Review article
UDC: 616-006:612.627:615.07

Peptide nucleic acid - sequence specific recognition in cancer diagnostics and gene therapy

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Cancer is increasingly recognized as a disease of genes and one of the therapeutic challenges in greatest need of breakthrough. When the first reports about antisenses were published, it was clear that an entirely new era of pharmacology was coming. Practical application of gene targeting to gene therapy is, however, restricted by its low efficiency: generally less than one modified cell in 100 000 treated cells. The optimism culminated with a recent achievements on peptide nucleic acids (PNAs), a man made mimic of natural nucleic acids, as one of the most promising new molecules for recognition of nucleic acids. We present data obtained on the PNAs utility in cancer diagnostics and cancer gene therapy.

KEY WORDS: *Peptide Nucleic Acids; Gene Therapy; Neoplasms*

Archive of Oncology 2001,9(1):33-37©2001, Institute of Oncology Sremska Kamenica, Yugoslavia

MECHANISMS OF ACTION

A decade of advances in gene targeting strategies, based on homologous recombination, has made it possible to alter genes on a routine basis to create cell lines or transgenic organisms with defined gene defects, and it has raised the hope of corrective gene therapy for human genetic diseases.

Oligonucleotides have been developed that selectively bind to sites on DNA (antigene or triplex forming oligonucleotides - TFOs), RNA (antisense oligonucleotides- asONs and ribozyme oligonucleotides) or proteins (aptamers).

Antigene oligonucleotides can be used to inhibit transcription of a targeted gene or to site specifically damage a gene to alter its sequence by stimulating mutation or homologous recombination. Antisense and ribozyme oligonucleotides can be used to direct the destruction of specific mRNAs or inhibit their translation. Aptamers can be used to interfere with or selectively stimulate the functions of particular proteins.

Antisense technology is the most advanced in its development as a genetic medicine, with several antisense reagents already in clinical trial (1,2). Antigene technology has a potential advantage, however, because there are generally one to two targets per cell as compared with the hundreds to thousands of mRNA targets for antisense oligonucleotides. Antigene technology, like antisense technology, can be used to restrict the expression of the target gene or to introduce permanent genetic change through mutation or by sensitizing the gene for targeted homologous recombination. Triplex formation- antigene action, in a regulatory region of a gene can block transcription initiation by inhibiting transcription-factor binding or by interfering with formation of the initiation complex. Alternatively, triplex formation within the transcribed sequence can inhibit transcription by blocking elongation.

RNAse H-mediated degradation of the target mRNA is the most documented and best characterized mechanism of antisense action. RNAse H recognizes RNA/DNA duplexes and cleaves the RNA strand. Oligomers that do not cleave the target by RNAse H can inhibit mRNA expression by interrupting splicing or by interfering with the translational machinery. The potentially active target sites for inhibition by asONs that do not activate RNAse H are limited to the 5'- untranslated region, the AUG (start codon) and splice sites within the pre-mRNA (1,3).

ABBREVIATIONS

dsDNA-double stranded deoxyribonucleic acid; mRNA-messenger ribonucleic acid; FISH- fluorescence in situ hybridization; PCR- polymerase chain reaction; Å - Angstrem

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The manuscript was received: 18. 11. 2000.
Provisionally accepted: 24. 01. 2001.
Accepted for publication: 07. 02. 2001.

SHORT HISTORY

PNA was introduced in 1991 by four Danish scientists at Copenhagen University. PNAs have achiral, pseudopeptide backbone consisting of N-(2-amino ethyl) glycine units, with purine or pyrimidine bases linked to each unit via a methylene carbonyl

linker. Therefore, chemically they are more proteins (peptides) than nucleic acids (Figure 1).

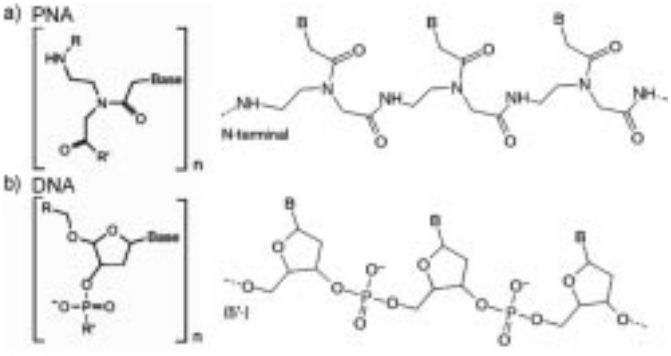

Figure 1. Basic chemical structures of PNA and DNA, compared. B-nucleotide base

Nonetheless, the amide (or peptide) bonds in PNA are sufficiently different from the α-amino acid peptide bonds present in proteins, resulting that PNA is biologically stable and not degradable by cellular proteases and peptidases. Unmodified PNAs are not charged at neutral pH (3-6).

Originally, PNAs were designed to recognize and bind to duplex DNA (antigene activity) in their major groove to form triple helices. Although certain PNA sequences can do this, it was soon discovered that PNA oligomers can bind to single stranded nucleic acids to form duplexes (antisense activity), either PNA-DNA or PNA-RNA, with affinity and specificity substantially exceeding that of comparable DNA or RNA oligonucleotides (7). Antisense binding of PNA to a specific region of a targeted messenger RNA (mRNA) occurs according to Watson- Crick base-pairing rules resulting in inhibition of gene expression. Although PNA-RNA duplexes are not substrates for RNAse H, efficient antisense inhibition of translation occurs, especially if triplex-forming PNAs are employed or the AUG translation initiation region was targeted.

Binding of homopyrimidine PNAs to homopurine targets in dsDNA usually does not result in a conventional PNA-$(DNA)_2$ triplex (Figure 2d), but in a strand displacement complex showing internal PNA_2-DNA triplex and a single stranded DNA loop (Figure 2a).

Triplex formation involving PNAs can occur either with PNA-DNA-DNA triplexes or the more favored PNA-DNA-PNA triplexes (Figure 2). The stability of the PNA interaction with dsDNA is such that strand invasion of DNA by PNAs is thermodynamically favored, and can take place *via* duplex, triplex or double duplex formation (7).

Numerous analyses of triplex structure using electrophoresis, chemical probes, affinity cleavage, enzymatic cleavage, infrared spectroscopy, X-ray fiber diffraction and NMR revealed two motifs for binding of the third strand. A parallel, in which the third strand occupies the major groove so that it has the same 5' to 3' orientation as the purine strand of the duplex to which it binds, and an antiparallel motif, in which the third strand is oriented oppositely to the purine strand. Parallel triplexes use Hoogsteen hydrogen bonds and antiparallel triplexes use reverse Hoogsteen hydrogen bonds to form base triads sequence specific recognition (2,3).

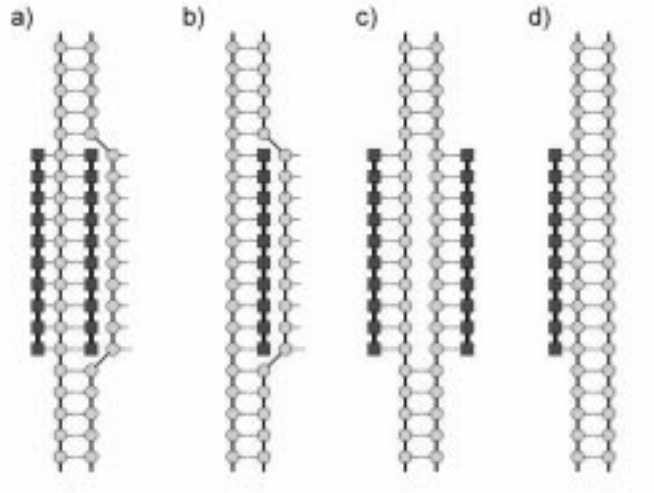

Figure 2. Targeting dsDNA with PNA results in a) strand displacement complex (triplex invasion) b) duplex invasion c) double duplex invasion or d) triplex, corresponding with PNA and targeted dsDNA nucleobase distribution. PNA-bold

The rather flexible PNA oligomer is able, to a great extent, to conformationaly adapt to its more rigid oligonucleotide complement. In PNA-RNA duplexes RNA strand is basically in A-form of helix conformation and in PNA-DNA duplexes DNA strand is close to B-form. PNA does, however, prefer a different helix conformation, termed P-form, which is characterized by a very large pitch (18 base pairs per turn) and a large diameter (28Å). This P-form conformation is seen in PNA_2-DNA triplexes and is fully developed in pure PNA-PNA duplexes (3,5,7).

Subsequent progress of PNA technology was hampered by the fact that PNA oligomeres are not efficiently delivered into the cell. PNAs and other uncharged oligonucleotide analogs are probably internalized through fluid phase endocytosis, while charged analogues enter the cell through receptor mediated endocytosis. Therefore initial PNA antisense experiments relayed on *in vitro* cell-free systems and microinjection and cell permeabilisation techniques (2,4).

PNA IN DIAGNOSTICS

The neutral backbone and correct interbase spacing in PNAs ensures that they bind to their complementary nucleic sequences with higher affinity and specificity compared with traditional oligonucleotides. Thermal stability of the PNA-DNA duplex is independent of the salt concentration in the hybridization medium. The thermal stability of PNA-DNA duplexes is strongly affected by the presence of imperfect matches. Single base mismatch in a PNA-DNA duplex lowers melting temperature by 15°C, compared with

11°C for an equivalent DNA-DNA duplex (8).

The examined nucleic acid target site is often inaccessible under conditions required for the probes to bind. Therefore, many nucleic acids (dsDNA and mRNA) cannot be easily analyzed using conventional DNA and RNA probes. PNA probes can bind under low salt conditions that make the targeted nucleic acid accessible. PNA probes also show greater affinity and higher sensitivity, leading to better target specificity. The PNA peptide-like backbone makes the PNA more stable. It is able to bind faster and from 50 to 100 times more tightly to DNA and RNA, than natural DNA. PNA probes are stable to changes in temperature, pH and enzyme action and have a wide range of label options. Their faster rate of hybridization results in faster assays and greater shelf life (8,9).

The favorable hybridization properties of PNA have inspired the incorporation of PNA into array and nucleic acid biosensor formats, but the performance of these still needs to be critically evaluated. Hybridization of DNA targets to a PNA array is detected by the specific presence of phosphorous in the hybridized DNA nucleotide (5,8).

Detection by mass spectrometry uses immobilization of biotinylated DNA targets (obtained by PCR) to a solid support and exploits the fact that PNAs are very sensitively analyzed by MALDI-TOF (Matrix-assisted laser desorption-ionization-time-of-flight) mass spectrometry. Thus, a mixture of PNAs, each specific for a certain mutation or gene, is hybridized to the immobilized DNA target. Following a stringent wash, the bound PNAs are analyzed by MALDI-TOF. If the PNAs have different mass (ascertained by incorporation of a mass-tag) they can unambiguously be detected and semi- quantified by mass position and signal amplitude (5,10).

PNA has proven successful in several in situ hybridization protocols especially in fluorescence in situ hybridization (FISH) (5,15). Originally this was demonstrated by staining, and quantifying the length of telomere ends of chromosomes. Telomere research has so far benefited the most from this technology. Superiority of PNA for this application can be ascribed to the neutral charge of the backbone, which reduces background binding and accelerates the hybridization rates. DAKO Telomere PNA fish Kits detect human (or other vertebrate) telomere sequences using a fluorochrome- conjugated PNA probe and are proposed for cytogenetic and chromosome aberrations involving studies.

PNA clamping was introduced as an elegant way of modulating PCR reactions for efficient and specific inhibition of amplicons only differing by a single base mutation. This is now gaining wider acceptance for detection of tumor mutations, suppressing the amplification of the wild type gene and the detection of low levels of oncogenes. Additionally, they can be used in clinical diagnostic studies of genetic variations or be constructed as detection beacons for real-time monitoring of PCR reactions (5,11).

PNA mediated- enriched PCR protocol has been developed for the sensitive detection of oncogenic mutations in body fluid samples from cancer patients. It combines an allele-specific PCR clamping step followed by a PCR-RFLP (restriction fragment length polymorphism) confirmatory step and provides rapid but sensitive detection of allelic subfractions (12).

Various examples of using PNAs for nucleic acid capture in sample preparation protocols have been published. PNAs can be used for sequence-specific capture of single stranded nucleic acids. Capture of dsDNA (DNA sample preparation for PCR analyses) can be accomplished using T7-bis-PNA that functions as a generic capture probe. A method for capture of dsDNA with extreme sequence specificity has been devised using two bis-PNA DNA openers (5).

The ability to identify a specific DNA or RNA strand is a powerful tool in the identification of microorganisms. Genetic identification of the species with PNA probes is particularly advantageous since not all members of the genus may be of significance (3,5).

PNA is finding solid ground within PCR and in situ hybridization techniques as well as in the new and very powerful detection methods such as 'sensors' and mass spectrometry. This may revolutionize genetic diagnostics. New results may accelerate development of efficient PNA antisense gene therapeutics, studies on gene target validation and functional genomics.

PNA IN CANCER GENE THERAPY

Triplex-mediated inhibition of transcription *in vitro* was first demonstrated in 1988 with the c-myc gene. This has been used for several other genes and promoter constructs like HER2/neu, interleukin-2 receptor, tumor necrosis factor and multi-drug resistance and has shown reduction of 50-90% in the mRNA or protein produced from the targeted gene (2).

PNAs targeted to the RNA part of telomerase are efficient inhibitors of telomerase activity. Several PNAs that are potent inhibitors of this enzyme at nanomolar concentrations have been identified. Such PNAs are candidates for anticancer drugs (3,13).

The antisense and antigene activity of PNA targeted to the human B-cell lymphoma (bcl)-2 gene was evaluated *in vitro*. Several PNAs complementary to different sequences of bcl-2 (start codon, 5' untranslated region and homopurine sequence on the coding strand of the bcl-2 DNA) were used. Results demonstrate the ability of PNA to selectively modulate both translation and transcription of bcl-2 *in vitro* and suggest its potential use for down regulation of bcl-2 expression in tumors (14).

PNAs have also been shown to effectively inhibit HIV reverse transcriptase when targeted to the RNA template, thereby suggesting this mechanism as a means of developing novel drugs to treat

HIV infections. PNAs are also demonstrated to inhibit DNA helicases and posses gene activating properties what can, hopefully, be utilized *in vivo* (3).

Antisense activity of PNAs was recently demonstrated in the prokaryote *Escherichia coli*, that to a limited extent, takes up 'naked' PNAs. It has been found that certain PNAs targeted to 23S ribosomal RNA show bacteriostatic activity in the high micromolar concentration range (5). These results indicate that PNA could be developed into 'genetic antibiotics' once methods have been devised to increase uptake and for effective target validation for development of traditional antibiotics.

Two papers have reported biological activity of antisense PNAs targeted to neural receptors when injected into the brain of rats, using both naked or PNA-peptide conjugates. These results are truly exciting and will hopefully inspire further exploration.

A recent report demonstrated a 10-fold increase in mutation frequency of a mouse gene targeted with a homopyrimidine *bis*-PNA indicating its significant binding to dsDNA *in vivo*. This confirmed that PNA reached its target in the mouse genome (3).

Identification of a family of small peptide motifs (12-20 amino acid residues in length) that "encode" cellular uptake when conjugated to a variety of biomolecules, including PNA, may provide the tool by which PNA can be delivered to eucariotic cells. Organ or cellular targeting of drugs or radioisotopes in vivo can be accomplished using cell-specific (cancer specific) antibodies. A stepwise delivery system, in which the antibody (*via* the biotin-streptavidin system) is administered, may be advantageous. It has been demonstrated using an antibody-PNA conjugate/PNA-(Te) chelate pair that the specific PNA-PNA recognition may be exploited in this type of targeting (5).

Dihydrotestosterone (T) covalently linked to PNA acts as a vector for targeting c-myc DNA to prostatic cancer cell nuclei which express the androgen receptor gene and facilitates cell-selective inhibition of c-myc expression. This suggests a strategy for cell-specific PNA anti-gene therapy in prostatic carcinoma (15).

In 1998 Vitravene (ISIS Pharmaceuticals, Carlsbad, CA, USA) became the first Food and Drug Administration (FDA)-approved antisense drug, currently used for the treatment of cytomegalovirus infection. Several other antisense compounds, including PNA, with indications for the treatment of cancer, viral infections and inflammatory diseases are currently being evaluated in clinical trials. The experience gained from these clinical trials will be applicable to the next generation of antisenses (Figure 3) (16).

CHALLENGES - PERSPECTIVES OF CANCER DRUGS

Oligonucleotides can be designed to inhibit any gene target, provided that the sequence is known. They can be synthesized using automated procedures, selected in vitro and can be modified to increase binding or improve function (2).

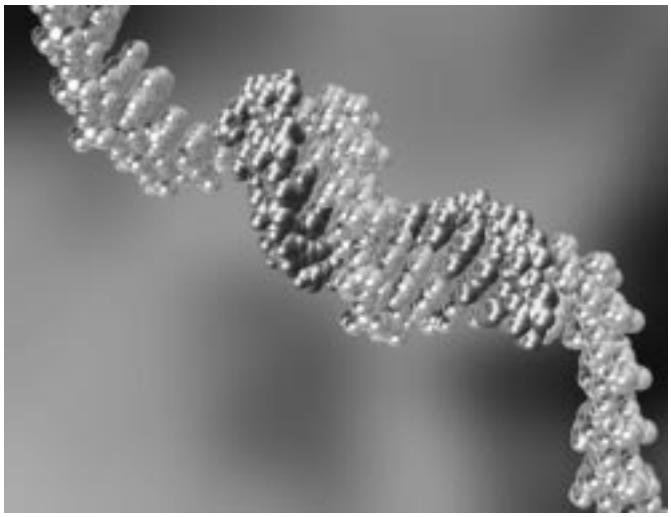

Figure 3. 12mer PNA-mRNA complex occurring according to Watson-Crick base paring rules. PNA-dark, mRNA-pale (Original computer rendered image)

For oligonucleotides to be used therapeutically it is necessary to improve uptake into cells and tissues, demonstrate specific binding, explore potential binding to closely related sites elsewhere in the genome, minimize immune and physiological responses and limit toxicity (2,9).

Finding effective methods to induce efficient uptake of oligonucleotides into cells is an active area of current research and crucial to the success of any oligonucleotide based pharmacology. In order to deliver oligonucleotides to the cells various procedures such as spontaneous uptake, electroporation, cationic lipids, adenovirus-polylysine complexes, and streptolysin have been used. The choice of method depends on the modification of the oligonucleotide.

Although the phenotypes of many diseases are well known, identification of the genes responsible for those phenotypes remains a major barrier in drug development. Determining function of novel genes identified by the human Genome-sequencing Project will be key in future drug discovery and drug development efforts, the most direct approach being by inhibition of a target gene.

Antisense oligonucleotides can be designed to determine the function of novel genes based on minimal sequence information. This enables the inhibition of one gene family member without affecting closely related members. The superb specificity of antisense technology enables the evaluation of the role of a single gene within a family of genes (1,17).

It is relatively simple to perform a large variety of backbone modifications on a PNA oligomer without seriously disrupting its hybridization efficacy. It is a unique possibility to explore profound medicinal chemistry without loosing affinity for the mRNA or the gene. This is not very common in drug development (3,18).

The structural and hybridization properties of PNA, combined with

their relative ease of synthesis and very high chemical and, most importantly, biological stability, make PNA an attractive candidate for developing effective antisense and antigene reagents and drugs. PNA technology has the potential to be utilized for virtually any biological testing including cancer research, diagnostics, genetic testing and drug discovery.

The current vigorous research efforts in each of these areas will determine their contribution in the 21st century.

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