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Stability of peptide nucleic acids in human serum and cellular extracts

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Abstract—The stability of a new type of DNA mimic, peptide nucleic acid (PNA) in human blood serum, *Eschericia coli* and *Micrococcus luteus* extracts and nuclear and cytoplasmic extracts from mouse Ehrlich ascites tumor cells was investigated using HPLC analysis. Under conditions that caused complete cleavage of a control peptide, adrenocorticotropic hormone fragment 4-10, no significant degradation of the PNAs, H-T_{10}-LysNH_2 and H-TGTACGTCACAACTA-NH_2, could be detected. Similarly, PNA H-T_5-LysNH_2 was found to resist attack by fungal proteinase K or porcine intestinal mucosa peptidase at concentrations exceeding those necessary to completely degrade a control peptide, H-Phe-Trp-Tyr-Cys-Phe-Trp-Tyr-Lys-Phe-Trp-Tyr-Lys-OH, by at least 1000- and 30-fold, respectively. Thus PNA is expected to have sufficient biostability to be used as a drug.

Key words: peptide nucleic acid (PNA); anti-sense/-gene drugs; biostability; human serum; cellular extracts; proteases

Oligonucleotides and their analogs are drug candidates which offer several mechanisms for sequence-specific nucleic acid targeting as anti-sense or anti-gene reagents [1]. Although most efforts have been devoted to natural oligonucleotides, and the closely related phosphorothioates and methylphosphonates [2], neither have yet proved to be optimal anti-sense/-gene targeting reagents. Novel oligonucleotide analogs and mimics are therefore high priority goals in medicinal chemistry [2, 3].

A new type of DNA mimic, termed PNA* was recently prepared [4–7]. PNAs are oligomers composed of *N*-(2-aminoethyl) glycine units to which nucleobases are attached (see Fig. 1). This backbone is structurally homomorphous to the (deoxy)ribose-phosphate backbone of nucleic acids. Subsequent studies have demonstrated that PNA binds tightly and selectively to DNA and RNA and may be well suited for the development of anti-sense and anti-gene drugs capable of sequence-specific targeting of both single-stranded and duplex nucleic acids [4, 8–15]. In view of the prospective use of PNAs as therapeutic agents it is worthwhile to determine the stability of PNA in bodily fluids and cellular extracts. We attempt to show that both extracellular (serum) and intracellular media, known to be very harmful to oligonucleotides and oligopeptides, are completely harmless to PNA oligomers.

Materials and Methods

The PNAs were synthesized as described previously [5–7].

Human blood taken from healthy volunteers (one male and three females of different ages) was mixed, clotted at room temperature and spun for 30 min in an Eppendorf centrifuge to obtain serum. Preparation of crude bacterial extracts from *E.coli* strain JH83 and *M. luteus* (strain from Dr S. Mindlin) was performed as described in Ref. 16. Crude cytoplasmic and nuclear extracts from mouse Ehrlich ascites tumor cells were prepared essentially as described in Refs. 17 and 18.

PNA (10–20 μL) and ACTH(4-10), dissolved in nanomolar quantities in 100 mM Tris–HCl buffer, pH 7.5, were mixed in a 1:1 ratio in Eppendorf test-tubes with either serum or one of the extracts and incubated at 37° for various periods of time. Subsequently, 10 μL of 1 M HCl were added to stop the reactions. Reaction mixtures were centrifuged to precipitate high-molecular mass substances

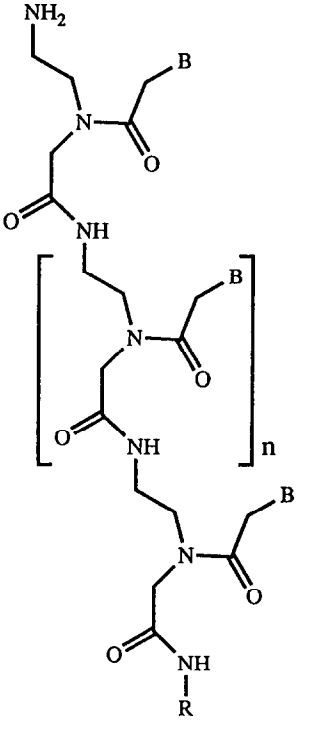

Fig. 1. Chemical structure of PNA. B is a nucleobase, which is thymine for oligothymidyl PNAs and thymine, cytosine, adenine or guanine for mixed sequence PNA. R = H for the latter PNA or positively charged lysinyl amide for oligothymidyl PNAs to improve their solubility, n is varied from 3 through 8 to 13 for the PNAs discussed here.

* Abbreviations: PNA, peptide nucleic acid; the PNAs are written from the N to the C terminal using normal peptide conventions; H, a free amino group; NH_2, a terminal carboxamide; ACTH(4-10), adrenocorticotropic hormone fragment 4-10.

and analysed by HPLC on a Jasco LC-800 or a Spectra-Focus instrument using Du Pont PEP-RP1 or Vydac C18 reversed-phase columns. Elution was performed at 40° and a flow-rate of 1 mL/min using 30 min linear 0–15% acetonitrile gradient in 0.1% trifluoroacetic acid/water for PNA and 50 min linear 10–25% acetonitrile gradient in 0.1% trifluoroacetic acid/water for ACTH(4-10). In the case of simultaneous separation of PNA and the control peptide the gradient was 0–15% (10 min) followed by 15–40% (40 min). The detector signal at 254 nm (PNA) and 215 nm [(ACTH(4-10)] was fed into a microcomputer to calculate peak areas. Some of the peaks inherent in the extract which did not change during incubation were used as internal standards to quantify peptide and PNA concentrations.

Results and Discussion

The decathymidyl PNA H-T_{10}-LysNH_2 contains all the major structural elements of PNA (Fig. 1): i.e., the *N*-(2-aminoethyl)glycine polyamide backbone, the N–C bond between nucleobases and the backbone, and the terminal lysinyl amide used to improve the solubility of the oligomer. Thus we consider this oligomer a reasonable model for PNA in general and have investigated its biostability using HPLC.

We studied the stability of PNA H-T_{10}-LysNH_2 in human serum (known to contain a large variety of proteolytic enzymes), in bacterial cell extracts from *M. luteus* and *E. coli*, and in nuclear extract from mouse Ehrlich ascites tumor cells. Serum is rich in nucleases, which efficiently degrade antisense oligonucleotides and their analogs [19] as well as containing many peptidases such as aminopeptidase [20–22] and dipeptidyl aminopeptidase [22, 23], which attack peptides from the amino terminal; carboxypeptidase [24, 25] and peptidyl carboxypeptidase activities [20–22, 26 27] which attack from the C end, as well as several endopeptidases, such as neutral endopeptidase [21, 22, 27–30]. Bacterial extracts were used due to the potential of PNAs to act as bacteriostatica. As a potential anti-gene agent, PNA stability in nuclei is also of interest. Thus we have studied its biostability in tumor nuclear extracts as well.

ACTH(4-10), a proteolytically well-characterized peptide [20], was incubated in parallel as a control of the presence of enzymes capable of cleaving peptide bonds. Figure 2 shows a typical chromatogram obtained after incubation of PNA H-T_{10}-LysNH_2 in serum. Although a large number of peaks originating from serum are present therein (nucleotides, peptides, etc.), the PNA peak is well separated from all other peaks and its intensity can be quantitated (the same was true for all extracts studied). The amount of PNA remained virtually unchanged during 2 hr of incubation, whereas HPLC analysis revealed a rapid degradation of ACTH(4-10) (with a half-life of about 5 min). This shows that our serum samples contained considerable enzymatic activity capable of cleaving natural peptide bonds. Similarly, ACTH(4-10) completely disappeared within 20 min in bacterial extracts, and was also substantially degraded within this same time period in ascites tumor cell extracts (Table 1), indicating the presence of proteolytic activities in these media as well. Even 2 hr of incubation and the resulting complete cleavage of ACTH(4-10) did not yield a detectable decrease of the decathymidyl PNA peak in any of the extracts studied. Thus, PNA H-T_{10}-LysNH_2 appears to be stable in media containing proteolytic enzymes which degrade oligopeptides.

We further investigated the stability of a PNA pentadecamer containing all four natural nucleobases and terminating in carboxamide (see Fig. 1). In addition to human serum, we incubated this PNA in a cytoplasmic extract of mouse ascites tumor cells. As seen from Table 1, PNA H-TGTACGTCACAACTA-NH_2 is stable in

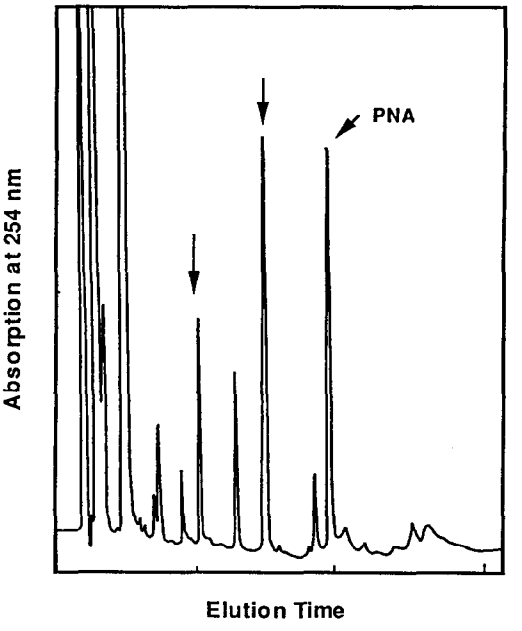

Fig. 2. Stability of PNA H-T_{10}-LysNH_2 in serum. A typical HPLC chromatogram of PNA/serum reaction mixtures after incubation for 2 hr at 37°. For experimental details see Materials and Methods. Some of the inherent blood peaks like the ones indicated by arrows, which did not change during incubation, were used as internal reference to quantify PNA concentrations.

human serum for a minimum of 2 hr and even after 20 hr of incubation the maximum decrease of the PNA detected was only 20% (data not shown). Similar stability of this PNA was observed in a eukaryotic cytoplasmic extract: we were unable to detect any significant degradation in 1 hr (data not shown) and a maximum decrease of 20% was seen following 2 hr of incubation in this medium, which rapidly degrades ACTH(4-10). Whether this apparent decrease in PNA (which is at the limit of the accuracy of the HPLC analysis) in cytoplasm is due to slow degradation or by its aggregation with cellular components remains to be determined.

We also studied the effect of digestion of PNA H-T_5-LysNH_2 by the isolated proteolytic enzymes, fungal proteinase K from *Tritirachium album* and peptidase from porcine intestinal mucosa. The results presented in Fig. 3 show that under conditions where a control peptide, H-Phe-Trp-Tyr-Cys-Phe-Trp-Tyr-Lys-Phe-Trp-Tyr-Lys-OH, which is more stable than ACTH(4-10) (about 50% remains in serum in 1 hr), is completely degraded, PNA is still intact. In fact, PNA H-T_5-LysNH_2 resisted treatment with enzyme concentrations 30 times (peptidase) or 1000 times (proteinase K) that required to degrade the control peptide. Moreover, this PNA is not attacked by bacterial protease type IV from *Streptomyces caespitosus* (data not shown).

Although natural amino acids (glycine) are part of the backbone, the amide bonds found in PNA are not like the ones found in natural peptides; no amide bond between two natural amino acids is present, and this is probably the reason why proteases and peptidases do not recognize these PNAs as a substrate. We thereby conclude that proteolytic enzymes such as those found in serum and cellular extracts as well as in at least some isolated proteases and peptidase cannot cleave the *N*-(2-aminoethyl)glycine

Table 1. PNA and peptide stability in cellular extracts and serum

Extracts:	*M. luteus*	*E.coli*	Ascites, nuclear	Ascites, cytoplasmic	Serum
PNA	307/295	261/259	291/306	ND	335/342
$H\text{-}T_{10}\text{-}LysNH_2$	(96%)	(98%)	(106%)		(102%)
PNA H-TGTACG	ND	ND	ND	334/267	240/222
$TCACAACTA\text{-}NH_2$				(80%)	(93%)
ACTH(4-10)	255/0	251/0	262/110	190/30	307/25
	(0%)	(0%)	(42%)	(16%)	(8%)

Initial/final quantities (arbitrary units) after incubation of PNAs for 120 min or ACTH(4-10) for 20 min were determined as peak areas by HPLC and the portion of remaining material (in brackets) was quantified as described in Materials and Methods. ND, not determined.

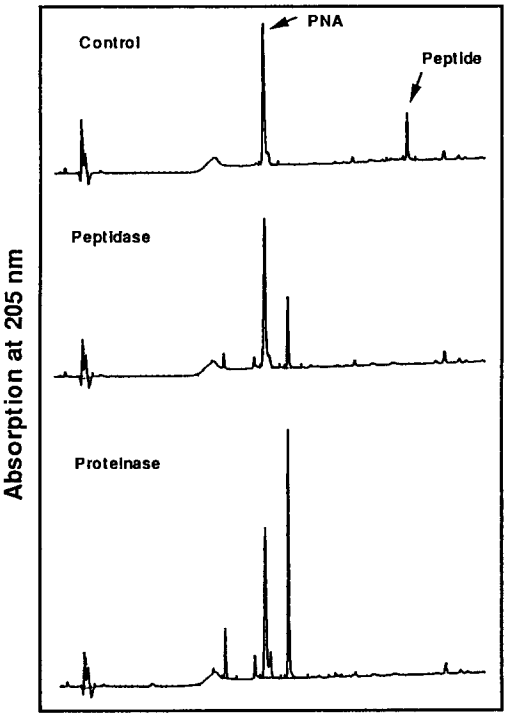

Fig. 3. Resistance of PNA to peptidase and proteinase K. HPLC chromatogram of control sample before enzyme treatment (upper panel) showing the peaks corresponding to PNA $H\text{-}T_5\text{-}LysNH_2$ and the control peptide H-Phe-Trp-Tyr-Cys-Phe-Trp-Tyr-Lys-Phe-Trp-Tyr-Lys-OH. Middle panel: HPLC trace after treatment with porcine mucosa peptidase (0.2 μg/uL, 30 min at 37°). Lower panel: HPLC chromatogram after treatment with proteinase K (0.02 μg/μL, 30 min at 37°). Experiments using porcine peptidase at 0.06, 0.6 or 2 μg/μL or proteinase K at 0.002, 0.2 or 2 μg/μL, respectively, gave the same results (PNA is stable and peptide is digested) except for the appearance of peaks originating from the peptidase/protease preparation observed at higher enzyme concentrations.

based peptide/polyamide backbone of PNA and that PNA is therefore expected to have sufficient biostability to be used as a drug.

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