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Peptide Nucleic Acids, Morpholinos and Related Antisense Biomolecules

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who facilitated its planning by assisting one
of the editors (Dr. Janson) during his leave from Yale University;
and to Dr. Stanley Miller, who first proposed the role
of PNA in prebiotic chemical evolution and the ancient history
of life on Earth, thereby raising the question if the recent
discovery of PNA was in fact a fortuitous rediscovery
of our common pre-DNA, pre-RNA origins.

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PREFACE

When this book project was first contemplated, some of the molecules and applications discussed in this volume (such as mammalian siRNA) did not yet exist, which speaks to the relative progress in the antisense field and the likelihood that further chemical modifications of existing classes of molecules will lead to even more enhanced and greater use of "gene tools" in the future. The original intention of the publisher was to devote an entire book to Peptide Nucleic Acid (PNA), which was an incipient but fast-growing field. Given the diversity of emerging antisense products, we felt that it would be more profitable to compare and contrast PNA with other available oligonucleotide homologues and to consider areas in which these biomolecules could be profitably applied to clinical and diagnostic applications. Because other books and research articles in the primary literature already provided specific protocols for use of PNA and related compounds, we preferred to take a broader review of the existing literature by some of the same innovators who developed the molecules and associated techniques.

There are currently a wide variety of research tools to choose from in the design of experiments utilizing gene knockdown and gene labeling, and the eight chapters in Part I address comparative strengths and weaknesses of various nucleoside homologues: standard modified DNA oligonucleotides, peptide nucleic acid (PNA), locked nucleic acid (LNA), morpholinos, and small interfering RNA (siRNA). In terms of unique properties, PNA is especially useful in situations where DNA binding affinity and resistance to nucleases is important such as gene-based diagnostics, or where another ligand is to be bound to DNA for site-specific mutagenesis, gene-specific drug delivery, or other demanding applications. Other currently popular molecules such as siRNA, LNA, and morpholinos are all efficient and versatile methods of knockdown for in vivo use, but each has distinct advantages and limitations. Some molecules are limited to acting on the RNA level (e.g., siRNA), while others work on the DNA or RNA level (e.g., LNA, PNA, mopholinos). After an overview of the basic characteristics of each "gene tool," the ten chapters in Part II address specific translational or clinical applications for PNA and related antisense biomolecules, such as anti-tumor or anti-AIDS therapies, gene activation, and gene repair.

The editors have aimed to present a balanced view of the methods available for gene targeting and modification, which will have broad appeal for either the research scientist or gene therapist. In the process we have omitted some techniques which originally appeared to have promise but which have subsequently been cast into serious doubt in terms of their specificity and effectiveness, such as DNA-RNA chimeraplasty. The molecules

discussed in this volume are widely considered to be beyond reproach in terms of their potential utility in the research setting, despite the fact that they are still proving themselves in the laboratory and have yet to enter the clinic. Because the same "gene tools" may not be equally effective in research and in the clinic—indeed, it is quite possible that the opposite will be true—we have aimed to strike a balance between the bench and the bedside.

Christopher G. Janson
Matthew J. During

Part I
Research Applications

CHAPTER 1

The Many Faces of PNA

Peter E. Nielsen

Introduction to PNA

Peptide nucleic acids or PNA (Fig. 1) were originally conceived as mimics of triple helix forming oligonucleotides designed for sequence specific targeting of double stranded DNA via major groove recognition.[1] It very quickly became clear that PNA is indeed a very potent structural mimic of DNA, capable of forming Watson-crick base pair dependent double helices with sequence complementary DNA, RNA or PNA.[2-4] It also turned out that triplexes formed between two homopyrimidine PNA strands and a complementary homopurine DNA (or RNA) target are exceptionally stable and that "triplex targeting" of double stranded DNA results in a strand displacement complex involving an internal PNA_2-DNA triplex rather than a "traditional" PNA-DNA_2 triplex.

These basic hybridization and structural properties combined with the simple and robust chemistry of the amino-ethyl-glycine-PNA, or aeg-PNA, has attracted attention from many areas of science, including bioorganic chemistry, drug development, molecular biology, genetic diagnostics, prebiotic evolution, and emergingly also materials science. Much of the development during the past ten years has been continuously reviewed over the years and many recent reviews have focused on specialized aspects of PNA applications and chemistry (viz. 5-12). In the present chapter, it is my goal to present an overview of this development stressing highlights, major break-throughs, and the most recent progress as well as future prospects.

PNA Chemistry

The PNA structure consists of three parts (Fig. 1). The backbone is composed of a glycine with an aminoethyl extension from the amine, thereby providing the correct internucleobase spacing, and the nucleobases are attached to the "glycine nitrogen" via an amide linkage as acetic acid derivatives. Thus PNA monomers are amino acids that can be oligomertized by conventional solid phase peptide chemistry using, e.g., Boc- or Fmoc-protection strategy.[13-15] Furthermore, the synthesis of PNA monomers (especially Boc-protected) is straightforward and allows access to a variety of nonnatural nucleobases.[16-20]

The simplicity of the PNA structure has inspired many chemists to explore other amide-based DNA mimics.[21] Because the PNA backbone structure has more degrees of freedom than DNA, it should also be possible to obtain derivatives that bind even tighter to DNA and RNA by conformationally constraining the backbone, most obviously by introducing cyclic structures in the backbone. A large variety of these have been prepared,[12] but only one seems to hold some promise, the aminoethyl proline or aep-PNA.[22] It is, however, too early to judge the potential of this aepPNA because the hybridization properties appear to be very context-dependent in a way that has not been fully elucidated.[23]

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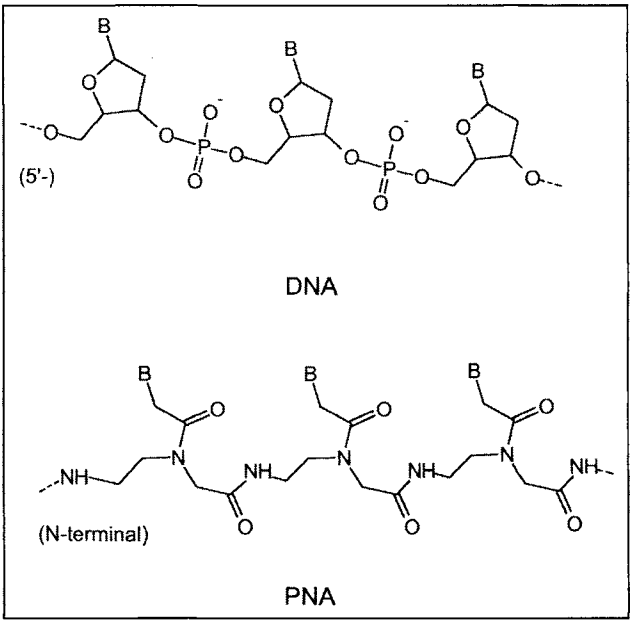

Figure 1. Chemical structures of PNA as compared to DNA. In terms of binding properties, the amino-end of the PNA corresponds of the 5'-end of the DNA. ©1995 Peter E. Nielsen.

Cellular Uptake of PNA

PNAs to be used for antisense or antigene applications are large (typically 2000-4000 MW) hydrophillic molecules and it is therefore not surprising that like most peptides, they are taken up very poorly by eukaryotic and prokaryotic cells. Thus efficient cellular delivery systems for PNAs are required if these are to be developed into antisense and antigene agents.

Several methods have been devised within the past few years to address this issue. Mainly four routes have been taken. One is to transiently disrupt the cellular membrane, e.g., by electroporation,[24] pore forming agents,[25] or physical scrape loading.[26] These methods have proven useful for PNA delivery in several systems, but are limited to cells in culture and also exert a significant stress on the cells. Alternatively, liposome vehicles can be exploited. Cationic liposomes are very efficient in delivering anionic oligonucleotides and DNA vectors to eukaryotic cells, but as PNA is an inherently charged neutral molecule loading of the cationic liposomes requires an extra trick. Corey et al developed a method by which they used a partly complementary oligonucleotide to complex the PNA and thus "piggy-back" it into the cells via the cationic liposomes.[27] This method is quite efficient as judged by the observed antisense effects,[28-30] but requires some optimization of the carrier oligonucleotide; there must be sufficient stability to assemble the PNA into liposomes, and yet it must be able to release the PNA once inside the cell. As an alternative, liposome affinity can be built into the PNA by conjugation to a fatty acid tail.[31] One disadvantage of this method is the limited aqueous solubility of such PNA-fatty acid conjugates as the carbonhydride moiety is enlarged. The adamantyl appears to be a useful compromise.[31,32] Such PNA-adamantyl conjugates may exhibit improved uptake by being more lipophilic than naked PNAs. Finally, a series of cationic small peptides have been identified that by an incompletely understood mechanism are able to transverse cellular membranes (Table 1). These peptides most often have a biological origin, being part of proteins that are exported/imported into cells,[33] and they have successfully been used to deliver large proteins into eukaryotic cells.[34] Such peptides can also conveniently be attached to PNAs

either by continuous synthesis or by chemical conjugation, and several groups have reported good cellular uptake and antisense efficacy using such conjugates.[35-37] We have recently conducted a study on the uptake of PNAs conjugated to the antennapedia or the Tat peptides in four different cell lines, but observed predominantly endosomal uptake or general toxicity at higher concentrations (5-10 μM), although significant differences between the cell lines were also evident.[37a] At this stage we are therefore not convinced that cell penetrating peptides such as pAnt, pTat or the NLS peptide are efficient, general carriers of PNA into eukaryotic cells, and this view is gaining independent support.[37b]

Antisense Applications

The PNA-RNA duplex is not a substrate for RNase H[38,39] and antisense activity of PNA oligomers must therefore rely on other mechanisms, most likely steric hindrance of the translational machinery itself, the ribosomes and various assembly factors, or of mRNA processing enzymes. Accordingly, cell free in vitro translation studies have shown that PNA oligomers targeting translation initiation are usually most potent,[39,40] as the ribosome scanning and assembly process is much less robust than the ribosome during elongation. It has been demonstrated that triplex forming PNAs targeting homopurine sites on the mRNA are able to arrest elongating ribosomes.[38,39] In some cases, simple duplex forming PNAs binding targets inside the reading frame have also been shown to inhibit in vitro translation, although the mechanism is not known.[41] Likewise, most of the antisense PNAs reported to down regulate gene expression in cells in culture were not targeted to the translation initiation region (Table 1). On the other hand, a recent study on a cellular model system targeting luciferase expression showed that of more than 20 PNAs designed to bind various regions of the mRNA, including both the translation initiation and the reading frame, only one PNA targeted to the far 5'-end of the mRNA showed significant antisense activity.[30] Thus a consensus at this stage is difficult to reach, although it seems most plausible that PNAs—as has been found for the analogous morpholino phosphoamidate antisense compounds —should be most potent as antisense gene expression inhibitors if targeted to or 5' of the translation initiation AUG site. Importantly, it was recently demonstrated that splice junctions are very sensitive targets for antisense PNAs as they are for MOE (methoxyl-ethoxy) oligonucleotides,[24] and MOE also do not activate RNase H.

Recently, a very convincing study demonstrated in vivo antisense inhibition of mRNA splicing in a variety of tissues in the mouse (24a), thereby increasing the prospects of in vivo drug applications of PNA.

Antigene Properties

Four modes of binding for sequence-specific targeting of double-stranded DNA by PNA have been identified (cf., Fig. 2). Three of these modes involve invasion of the DNA duplex by PNA strands. It is possible either for a single PNA (homopurine) strand to invade ("duplex invasion") via Watson-Crick base pairing,[42] or alternatively, invasion may be accomplished by two pseudo-complementary PNA strands, each of which binds one of the DNA strands of the target ("double duplex invasion").[20] These pseudo-complementary PNAs contain modified adenine and thymine nucleobases (Fig. 3) that do not allow stable hybridization between the two sequence complementary PNAs, but do permit good binding to the DNA.[20] The third invasion ("triplex invasion") requires a homopurine DNA target and complementary homopyrimidine PNAs that bind the purine DNA strand through combined Watson-Crick-Hoogsteen base paring (Fig. 4) via formation of a very stable PNA_2-DNA triplex.[43] For most applications, the two PNA strands are connected in a bis-PNA designed such that the one strand is antiparallel (WC-strand) and the other strand is parallel (H-strand) to the DNA target. Furthermore, the most efficient binding at physiological pH is obtained when cytosine in the PNA H-strand is replaced by pseudo-isocytosine which mimics N3-protonated cytosine[16] (Fig. 4).

Although PNA triplex invasion complexes are extremely stable once formed, their rate of formation is very sensitive to the presence of cations that stabilize the DNA double helix, and

Table 1. PNA cellular delivery and ex vivo effects

PNA	Target	Method	Modification	Cell Type/Line	Assay	Reference
21-mer	Galanin receptor (ORF)	Direct delivery	Peptide conjugate (penetratin[1]/ transportan[2])	Human melanoma Bowes	Receptor activity/protein level (Western blot)	35
16-mer	Pre-pro-oxytocin	Direct delivery	Peptide conjugate (retro-inverso penetratin[3])	Primary rat neurons	mRNA level (RT-PCR) Immunocytology	36
14-mer (homopyrimidine)	Nitric oxide synthase	Direct delivery	PNA peptide conjugate $(Phe\text{-}Leu)_3$	Mouse macrophage RWA264.7	Enzyme activity	Scafi et al, FEBS Lett 1999; 451:264
17-mer	c-myc (ORF-sense)	Direct delivery	NLS peptide[4] conjugate	Burkitt's lymphoma	Protein level (Western blot)/ cell viability	37
15-mer	PML-Rar-α (AUG)	Cationic liposomes	Adamantyl conjugate	Human lymphocyte (APL) NB4	Protein level (Western blot)/ cell viability	32
13-mer	Telomerase (RNA)	Cationic liposomes	PNA/DNA complex	Human prostate cancer DU145	Telomerase activity	27
13-mer	Telomerase (RNA)	Cationic liposomes	PNA/DNA complex	Human mammary epithelial (immort.)	Telomerase activity/cell viability/telomere length	28
13-mer	Telomerase (RNA)	Electroporation	PNA/DNA complex	AT-SV1, GM05849	Telomerase activity/cell immortality	29
11/13-mer	Telomerase (RNA)	Direct delivery	Peptide conjugate (penetratin[3])	JR8/M14, human melanoma	Telomerase activity/cell viability	Villa et al, FEBS Lett 2000; 473:241
11-mer	none	Direct delivery	Mitochondrial uptake peptide[5]	IMR32, HeLa, a.o.	Only uptake	Chinnery et al, Gene Ther 1999; 6:1919

Table continued on next page

Table 1. Continued

PNA	Target	Method	Modification	Cell Type/Line	Assay	Reference
		Direct delivery	PNA dihydro-testosterone conjugate	Prostatic carcinoma		Boffa et al, Cancer Res 2000; 60:2258
11-18-mer	Luciferase (5′-UTR)	Cationic liposomes	PNA/DNA complex	HeLa	Luciferase activity	30
15-mer	IL-5Rα (splice site)	Electroporation	None	BCL_1 lymphoma	RNA synthesis (splicing)	24
11-mer	mitochondrial DNA	Direct delivery	PNA-phosphonium conjugate	143B osteosarcoma/ fibroblasts (human)	Biotin uptake/ MERRF DNA	Muratovska et al, Nucl Acids Res 2001; 29:1852
13-mer	Telomerase (RNA)	Direct delivery	PNA-lactose conjugate	HepG2 hepatoblastoma	Fluorescence uptake/ telomerase activity	Zhang et al, Bioorg Biomed Lett 2001; 11:1269
15-mer	HIV-1 *gag-pol*	Direct delivery	None	H9	Virus production	65
7-mer bis-PNA	ribosomal RNA α-sarcin loop	Direct delivery	None	*E. coli*	Growth inhibition	55
10-15-mer	β-lactamase β-galactosidase (AUG)	Direct delivery	None	*E. coli*	Enzyme activity	56
10-mer	*acpP* (AUG) α-sarcin loop	Direct delivery	Peptide conjugate (KFF^6)	*E. coli*	Growth inhibition	59
17-mer	NTP/EhErd2 (AUG)	Direct delivery	None	*Entamoeba histolytica*	Enzyme activity	66
Triplex forming bis-PNA		Electroporation	None	Mouse fibroblasts	Mutation induction	Faruqi et al, PNAS 1998; 95:1398
Triplex forming bis-PNA	Globin gene (dsDNA)	Electroporation	None	Monkey kidney CV1	mRNA level (RT-PCR)	52

1: penetratin (pAntp): RQIKIWFQNRRMKWKK; 2: transportan: GWTLNSAGYLLGKINLAALAKKIL; 3: retro-inverso penetratin: (D)-KKWKMRRNQFWVKVQR; 4: Nuclear localization signal (NLS): PKKKRKV; 5: MSVLTPLLLRGLTGSARRLPVPRAKIHSL; 6: KFFKFFKFFK

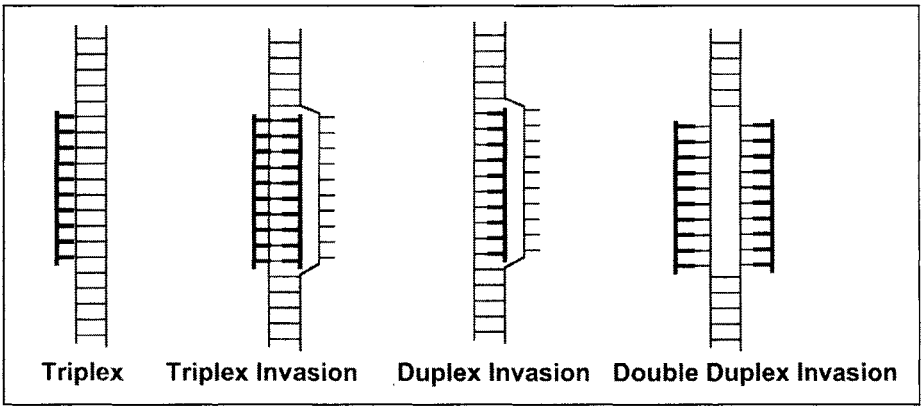

Figure 2. Various structural modes for binding of PNA oligomers to sequence complementary targets in double stranded DNA. ©1996 Peter E. Nielsen.

with simple PNAs triplex invasion at physiological conditions is hardly detectable. The rate of binding can, however, be increased several orders of magnitude by using cationic PNAs which are conveniently made by incorporation of oligo-lysines - or other cationic peptides - at the N- or C-terminal of the PNA,[44,45,45a] or by conjugating the DNA intercalator, 9-aminoscridine to a PNA.[45b] Such cationic bis-PNAs are indeed able to invade their target at physiological ionic strength. Also they have maintained the excellent sequence discrimination exhibited by triplex invasive binding in general. A single mismatch in the DNA target can decrease the binding efficiency 2-3 order of magnitude and like the binding itself,[46] this discrimination is kinetically controlled.[45]

The very high stability of PNA triplex invasion complex as well as the exquisite sequence specificity of the binding has—despite possible obstacles of target accessibility in vivo because

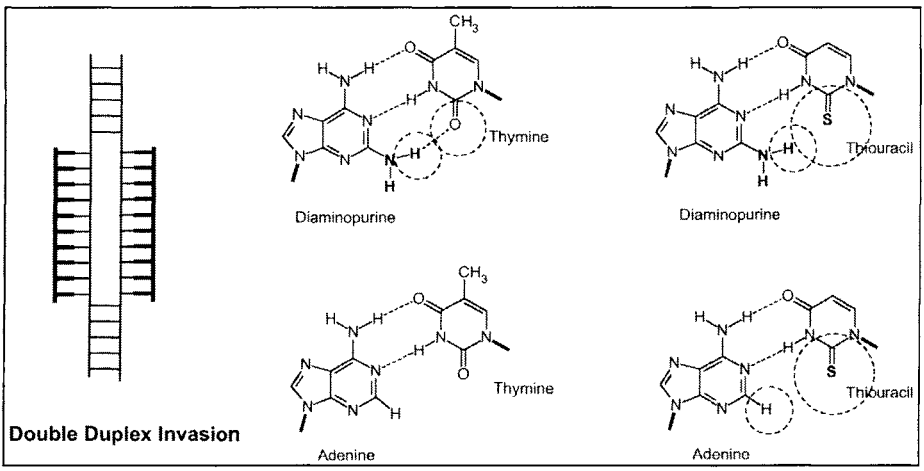

Figure 3. Double duplex invasion of pseudo complementary PNAs. In order to obtain efficient binding the target, and thus the PNAs, should contain at least 50% AT (no other sequence constraints), and in the PNA oligomers all A/T base pairs are substituted with 2,6-diaminopurine/2-thiouracil "base pairs". This base pair is very unstable due 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. ©1999 Peter E. Nielsen.

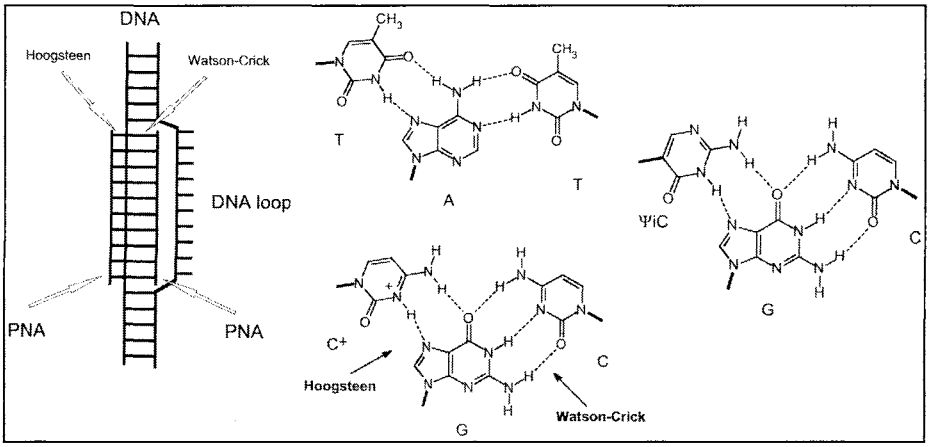

Figure 4. Triplex invasion by homopyrimidine PNA oligomers. One PNA strand binds via Watson-Crick base pairing (preferably in the antiparallel orientation), while the other binds via Hoogsteen base pairing (preferably in the parallel orientation). It is usually advantageous to connect the two PNA strands 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". ©2000 Peter E. Nielsen.

of high ionic strength and chromatin structure—inspired several groups to explore the possibility of using PNA as antigene reagents to control gene expression at the DNA level. Employing cell free in vitro systems, it has been demonstrated that PNA triplex invasion complexes effectively block the access of DNA binding proteins, such as transcription factors,[47] restriction enzymes,[48] or DNA methylases.[49] Furthermore, such complexes, in contrast to, e.g., triplexes formed by oligonucleotides, are capable of arresting elongating RNA polymerases,[38,50] especially if the PNA triplex is bound to the DNA template strand.[48]

Most interestingly, the displaced DNA single strand in a triplex invasion complex can be used by RNA polymerases as an initiation site for transcription[51] in which case the PNA functions as an artificial transcription factor with the PNA target on the DNA acting as an artificial promoter. This observation opens the exciting prospects of developing reagents and drugs that activate specific genes. This topic is discussed further in this book in chapters by Glazer and Janson.

Several recent experiments have indicated that both specific gene mutation[25] and activation of genes[52] may be possible by administering specific PNAs to cells and thus eventually to animals and humans. It may seem surprising that PNAs apparently are able to invade duplex DNA under physiological conditions, especially considering the high ionic strength (140 mM). However, at least some of the explanantion may be found in the observations that negative supercoiling[53] and the transcription process itself[54] dramatically facilitate PNA invasion into the DNA helix.

Antimicrobial PNAs

Many traditional antibiotics (e.g., tetracycline, chloramphenicol) interfere with microbial protein synthesis by specifically binding to (prokaryotic) ribosomes, and it seems that the ribosomal RNA is an integral part of the antibiotic binding site as it is for ribosome catalytic function. Therefore "antisense" targeting of ribosomal RNA could be a sound principle for development of novel antibiotics that are not sensitive to existing resistance mechanisms employed by microorganisms.

Following these ideas it was recently shown that bis-PNAs targeted to purine sequences of the peptidyl transferase center and the α-sarcin loop of 23S *E. coli* ribosomal RNA (Fig. 5) are

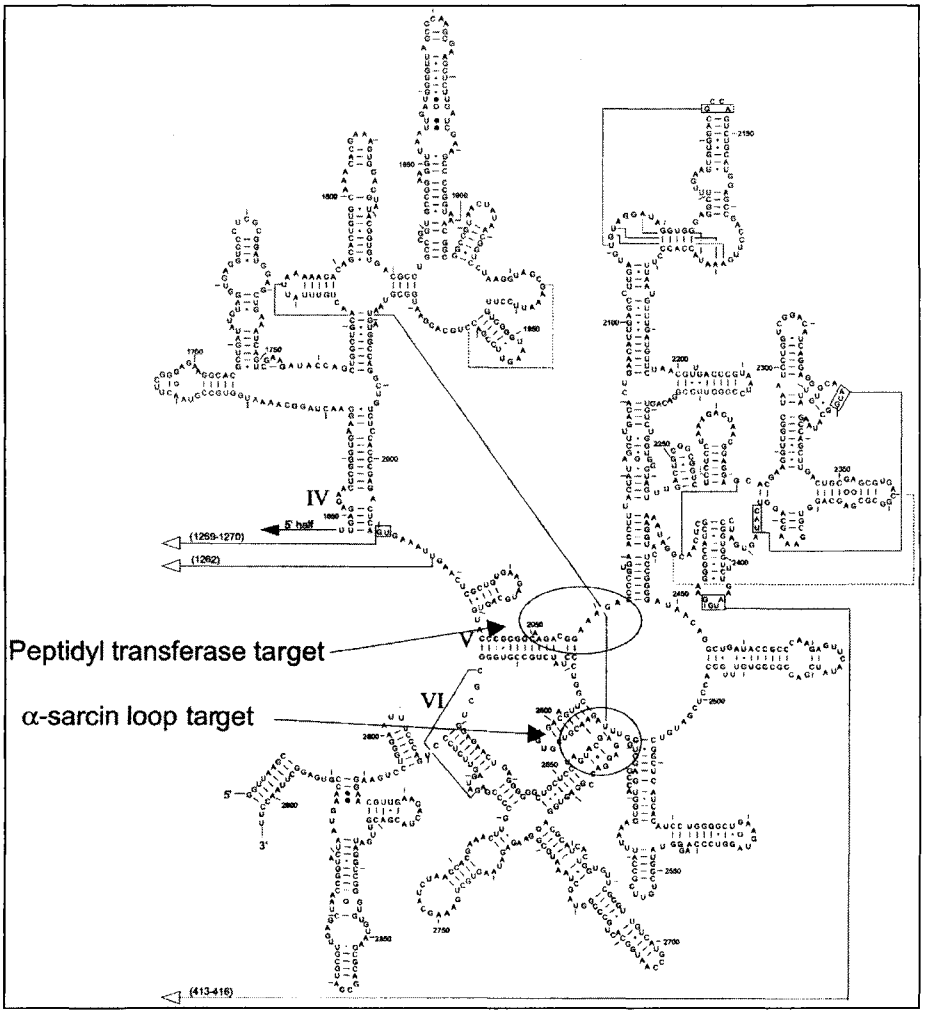

Figure 5. Sequence of part of the 23S ribosomal RNA from *E. coli*. Two purine rich targets that have been found to be sensitive to tageting by bis-PNAs are indicated. These targets are found in two functional regions: The peptidyl transferase center, and the α-sarcin loop. ©2000 Peter E. Nielsen.

indeed able to inhibit in vitro cell free translation as efficiently as tetracycline. Furthermore, the anti-α-sarcin PNA (Fig. 6) was, albeit with low efficiency due to poor uptake in bacteria, able to inhibit bacterial growth at low micromolar concentrations.[55]

These results supported by analogous experiments in which the AUG-translation initiation region of the β-galactosidase and β-lactamase genes were targeted by PNA[56] clearly demonstrated that PNA oligomers can reach their target within (*E. coli*) bacteria, but they also show that potency is severely limited by poor cellular uptake.[57] Fortunately, many peptides are known that interact with and permeabilize bacterial cell-walls and membranes, and some of these are even being developed into antibacterial drugs in their own right. Furthermore, a very simple synthetic peptide was recently discovered that has the permeability effect without being pronouncedly bacteriocidal.[58] Simple conjugation of this peptide to antibacterial PNAs dramatically increased their potency (Table 2) both for the anti-α-sarcin PNA as well as for a

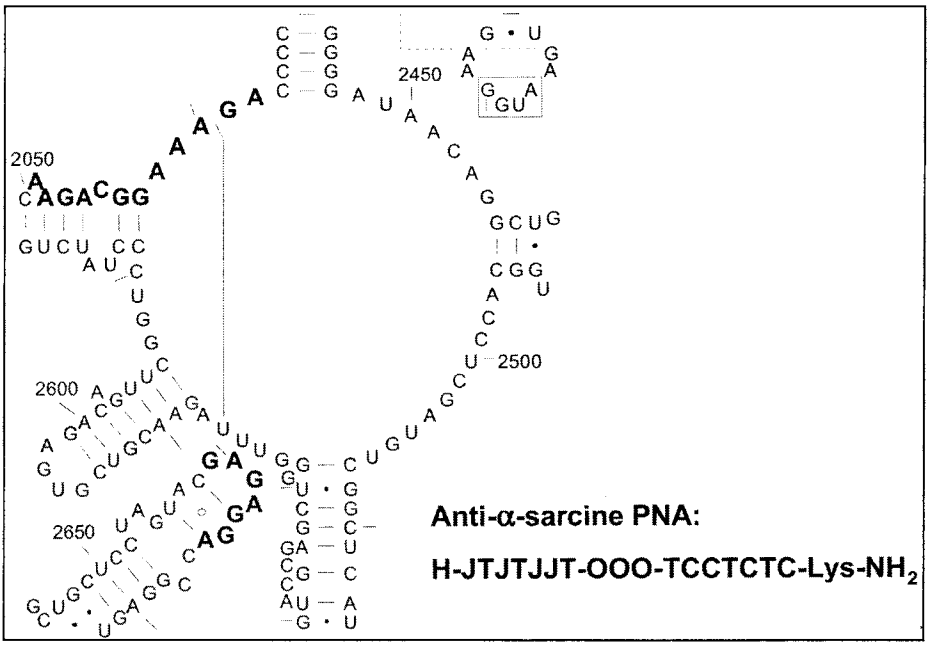

Figure 6. Close-up view of the peptidyl transferase center and the α-sarcin loop targets (in bold). The bis-PNA targeting the α-sarcin loop is also shown. The linker is composed of three 8-amino-3,6-dioxaoctanoic acid (O) units. ©2000 Peter E. Nielsen.

bacteriocidal antisense PNA targeting the essential acpP gene.[59] This approach therefore raise hopes for development of a novel class of antibacterial agents.

PNAs targeted to HIV RNA are very potent inhibitors of reverse transcription of this RNA.[60-62] Thus it may be possible to develop specific antisense-HIV drugs by targeting very evolutionarily conserved regions of the HIV genome.[60-64] Most encouragingly, it was recently reported that such PNAs albeit at very high concentrations (30-100μM) are able to inhibit HIV replication in cells in culture.[65] Obviously, improving the cellular uptake of the PNAs is also required in this case. Indeed, it was recently demonstrated that this is (to a limited degree) possible using PNA-peptide (transportan) conjugates.[65a]

Finally, PNA oligomers have successfully been used for antisense targeting in amoebae.[66] Interestingly, it was found that naked PNA oligomers could be used. This is probably due to the very efficient vacuolar feeding behavior of this organism and these results broaden the scope for developing anti-infective PNA drugs to include decease caused by these types of microorganisms.

Genetic Information Carrier

PNA polymers, in principle, carry genetic information. Because PNA is not recognized by biological "decoding systems" such as RNA (or DNA) polymerases or ribosomes, this information is not available to living organisms. However, a formal possibility exists that in a prebiotic world where RNA or DNA was not yet invented and therefore our biological world had not yet evolved, a predecessor of RNA as genetic material could have been a PNA like material, i.e., a peptide based genetic material.[67] It is quite easy to envisage how these types of molecules could have formed from simple organic and inorganic precursors under "primordial soup" conditions such as those proposed to be responsible for the production of amino acids and nucleobases on the primitive earth, as the pioneer chemical evolutionist Stanley Miller has suggested.[68]

Table 2. Targeting of antibacterial PNAs

PNA	Target	MIC (μM)
H-jtjtjjt-(eg1)$_3$-tcctctc-lysNH$_2$	α-sarcin-loop	5
H-KFFKFFKFFK-eg1-jtjtjjt-(eg1)$_3$-tcctctc-lysNH2	α-sarcin-loop	0.7 3.0*
H-KFFKFFKFFK-NH$_2$		> 3
H-KFFKFFKFFK-eg1-ctcatactct-NH$_2$	*acp*P	0.2 1.0*
H-KFFKFFKFFK-eg1-tcactatctc-NH$_2$	*acp*P mm	3

10% LB; *Mueller-Hinton broth

In support of the possibility of such a preRNA/peptide nucleic acid world scenario are experiments demonstrating that "chemical transfer" of information, "chemical replication" of PNA is, in principle, possible.[69] Likewise it is possible to transfer information from PNA to RNA which would be a requirement for a transition from a PNA to an RNA world.[69-71] It is also worth recalling that such an ancient peptide-based genetic material could be achiral like PNA, thereby leaving the introduction of homochirality in living matter to a later stage in evolution. Simple model ligation experiments have hinted at a mechanism by which homochirality may be introduced as part of the evolutionary and gradual transition from a PNA to an RNA world.[72] A prebiotic PNA world is purely speculative, but the structural and chemical properties of PNA force us not to immediately discard these types of polymers as evolutionary predecessors of our modern biology genetic material(s) DNA (and RNA); it is ironic that a molecule which we discovered in the 1990s and synthesized de novo based on theoretical considerations may have existed long ago as the precursor to all life on Earth.

PNA in Diagnostics

PNA has also found widespread applications in genetic diagnostic techniques. In particular two techniques have benefited from the unique properties of PNA. PNA is not a substrate for DNA metabolizing enzymes and specifically not for DNA polymerases. Therefore PNA oligomers cannot act as primers in PCR techniques. On the contrary, they are very specific blockers of PCR amplification as they compete with DNA primers for binding to the template.[73] This finding has been exploited to specifically suppress amplification of a "background" gene (e.g., wild type) in assays for detection of single base gene mutations or other genetic variations.[74-78] The suppression has also sufficient efficiency to allow specific amplification of e.g., oncogenes on a background of up to 10^4 fold excess of the wild type gene.[74,77]

Furthermore, very specific and sensitive in situ hybridization (FISH) assays have been developed using PNA probes. These include assays for quantitative measurements of telomere size,[79] chromosome painting techniques,[80] as well as sensitive diagnostic methods for detection of virus and bacteria in medical (tissue) samples[81-83] and in environmental (water) samples.[84-88]

As a further development of PNA hybridization probes, PNA fluorescent dye conjugates, "light-up" probes[89-91] that (at least for certain sequences) exhibit significantly increased fluorescence upon hybridization to DNA was recently introduced. Provided that this can be developed into a general principle such probes could be very useful in diagnostics, as they represent a much simpler version of the molecular beacon approach.

The nonionic characteristic of PNA oligomers and thus their lack of migration in an electric field was cleverly exploited by Igloi to develop an "in gel retardation" technique.[92-93] Gels

are polymerized to contain certain PNA oligomers and upon subsequent electrophoresis, DNA fragments containing regions of complementarity to these PNAs will be retarded. By this method large DNA fragments differing by only one nucleobase (point mutations) may be efficiently separated. Finally, PNA oligomers can be used for affinity capture of large DNA or RNA molecules [94-97] for sample preparation and/or further analyses. Also, a number of companies are developing PNA gene arrays as an alternative to the existing DNA photolithography technology, which may facilitate high-throughput genomic analysis.

Prospects

I hope that this brief account has illustrated some of the important characteristics and uses of PNA. Hopefully, it will inspire the implementation and further optimization of these applications, or even more importantly, will give inspiration to develop novel uses of PNA. As an area of further medical applications, I should mention the recent application of PNA conjugates for cell or nuclear targeting and also the delivery of PNA-DNA vectors for gene therapy.[98-100]

Acknowledgements

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