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Peptide Nucleic Acid (PNA) - more than anyone could imagine at the first sight

ABSTRACT

Peptide nucleic acid (PNA) is a nucleic acid analog and a fully synthetic DNA/RNA-recognizing ligand with a neutral peptide-like backbone. PNAs have several characteristics required for a good antisense/anti-gene oligonucleotide molecule. The extraordinary properties of PNA may potentiate the incoming entirely new era of routine diagnostic tests and analyses, including broad spectrum of clinical assays and environmental tests that will utilize the PNA technology. PNA will also perform a great impact on areas of in situ hybridization, cytogenetics and industrial microbiology. In this paper we present some recent achievements on peptide nucleic acids and discuss, from the viewpoint of literature, what the potential is and what the limitations of such compounds are. We also try to realize what possible applications in the field of oncology are, what the restrictions to use them are, and how PNA can influence the existing diagnostic methods and, hence, our present knowledge.

Key words: *Peptide nucleic acid (PNA); Antisense; Anti-gene oligonucleotides; Cellular delivery; Diagnostics*

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INTRODUCTION

In this paper we present some recent achievements on peptide nucleic acid, a man-made mimic of natural nucleic acid. We discuss, from the stand-point of literature, what the potential is and what the limitations of such compounds are. We also try to realize how PNA can influence the existing diagnostic methods and consequently our present knowledge, as well as what possible oncological applications are and what the restrictions to use them are.

What is PNA?

Peptide nucleic acid (PNA) is a nucleic acid analog with an achiral polyamide backbone consisting of N-(2-aminoethyl)glycine units. The purine or pyrimidine bases are linked to the each unit via a methylene carbonyl linker to target the complementary nucleic acid (Figure 1).

PNA is a fully synthetic DNA-recognizing ligand with a neutral peptide-like backbone that is structurally homomorphous to the deoxyribose phosphate backbone of DNA, and with purine- and pyrimidine-based nucleobases (i.e., adenine, cytosine, thymine and guanine). PNA binds in a sequence-specific manner to complementary RNA or DNA, in a parallel or antiparallel orientation, and hybridization of PNA to complementary chain occurs following the Watson-Crick base-pairing rules (H-bonding between the nucleobases).

The uncharged nature of the PNA oligomers enhances the stability of the hybrid PNA-DNA/RNA duplexes in comparison to the natural homoduplexes. The non-natural character of the PNA makes these oligomers highly resistant to various protease and nuclease attacks. These favorable properties of PNA oligomers suggest that they could be efficient antisense or anti-gene agents. Indeed, peptide nucleic acids have already been applied to block protein expression on the transcriptional and translational level, and PNA oligomers microinjected into the nuclei of cultured cells demonstrate a strong antisense effect. However, PNA oligomers are not efficiently delivered into the cell cytoplasm through the membrane like the "normal" nucleic acid, and until recently this has obstructed the application of PNA oligomers as antisense reagents (1).

Antisense properties of PNA oligonucleotides

Antisense is just one of the several properties (anti-gene, ribosime and aptamere) that one oligonucleotide molecule may have. Antisense

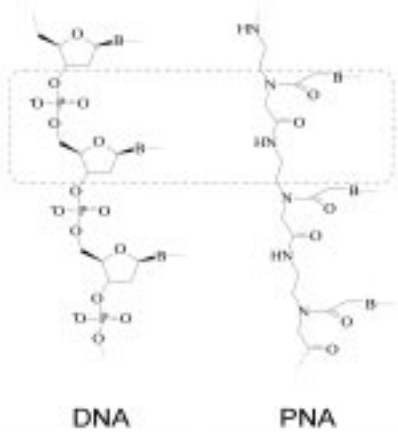

Figure 1. Structure of natural nucleic acid and synthetic peptide nucleic acid. The purine and pyrimidine nucleobases are marked with "B". The basic subunits (a nucleotide monomers) of both DNA and PNA are separated by frame.

agents include compounds which specifically form hybrids with complementary sequence of the target mRNA or single-stranded DNA. If nucleotide oligomere forms triple helix by hybridization with a double-stranded DNA, it is referred to as anti-gene properties of oligonu-

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cleotide molecule. All of these properties principally lead to the blockade of targeted macromolecule function (2-4).

There are at least two mechanisms by which e.g. antisense oligodeoxyribonucleotides (asODN) block mRNA function: by making an mRNA-asODN hybrid duplex ready for cleavage by ribonucleotidase H (RNaseH) (when an asODN is targeted to the coding region of the mRNA) (Figure 2a) or by binding to the 5'-untranslated region of the mRNA, which inhibits its binding or movement on the 40 S ribosomal subunit.

Another strategy is to direct oligonucleotides to DNA as a way to inhibit gene transcription and it is called the "anti-gene" approach. The molecular basis for this approach is the ability of thymine and protonated cytosine to form a type of hydrogen bond called Hoogsteen hydrogen bonds with A-T and C-G Watson-Crick base pairs, respectively, producing a "triple helix". Triple helixes may form by interactions between two pyrimidines and one purine (the "pyrimidine motif") or by recognition of a Watson-Crick double helix by a purine oligonucleotide ("purine motif"). Both motifs involve binding of oligonucleotides in the major groove of DNA (5,6) (Figure 2b).

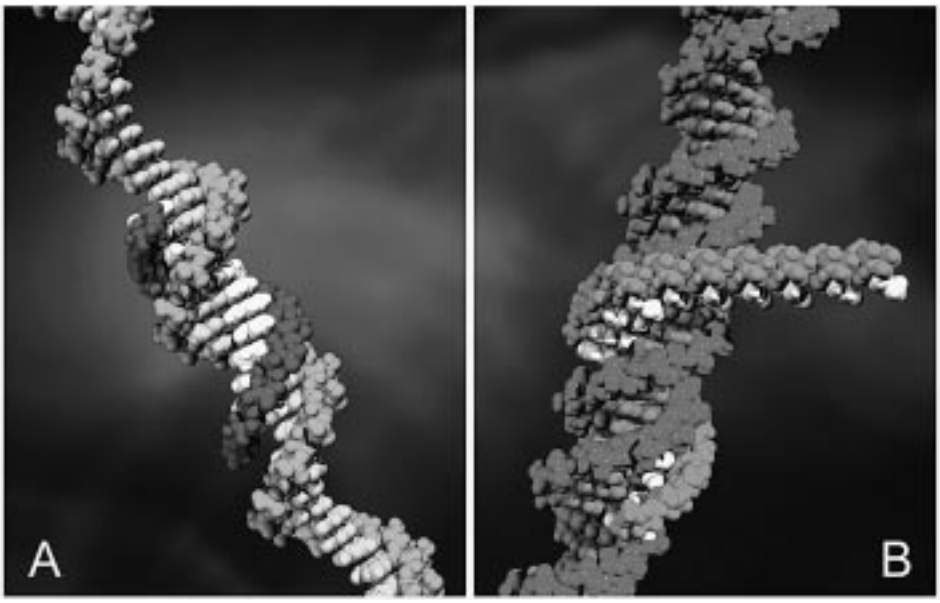

Figure 2. Computer simulation of antisense and anti-gene reaction of PNA. a) 12-mer PNA oligonucleotide (red and white) directed to the coding region of the mRNA (green and yellow). b) The "anti-gene" approach: 17-mer PNA (magenta and white) designed to recognize a specific sequence in double-stranded genomic DNA (blue and orange) form a triple helix by binding in the major groove of DNA.

The chemical backbones of the conventional antisense oligonucleotides (which are consisting of ribo- or deoxyribonucleotides) usually have to be modified so as to render them less susceptible to ribonucleases than the naturally occurring phosphodiesters. PNA antisense oligonucleotides do not have such a problem, because their backbone is totally unknown to cell systems, which means that PNA has no natural predators in the cell.

A minimum length of the conventional antisense required for specific base-sequence recognition is 11 nucleotides for those rich in GC bases because of the abilities of G-C base pairs to form three hydrogen bonds and at least 15 for good binding to AT-rich sequences because of the lower binding affinity afforded by only two hydrogen bonds. The length of the antisense PNA can be as short as 6 nucleotides and probably less (6).

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

Since 1992, when the first study about the potential application of PNA was published, until now many investigations were performed on microinjected PNA, cell or tissue delivery of the unmodified PNA and carrier mediated uptake of PNA antisense oligonucleotides.

Several groups have used a rabbit reticulocyte cell-free translational system or rat hepatocyte nuclear extract to study antisense/anti-gene effects of PNAs in vitro. Using this technique, the inhibition of the mRNA translation for many important proteins by PNAs have been demonstrated. In all reported cases down-regulation of the gene expression was strong, dose-dependent and specific. Further, the 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.

PNA can bind to RNA or DNA even under conditions where nucleic acid structure is disfavored and target is unaccessible for all other antisense agents. Due to this extraordinary capabilities, new studies on PNAs that inhibit telomerase have demonstrated that unexpected regions of this enzyme can serve as targets for these inhibitors. PNAs have, therefore, a great potential for mapping nucleic acid structure and predictably regulating biological processes (7,8).

Recently published studies have shown anti-gene activity of PNAs in the cells, showing its potential as possible regulator of gene expression. Synthetic homopyrimidine PNAs can bind complementary targets in double-stranded DNA, generating strand-displacement complexes proximal to the target, and so offering an opportunity to modulate expression of the specific gene. However, several issues remain to be addressed before these attributes can be exploited in vivo (9,10).

In 1998, the first in vivo results describing antisense effects of unmodified PNA directed against various targets were published. PNA treated animals showed substantially reduced expression of the given gene (e.g. some receptor, with dramatically reduced responses to the added respective ligand, e.g. morphium or some other opioids). These effects where reversible and responses returned to normal 1-2 weeks after the final injection (1).

PNA in diagnostics

Traditional probe-based diagnostic tests use small strands of DNA or RNA - called probes - to seek out and identify complementary DNA or RNA chains within a patient cells or environmental sample.

How do these probes work? Probes are short pieces of nucleic acid that can be chemically synthesized. They are designed to bind tightly and specifically to DNA and RNA molecules in the sample that is being tested. The probe is labeled so, when it binds to the target DNA or RNA, it can be seen and identified. The probes can be tagged with a variety of labels, allowing multiple targets to be detected in a single test. The ability to identify specific DNA or RNA strands is a powerful tool which provides the fundamental basis for understanding health and disease, and also for the diagnosis of diseases, including sexually transmitted diseases, genetic disorders and cancers. Although probe-based testing is becoming more widespread, issues of accuracy and lack of simplicity still limit its use.

Many nucleic acids (e.g., double-stranded DNA and ribosomal RNA) cannot be easily analyzed using conventional DNA and RNA probes because the target site within the nucleic acid is inaccessible under conditions required for the probes to bind (i.e., low salt denaturing conditions). PNA probes, however, do not suffer this drawback since PNA can bind under conditions

that make the targets accessible. So, PNA probes bind to RNA and DNA under conditions where nucleic acid structure is disfavored whereas DNA probes do not. Thus, PNAs can be used to interrogate virtually any target in complex macromolecules containing intramolecular stem-loop structures such as ribosomal RNA.

As an added benefit, the binding of PNA is also stronger and more specific. The PNA peptide-like backbone makes the compound more stable. They have a faster rate of hybridization with the DNA or RNA and are capable to bind 50 to 100 times more tightly than natural DNA. PNA probes are stable to temperature, stable to pH and enzymes and have a wide range of label options. As a result, PNA has been shown to render the entire testing process faster, more specific and more sensitive than currently available DNA/RNA probe methods, and the assays are more robust so they have a greater shelf life (11).

Even a single-base substitution in a human gene can result in deleterious effects in humans. A rapid and accurate detection of genetic variants, including single-base mismatches, is essential for the detection of genetic diseases. PNA has the potential to detect single-base substitution in sample DNA, which was demonstrated using the mutations associated with cystic fibrosis as a model system. It was shown that capillary electrophoresis in free solution at high temperature of a hybridization mixture containing PNA of a 15-mer sequence complementary to the wild-type gene can detect a single-base substitution in the added sample of DNA. This result was achieved in only a few minutes and the PNA can distinguish normal and mutant sequences in the gene.

There is a real need for developing a nucleic acid-based diagnostic technique for the detection of genetic diseases, which will include the ability to ascertain whether an individual is heterozygous or homozygous allelic for a genetic disease. The ultimate goal is to develop arrays of PNA probes tethered to the surface of a solid support in a regular pattern and labeled with a specific fluorophore that will result in enhanced detection simplicity and sensitivity. This will avoid the use of a separation technique, such as capillary electrophoresis, and afford us a simple fluorescent "dipstick" assay for the detection of genetic aberrations (12).

PNA is the first new probe technology in more than 10 years that so dramatically expands the range of accessible sample types and assay formats while also improving test specificity and sensitivity. It is a totally different pioneering technology (11).

Transport of PNA through the plasma membrane

PNA was shown to be poorly taken up by the cells, whereas transformed fibroblasts internalized all analogues better than non-transformed cells. Some 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. It is interesting that a synthetic PNA-DNA chimeric molecule was internalized by cells as efficiently as normal oligos and, furthermore, in contrast to PNAs, were able to activate RNaseH. By the way, 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 (1).

Some studies have shown, using an liposomal model system for the plasma membrane, that PNAs do not readily diffuse through a membrane barrier. Liposomal delivery is often used for transfection with oligonucleotides, but it has not been successfully used for PNA transport. Indeed, the neuron is the only cell type to date that has been shown to efficiently internalize unmodified PNAs. Some other studies, for example, showed that only the conjugation of the PNA to an anti-transferrin antibody allowed it to be transported through the blood-brain barrier. That demonstrates the need for an effective transporter of these molecules for other cell types and tissues, and interesting solutions to the PNA uptake problem were proposed. 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 and several potential delivery systems have been developed. Some of them are: a short hydrophobic peptide with the sequence biotinyl-FLFL and all-D-amino acid insulin-like growth factor 1 (IGF1) mimicking peptide which was specifically taken up by cells expressing the IGF1 receptor. Some peptides that translocate over the plasma membrane in an energy and endocytotic independent manner, have been designed and synthesized. Some of them that have been successfully used to transport PNAs over the membrane of cells in culture are penetratin or penetratin analogues and a chimeric peptide transportan. PNA was conjugated to the penetratin or transportan (cell penetrating constructs) via a disulfide bridge. The conjugation of a transporter peptide to PNA greatly improved uptake in neurons. The first results in this field are promising and the recent achievements in PNA transport have fueled the interest in in vivo application of PNA (1).

DISCUSSION

If we compare, somehow, a living cell with a system such as e.g. a computer (such an analogy can be established because both are highly organized systems with a minimum of entropy, processing a large amount of information), we would realize the importance of the accurate information. Computer programmers know very well that wrong implementation of data can result in an infinite back-loops and system breakdown. A similar situation is with cancer, which is actually the result of alteration of the original program code of the cell (that is called here a genetic code). Therefore, we can say that cancer is some kind of software problem of the living organisms. Antisense oligonucleotides (especially PNA antisenses) and their extreme fidelity in recognition allowed us to switch on or switch off a particular subprogram (gene) and to regulate disturbed order. Antisenses can not increase the level of a gene expression i.e. synthesis of proteins, unless they are targeting some inhibitor of those proteins. However, the possibility to down-regulate or completely shut down a particular gene is quite powerful tool to fight the disease such as cancer.

The PNAs are much better antisense reagents than all other present kinds of oligonucleotides. The only problem still remains how to transport the PNAs across the plasma membrane. How to cross the barrier? How to win something that is improving itself for a very long time to be invincible? Maybe there is something that we can learn from viruses. By solving the problem of PNA internalization two big gates will open: PNA diagnostics and PNA antisense therapy for cancer and many other diseases, and we think that these goals are worth every single spent nucleotide.

CONCLUSION

Peptide nucleic acid (PNA) is completely synthetic nucleic acid analogue with excellent antisense/anti-gene properties. PNA can bind to RNA/DNA under conditions where other conventional antisense oligonucleotides can not. They are immune to proteases and nucleases, more stable to pH and temperature and capable to bind 50 to 100 times stronger to complementary sequence than any other antisenses designed to target the same area. These extraordinary properties of PNA have a potential to open an entirely new era of routine diagnostic tests and analyses, including broad spectrum of clinical assays and environmental tests that will utilize the PNA technology.

PNA will perform a great impact on the development of probe technologies for use in the diagnostics of human infectious diseases, genetic disorders and cancers as well as food, beverage and water testing. And this will certainly not avoid the areas of in situ hybridization, cytogenetics, some novel assay formats, the field of microbiology for rapid direct detection, identification and routine monitoring of toxic, infectious disease causing bacteria, viruses and industrially important microorganisms etc.

The major problem with PNA is low membrane penetrability, but recent achievements in this field are promising.

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