inhibition of replication of mutant mitochondrial DNA.

Good and Nielsen have recently published studies (23,24) showing that PNA can inhibit reporter gene expression in *E.coli*, probably through an antisense mechanism. The inhibition was shown to be specific and concentration dependent, and was more efficient in antibiotic permeable bacteria than in the wild type strain.

Some studies have shown anti-gene activity of PNAs in the cells showing its potential as possible regulator of gene expression. Vickers *et al* (25) studied the ability of 15-mer PNA to specifically block interaction of the transcription factor NF-kB with its binding site in the IL2-Rα promoter. Complete inhibition of transcription was shown when the cells were transfected with an IL2-Rα plasmid pre-incubated with PNA, while the treatment of cells with PNA after the transfection failed to modulate the transcription.

Boffa *et al* (26) reported that the 18-mer PNA complementary to the poly-CAG triplet area (27) of the androgen receptor and a TATA binding protein has a specific anti-gene activity in permeabilized prostatic cancer cells (28). Furthermore, the same authors showed that 17-mer PNA complementary to the sense strand of the second *myc* exon inhibits transcription in permeabilized COLO320-DM cells.

3.2. CARRIER MEDIATED UPTAKE OF PNA

Wittung *et al* have shown, using an liposomal model system for the plasma membrane, that PNAs do not readily diffuse through a membrane barrier (29). Furthermore, Bonham *et al* showed that incubating CV-1 cells with FITC-labeled PNA resulted only in cytoplasmic vesicular staining (12). Indeed, the neuron is the only cell type to date that has been shown to efficiently internalize unmodified PNAs, demonstrating the need for an effective transporter for these molecules for other tissues. To this end, several potential delivery systems have been developed.

Liposomal delivery that is often used for transfection with oligonucleotides has, to our knowledge, not been successfully used for PNA transport.

An interesting solution to the PNA uptake problem was proposed by Uhlmann *et al*. They showed that a synthetic PNA-DNA chimeric molecule was internalized by cells as efficiently as normal oligos (30) and, furthermore, in contrast to PNAs, were able to activate RNAse H (31).

Recent studies by several groups have shown that coupling of PNA to different carriers will improve their uptake into cells. Among these, several peptide sequences have been shown to be able to carry PNA oligomers across the cell membranes.

A short hydrophobic peptide with the sequence biotinyl-FLFL coupled to a PNA trimer has been shown to internalize into human erythrocytes and Namalwa cells (32). However, no data on transport of longer PNAs has been shown. Basu and Wickström (33) showed that PNA conjugated to an all-D-amino acid insulin-like growth factor 1 (IGF1) mimicking peptide was specifically taken up by cells expressing the IGF1 receptor, although no antisense activity was described.

In recent years, some peptides that translocate over the plasma membrane in an energy and endocytotic independent manner, have been designed and synthesized. An extensively studied sequence, derived from the third helix of the Antennapedia homeodomain (34), is called penetratin (for review cf. (35)). Penetratin or penetratin analogs have been used by us (36) and others (22,37) to transport PNAs over the plasma membrane of cells in culture. Moreover, we used the chimeric peptide transportan as an alternative transport peptide, showing that penetratin is not the only transport peptide that can mediate PNA transport (36). The conjugation of a transporter peptide to PNA greatly improved uptake in neurons (22) and was necessary for any significant uptake in Bowes (36) and DU-145 cells (37).

In their study (22) Aldrian-Herrada and co-workers synthesized antisense PNA against the starting codon region of prepro-oxytocin mRNA. Treatment of cultured magnocellular oxytocin neurons with antisense PNA or vector peptide-PNA conjugate resulted in reduced immunohistochemical signal for prepro-oxytocin and reduced amount of oxytocin mRNA in a dose- and time-dependent manner. The mechanism behind the decrease of mRNA levels is not clear yet, but the authors suggest that the PNA-induced RNA degradation could occur in RNaseH independent metabolic pathways.

In our work (36), we synthesized 21-mers antisense PNA complementary to positions 1-21 and 18-38 of the coding region of human galanin receptor type 1 (hGalR1) mRNA. Since unmodified PNA uptake by Bowes melanoma cells was not significant we used the cell penetrating constructs where PNA was conjugated to the penetratin or transportan via a disulfide bridge. After the treatment of cells with antisense constructs the inhibition of ^{125}I-galanin binding was measured and the maximal effect was obtained with a construct targeting area 18-38 in hGalR1 mRNA. The PNA constructs were more potent than phosphorothioates and phosphodiester oligomers designed to target the same area in the coding region of hGalR1 mRNA. The down-regulation of hGalR1 level was dose-dependent and specific since no effect was observed after the treatment of cells with scrambled PNA construct.

In addition we used the PNA complementary to region 18-38 of the rat galanin receptor type 1 in vivo experiments. The intrathecal administration of antisense construct into the spinal cord of rats reduced the effect of intrathecal galanin approximately 100-fold. Again, the effect was specific since treatment with scrambled PNA construct did not affect the galanin inhibition of the flexor reflex.

4. PERSPECTIVES

It seems that the period in antisense research described as the "end of the beginning" (Crooke), fairly describes the situation in PNA application as well. The stability and high affinity of PNA oligomers have been demonstrated as well as their in vitro applicability. The recent achievements in PNA transport have fueled the interest in *in vivo* application of PNA, and, the first results in this field are promising.

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