Gene Ther Mol Biol Vol 5, 47-53, 2000

PNA (peptide nucleic acid) anti-gene/antisense can access intact viable cells and downregulate target genes

Review Article

Lidia C Boffa*, Elisabetta M.Carpaneto, Benedetta Granelli, Maria R. Mariani

National Cancer Institute, IST, L.go R. Benzi 10, 16132 Genoa, Italy

* **Correspondence**: Lidia Boffa, National Cancer Institute, IST, L.go R. Benzi 10, 16132 Genoa, Italy, Tel: -39-010-5600214; Fax: -39-010-5600217; E-mail: boffa@hp380.ist.unige.it

Key words: Peptide Nucleic Acid (PNA), anti-gene, antisense, cell /nuclear localization vectors, gene downregulation

Abbreviations: Androgen Receptor, (AR); blood-brain barrier, (BBB); Hydrophobic tetrapeptide, (FLFL); insulin growth factor, (IGF1); Nitric Oxide synthase, (iNOs); Nuclear Localization Signal peptide, (NLS); Peptide Nucleic Acids, (PNA); periaqueductal gray, (PAG)

Received: 27 June 2000; accepted: July 2000; electronically published: February 2004

Summary

In this paper we summarize our recent data on the anti-gene properties of PNA constructs both with a classical Nuclear Localization Signal peptide, that appears to be effective in all the cell lines tested, and with other vectors designed to be specific for cells carrying their receptors on the nuclear membrane. We discuss the cellular localization pattern of PNA constructs, the consequent regulatory effects on the target gene and their influence in the cellular metabolism. The data are discussed from the perspective of the very recent literature on the access of PNAs antisense/anti-gene in intact viable cells and their consequent regulatory effect in the form of: (i) PNA linked to cellular/nuclear peptidic localization vectors; (ii) PNA linked to non peptidic vectors; (iii) unmodified PNA.

I. Introduction

Peptide Nucleic Acids (PNA) are a recent development in the field of oligonucleotides analogues. PNAs were originally conceived as oligonucleotide homologues that could be used in the sequence-specific targeting of double-stranded DNA (Nielsen et al, 1991; Egholm et al, 1992). They are constructed in such a way to have a neutral charge, achiral, pseudo peptide backbone of N-(2-aminoethyl) glycine polymer. Each unit is linked to a purine or pyrimidine base to create the specific sequence required for hybridization to the targeted polynucleotide. Therefore PNA is chemically more closely related to peptides and proteins than to nucleic acids.

In vitro PNA/DNA and PNA/RNA duplexes are, in general, thermally more stable than the corresponding DNA/DNA or RNA/RNA duplexes and PNA2/DNA triplexes formed between homopyrimidine PNAs and sequence complementary homopurine DNA show even higher stability (Egholm et al, 1993; Nielsen et al, 1994). Although PNAs may bind complementary oligonucleotide or PNA targets in both orientations (parallel and antiparallel) with significant efficiency, the most stable duplexes are formed with an antiparallel Watson–Crick orientation (the PNA N-terminus facing the 3' end of the oligonucleotide) and, in case of triplex helix formation, the Hoogsteen PNA strand should be parallel to the DNA or RNA target. Structural information on the four possible PNA complexes has been obtained by NMR spectroscopy for PNA/RNA (Brown et al, 1994) and for PNA/DNA duplexes (Eriksson and Nielsen, 1996) and by X-ray crystallography for PNA2/DNA triplex (Betts et al, 1995) and for PNA/PNA duplex (Rasmussen et al, 1997).

The overall conclusion from these studies is that the rather flexible PNA oligomer is able to a large extent to adapt its conformation to its rigid complementary oligonucleotide. In terms of sugar conformation, in PNA/RNA duplexes the RNA strand is basically A-form and in PNA/DNA duplexes the DNA strand is close to B-form.

The advantages of using PNA oligomers over the conventional antisense oligonucleotides, that have been

used for some time to try to downregulate target gene expression, are numerous partially due to the high flexibility and absence of charge of the artificial backbone: they are resistant to nucleases and proteases (Demidov et al, 1994) and consequently have a longer life span in the cellular environment than any other oligonucleotide; they can invade duplex DNA and hybridize with complementary sequences with such a superior thermal stability (Giesen et al, 1998), resulting from the decrease in electrostatic repulsion, so that they can successfully compete and eventually displace the natural complementary strand; they have a higher mismatch discrimination therefore forming strong, selective duplexes upon binding to complementary DNA or RNA sequences (Almarsson et al, 1993; Egholm et al, 1993). In cell chromatin the PNA/DNA hydrogen-bonded double is further stabilized by three factors: in the cellular environment PNA/DNA hybrids are more stable than their homologues DNA/DNA since they are ionic strength independent (Jensen et al, 1997); PNA/DNA binding is more tight if DNA is supercoiled than if it is linear; PNA/DNA hybridization is favored in transcriptionally active, open chromatin (Bentin and Nielsen, 1996; Boffa et al, 1997). The greater stability of PNA hybrids with the complementary DNA of transcriptionally active chromatin was used as a tool for selection and characterization of active chromatin fragment as large as 23 kb (Boffa et al, 1995).

Experiments with permeabilized cells and isolated nuclei (Boffa et al, 1996, 1997) have shown that complex sequence PNAs are highly effective in blocking transcription of the targeted gene without inhibiting RNA synthesis in unrelated genes.

Unfortunately at 37ºC PNAs easily enter the cells by endocytosis but are readily sequestered by cytoplasmic vesicles (endosomes and lysosomes) before they can enter the nucleus (Bonham et al, 1995).

II. Review

A. PNA linked to cellular/nuclear peptidic localization vectors

Cellular and nuclear localization signal peptides have successfully been used for some time to carry bulky uncharged molecules into live cells. Several recent reports have demonstrated that PNAs conjugated to such vectors are quite efficiently taken up by some eukaryotic cells.

1. SV 40 Nuclear Localization Signal peptide (NLS)

PKKKRKV is a basic Nuclear Localization Signal peptide (NLS) that was shown first to mediate the transfer of SV40 large T antigen across the nuclear membrane (Kalderon et al, 1984) and later to facilitate the nuclear delivery of large proteins (Gorlich and Mattaj, 1996).

Our Laboratory first described the construct of this NLS with a PNA (Boffa et al, 1997) and observed that it was capable of remarkably decreasing the time of access of PNA to nuclei of permeabilized cells *in vitro* without altering their anti-gene effects. Permeabilized cells were exposed for increasing time to a 17mer anti-gene PNA (+/- NLS) complementary to a unique sequence at the beginning of the second exon of c-myc oncogene. In human adenocarcinoma derived cell line (COLO320-DM) at short time of exposure, we described a selective c-myc transcriptional inhibition that was significantly higher upon exposure to the anti-gene PNA-NLS than to the unmodified matching PNA (**Figure 1**).

In particular, all cells were subjected to run on transcription assay in the presence of [α-^{32}P]UTP. Total RNA was purified and the newly synthesized, radioactively labeled mRNA was analyzed by hybridization for its content in specific sequences, located not only at the PNA/DNA binding site but also upstream and downstream from it, that were previously blotted on an appropriate membrane. We showed that the PNA binding to its target sequence in the c-myc gene strongly inhibited the sense transcription of 4 sequences downstream from the PNA/DNA hybridization site and that the extent of this inhibition depended on the distance of the sequences from the PNA block.

Recently (Cutrona et al, 2000) we have described the anti-gene effect of the above described construct in live cultured cells. When Burkitt's lymphoma derived cell lines (BL) were exposed to the c-myc anti-gene PNA-NLS this molecule was localized predominantly in the cell nuclei. The PNA nuclear localization was not only due to the basic nature of the peptide, but also to the specific amino acids sequence. In particular previous studies have determined that the nuclear localization function of the peptide was strictly dependent on the presence of lysine as the third amino acid (termed Lys^{128}, as from the original sequence of SV40 NLS) (Colledge et al, 1986). In order to demonstrate that only the correct original NLS sequence can specifically confer a cellular/nuclear localization to the bound PNA, we designed a scrambled NLS (KKVKPKR) mutated at the third amino acid to be used as a negative control (NLS_{scr}). BL cells were exposed in culture to PNA-myc +/-NLS or NLS_{scr} tagged with a fluorophore Rhodamine (Rho) (Rho-PNA-myc, Rho-PNA-myc-NLS or Rho-PNA-myc-NLS_{scr}) and analyzed by confocal microscopy. Maximum cellular fluorescence intensity was obtained at 24 h (**Figure 2**). Rho-PNA-myc (a) and Rho-PNA-myc-NLS_{scr} (b) were localized in the cytoplasm, while Rho-PNA-myc-NLS (c) was clearly detectable in the cell nuclei.

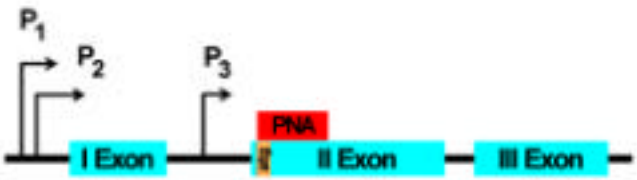

Figure 1. c-myc and its specific anti-gene PNA

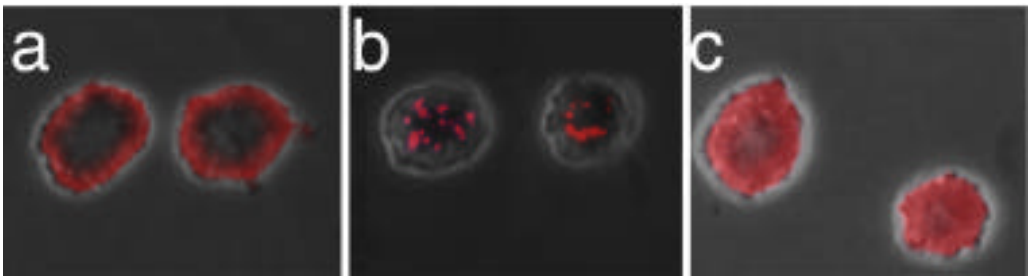

Figure 2 Delivery of PNA-NLS to intact nuclei of BL cells. BL cells were exposed to Rho- PNA-myc (**a**), to Rho- PNA-myc-NLS (**b**) or to Rho- PNA-myc-NLS_{scr} (**c**) and analyzed by confocal microscopy. The pictures show the section crossing the middle of the nuclei with the phase contrast and fluorescence images superimposed.

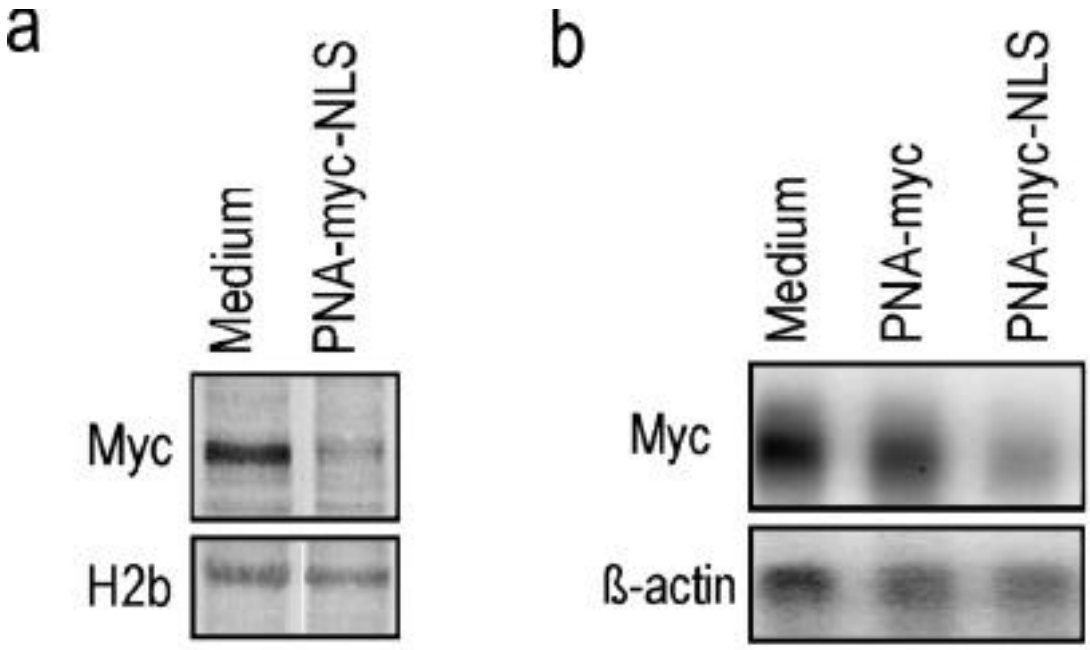

Figure 2. Inhibition of c-myc expression by PNA-myc-NLS. **a**. MYC expression determined by Western blot following exposure of BL cells to the indicated PNAs for 18h. **b**. Northern blot analysis of c-myc mRNA expression in BL cells exposed to the indicated PNAs for 18h.

The PNA myc-NLS access to the cell nuclei caused a rapid consequent downregulation of c-myc transcription as from the time course of MYC expression determined by Western blot and Northern blot analysis of c-myc mRNA (**Figure 3**).

PNA-myc-NLS-treated BL cells displayed a largely impaired growth capacity and a decrease in ^{3}H-thymidine incorporation compared to those treated with control PNA-myc (not shown). Concomitantly, there was a substantial reduction in the proportion of cells in the S or G_2M phases of the cell cycle as determined after 36 h following PNA treatment (**Figure 4a**). The presence of PNA-myc-NLS caused a decrease in viability of the cells in culture that became particularly evident by 72 h. Cell death, however, was unrelated to apoptosis that was not above the control values, as measured by Annexin-V staining (**Figure 4b**).

A PNA-NLS construct was also shown to increase the transfection efficacy of plasmids containing the PNA complementary sequences (Branden et al, 1999).

2. Antennapedia peptide

A 16-amino acid peptide corresponding to the third helix of DNA binding domain of Antennapedia was shown to be able to translocate bulky or uncharged molecules through biological membranes and such internalization does not depend on classical endocytosis (Derossi et al, 1996).

This peptide coupled to PNA (Simmons et al, 1997) was shown to confer permeability through cellular membranes. In particular the intracellular delivery property of this peptide was verified when conjugate with few different PNAs:

i) antisense PNAs targeting the galanin receptor were shown to downregulate its expression by receptor

activity assays and by Western blot (Pooga et al, 1998) in Bowers cells. Furthermore intrathecal injection of such a PNA conjugate into the brain of living rats reduced receptor activity in the brain, implicating *in vivo* antisense effects. Also, the behavioral response of the rats was compatible with a decreased galanin receptor level;

ii) antisense PNA to the prepro-oxytocin mRNA selectively and significantly decreased mRNA and protein product in neuronal cells in culture (Herrada et al, 1998);

iii) two antisense PNAs to telomerase mRNA were selectively effective in downregulating the telomerase activity in human melanoma cells (Villa et al**,** 2000).

In all cases the antisense was not only efficiently delivered to the cells but eventually migrates into the nuclei.

3. D-peptide analog of insulin growth factor (IGF1)

IGF1 PNA conjugates displayed a much higher cellular uptake than unmodified PNAs but the uptake was in correlation with the level of expression of the IGF1 gene in the cells. In fact, in Jurkat cells that do not express the gene there is no PNA uptake, while in p6 cells, where the gene expression is high, a relevant uptake is detectable (Basu and Wickstrom, 1997) suggesting for the first time a possible cell-specific, tissue specific application of PNAs as gene-regulatory agents *in vivo*.

4. Hydrophobic tetrapeptide (FLFL)

FLFL linked to PNA caused not only PNA internalization, but also remarkable stability of the complex in the cellular environment in Namalwa cells (Scarfì et al, 1997). This peptide could also internalize inducible Nitric Oxide synthase (iNOs) cDNA complementary DNA and significantly reduce the level of the enzyme in Macrophages in culture (Scarfì et al, 1999).

B. PNA linked to non peptidic vectors

1. Dihydrotestosterone (T)

T covalently linked to PNA acts as a vector (**Figure 5**) for targeting a c-myc anti-gene PNA selectively to cell nuclei of prostatic cancer LNCaP cells, which express Androgen Receptor (AR) gene, but not to DU145 cells, in which the AR gene is silent (Boffa et al, 2000).

T vector was covalently linked to the N-terminal position of a PNA complementary to a unique sequence of c-myc oncogene (PNAmyc-T). A fluorophore (Rho) was also attached at the C-terminal position to localize the vector-free PNA and the PNA myc-T conjugate (PNAmyc-Rho, PNAmyc-T-Rho) within the cells. The cellular uptake was monitored by confocal fluorescence microscopy. PNAmyc-Rho was detected in the cytoplasm of both prostatic cell lines, whereas PNAmyc-T-Rho was present only in nuclei of LNCaP (AR+) cells. The effects of the complete set of PNAs on expression of the c-myc gene in LNCaP and DU145 cells was monitored by Western blots of the MYC protein content of cell lysates: The results of a series of these analyses proved that in LNCaP cells only PNAmyc-T induced a significant and persistent decrease of MYC expression (**Figure 6**).

2. OX26 murine monoclonal antibody to the rat transferrin receptor

OX26 linked to the antisense PNA for the rev gene mRNA of the human HIV1 virus, not only was able to cross the blood-brain barrier (BBB) but also retained the capacity to bind the target mRNA, if injected in rats (Pardridge et al, 1995). This model system has recently been proposed also in the treatment of Alzheimer's disease (Pardridge et al, 1998).

3. Spermine

Covalent conjugation of Spermine to PNAs was shown to increase PNA solubility with consequent increase in cellular accessibility and a 2-fold acceleration of the rate of molecular association with complementary DNA (Gangamani et al, 1997).

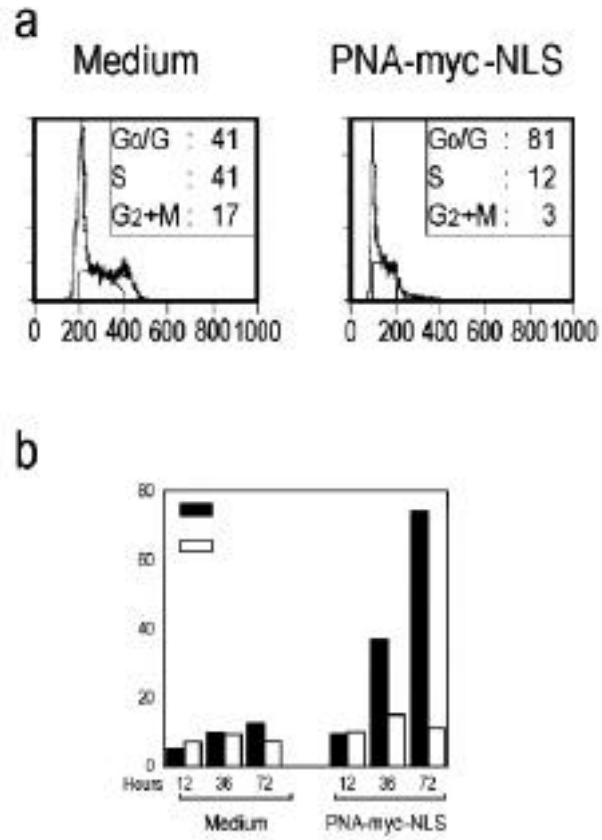

Figure 4. PNA-myc-NLS effects on completion of a productive cell cycle and apoptosis **a)** Flow-cytometric analysis of the cell cycle upon 36-h exposure to the indicated PNAs. **b**. BL cells were incubated with the indicated PNAs for different times. Apoptotic cells and dead cells were detected by Annexin-V binding and PI incorporation.

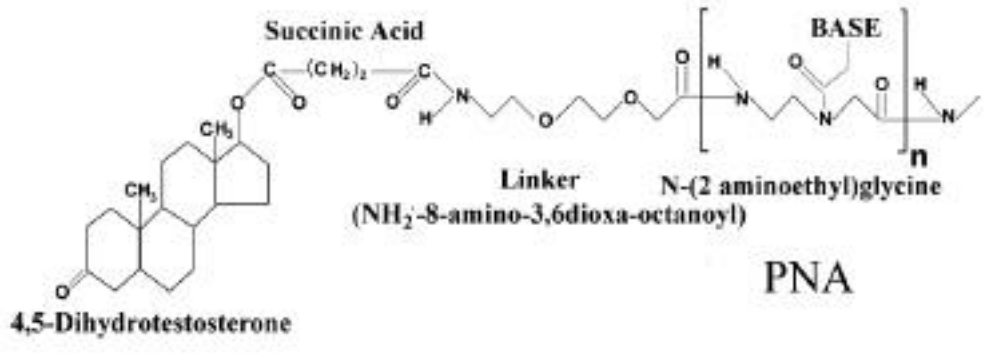

Figure 5 Chemical structure of PNA-dihydrotestoterone construct. Dihydrotestoterone was bound through a flexible linker to the N terminal position of PNA in order to confer the construct a better accessibility to nucleic acid in chromatin.

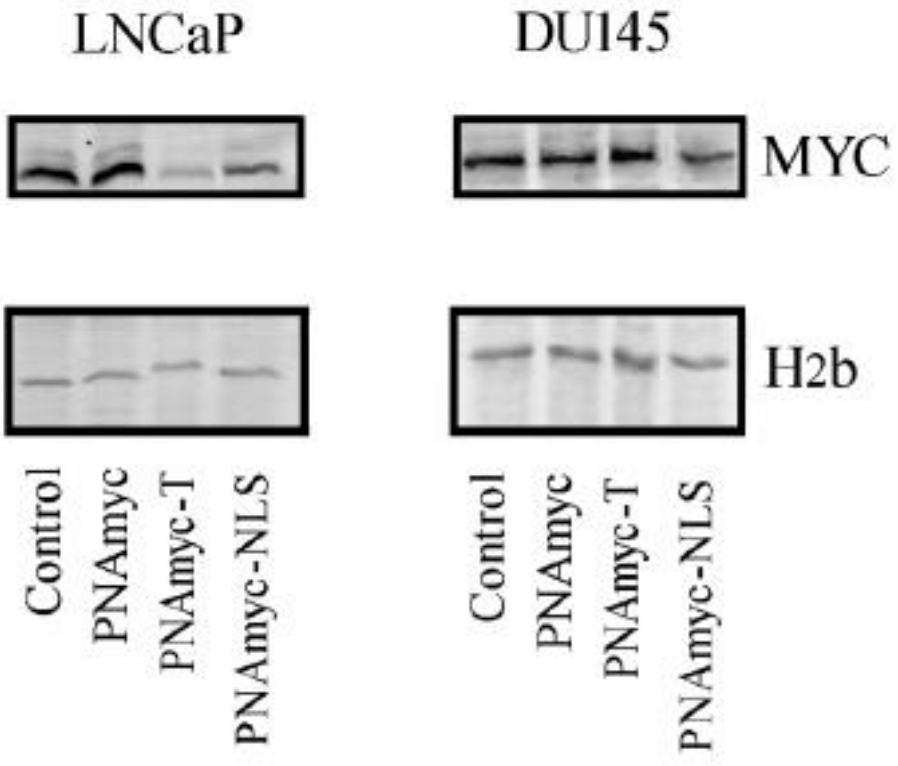

Figure 6. Effect of the different specific PNA constructs on c-myc expression. LNCaP and DU145 cells were treated for 24h with PNA-myc, PNA-myc-T, and PNA-myc-NLS. In this picture is shown a Western blot analysis of MYC. These findings suggest a strategy for targeting of cell-specific anti-gene therapy in prostatic carcinoma.

4. Adamantyl group

This group is probably the most innovative PNA vector. Adamantyl is a lipophilic group that when covalently attached to PNA (Ardhammar et al, 1999) shows at least a 3-fold improvement in PNA cellular uptake in a variety of cell lines in culture (Ljungstrom et al, 1999).

C. Unmodified PNA

PNAs have been shown to enter to a small extent in cultured cells, for example neurons, probably through a mechanism of endocytosis (Aldrian-Herrada et al, 1998). Antisense PNAs uptake by cultured human myoblasts was shown to cause a specific inhibition of replication of mutant mitochondrial DNA (Taylor et al, 1997). In the literature there are recent reports of antisense effects of

unmodified PNAs uptake in live mice brain. The first study (Tyler et al, 1998) was on a 14-mer PNA directed against the neurotensin, NTR1, (position +103) and mu opioid (position -70) receptors mRNAs. PNAs 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 receptors PNA treated animals showed dramatically reduced responses to neurotensin and morphine, respectively. Furthermore, hypothermia induced by neurotensin was substantially reduced. These effects where reversible and specific. A similar pattern of results was obtained in a subsequent study (Tyler et al, 1999) with NTR1 antisense (injected intraperitoneally) and sense (injected directly into the PAG of rats) PNA. The PNA uptake into the brain was detect by a gel shift assay. A 50% decrease of NTR1 mRNA level in brain induced only by the specific sense PNA, determined by quantitative PCR, suggested an anti-gene mechanism.

Moreover a recent study described the non toxicity of an antisense PNA targeted toward mRNA of the opioid receptor gene injected repeatedly in mice (Fraser et al, 2000).

III. Conclusion

We hope to have been able to clearly illustrate the recent body of evidence in support of the novel anti-gene/antisense PNAs-cellular/nuclear vector constructs capacity to access intact viable cells and consequently to downregulate target genes. These new modified PNAs could really provide an innovative and effective approach to the in "vivo" antisense and anti-gene therapy.

Acknowledgments

We acknowledge the support of the Italian Association for Cancer research (AIRC) and National Research Council (CNR "Biotechnologies").

References

Aldrian-Herrada G, Desarmenien M.G, Orcel H, Boissin-Agasse L, Mery J, Brugidou J and Rabie A. (**1998**) .A peptide nucleic acid (PNA) is more rapidly internalized in cultured neurons when coupled to retro-inverso delivery peptide. The antisense activity depresses the target mRNA and protein in magnocellular oxytocin neurons. **Nucleic Acids Res**. 26,4910-4916.

Almarsson O, Bruice T.C, Kerr J .and Zuckermann R.N (**1993**) Molecular mechanics calculations of the structures of polyamide nucleic acid DNA duplexes and triple helical hybrids. **Proc Natl Acad Sci USA**. 90 ,7518-7522

Ardhammar M, Norden B, Nielsen P.E, Malmstrom B.G and Wittung-Stafshede P. (**1999**) *In vitro* membrane penetration of modified peptide nucleic acid (PNA). **J Biomol Struct Dyn** 17,33-40

Basu S and Wickstrom E (**1997**) Synthesis and characterization of a peptide nucleic acid conjugated to a d-peptide anslog of insulin-like growth factor 1 for increased cellular uptake. **Bioconjug Chem** 8, 481-488.

Bentin T and Nielsen P.E (**1996**) Enhanced peptide nucleic acid binding to supercoiled DNA: possible implications for DNA "breathing" dynamics. **Biochemistry** 35, 8863-8869.

Betts L, Josey J.A, Veal J.M and Jordan S.R(**1995**) A nucleic acid triple helix formed by a peptide nucleic acid-DNA complex. **Science**, 270, 1838–1841.

Boado R.J, Tsukamoto H and Pardridge W. (**1998**) Drug delivery of antisense molecules to the brain for treatment of Alzheimer's disease. **J Pharm Sci** 87,1308-1315

Boffa L.C, Carpaneto E.M and Allfrey V.G (**1995**) Isolation of active genes containing CAG repeats by DNA strand invasion by a peptide nucleic acid. **Proc Natl Acad Sci USA**. 92, 1901-1905.

Boffa L.C, Carpaneto E.M, Mariani M.R, Louissaint M and Allfrey V.G (**1997**) Contrasting effects of PNA invasion of the chimeric DMMYC gene on transcription of its myc and PVT domains. **Oncology Res**. 9, 41-51

Boffa L.C, Morris P.L, Carpaneto E.M, Louissaint M and Allfrey V.G. (**1996**) Invasion of the CAG triplet repeats by a complementary peptide nucleic acid inhibits transcription of the androgen receptor and TATA-binding protein genes and correlates with refolding of an active nucleosome containing a unique AR gene sequence. **J. Biol. Chem** 271, 13228-13233,

Boffa L.C, Scarfi' S, Mariani M.R, Damonte G,. Allfrey V.G, Benatti U and. Morris P.L. (**2000**) Dihydrotestosterone as a Selective Cellular/Nuclear Localization Vector for Anti-Gene Peptide Nucleic Acid in Prostatic Carcinoma Cells. **Cancer Res** 60, 2258-2262.

Bonham M.A, Brown S, Boyd A.L, Brown P.H, Bruckenstein D.A, Hanvey J.C, Thomson S.A, Pipe A, Hassman F, Bisi J.E, Froehler B.C, Matteucci M.D, Wagner R.W, Noble S.A and Babiss L.E. (**1995**) An assessment of the antisense properties of RNase H-competent and steric-blocking oligomers. **Nucleic Acids Res** 23, 1197-1203.

Branden L.J., Mohamed A.J and Edward Smith C.I (**1999**)A peptide nucleic acid nuclear localization signal fusion that mediates nuclear transport of DNA. **Nat Biotechnol**. 17, 784-787.

Brown S.C, Thomson S.A, Veal J.M and Davis D.G (**1994**) NMR solution structure of a peptide nucleic acid complexed with RNA. **Science** 265, 777–780

Colledge W.H, Richardson W.D, Edge M.D and Smith A.E. (**1986**) Extensive mutagenesis of the nuclear localization signal of simian virus 40 large T antigen. **Mol Cell Biol**. 6, 4136-4138

Cutrona G., Carpaneto E.M, Ulivi M, Roncella S, Landt O, Ferrarini M and Boffa L.C.(**2000**) Effects in live cells of a c-myc anti-gene PNA liked to a nuclear localization signal. **Nat Biotechnol** 18, 300-303.

Demidov V.V, Potaman V., Frank-Kamenetskii M.D., Buchardt O, Egholm M.E.and Nielsen P.E (**1994**) Stability of Peptide Nucleic Acids in human serum and cellular extracts. **Biochem Pharmacol** 48, 1310-1313.

Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G and Prochiantz A (**1996**) Cell internalization of the third helix of Antennapedia homeodomain is receptor indipendent **J Biol Chem** 271, 18188-18193.

Egholm M, Buchardt O, Christensen L, Behrens C, Freier S, Driver D.A, Berg R.H, Kim S.K, Norden B and Nielsen P.E. (**1993**) PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. **Nature** 365**,** 566-568.

Egholm M, Buchardt O, Christensen L, Behrens C, Freier S.M, Driver D.A, Berg R.H, Kim S.K, Nordén B, and Nielsen

P.E (**1993**) PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen bonding rules. **Nature** 36, 556–568.

Egholm M, Buchardt O, Nielsen P.E and Berg R.H (**1992**) Peptide Nucleic Acids (PNA). Oligonucleotide analogues with an achiral peptide backbone. **J Am Chem Soc** 114, 1895-1897.

Eriksson M, and Nielsen P.E (**1996**) Solution structure of a peptide nucleic acid-DNA duplex. **Struct Biol** 3, 410–413.

Fraser G.L, Holmgren J, Clarke P.B ,.and Wahlestedt C. (**2000**) Antisense inhibition of delta–oppioid receptor gene function *in vivo* by peptide nucleic acids. **Mol Pharm** 57,725-731..

Gangamani B.P, Kumar V.A and Ganesh N.K. (**1997**) Spermine conjugated peptide nucleic acids (spPNA): UV and fluorescence studies of PNA-DNA hybrids with improved stability. **Biochem Biophys Res Commun** 240, 778-782.

Giesen U, Kleider W, Berding C, Geiger A, _rum H and Nielsen PE (**1998**) A formula for thermal stability (Tm) prediction of PNA/DNA duplexes. **Nucleic Acids Res** 26, 5004–5006.

Gorlich D and Mattaj I.W (**1996**). Nucleocytoplasmic transport**. Science** 271, 1513-1518.

Jensen K.K, Orum H, Nielsen P.E and Norden B. (**1997**) Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studied with the BIAcore technique **Biochemistry** 36, 5072–5077

Kalderon D, Roberts B.L Richardson W.D and Smith E.A (**1984**) Short amino acid sequence able to specify nuclear localization. **Cell** 39, 499-509.

Ljungstrom T, Knudsen H and Nielsen P.E (**1999**)Cellular uptake of adamantyl conjugated peptide nucleic acids. **Bioconjug Chem** 10,965-972.

Nielsen P.E, Egholm M and Buchardt O (**1994**)Evidence for (PNA)2/DNA triplex structure upon binding of PNA to dsDNA by strand displacement. **J Mol Recognit** 7, 165–170.

Nielsen PE, Egholm M, Berg R.Hand Buchardt O (**1991**) Sequence selective recognition of DNA by strand displacement with a thymine-substituted polyamide. **Science** 254, 1497–1500.

Pardridge W, Boado RJ and Kang Y.S (**1995**) Vector-mediated delivery of polyamine ("peptide") nucleic acid analogue through the blood barrier *in vivo*. **Proc Natl Acad Sci USA** 92,5592-5596

Pooga H, Soomets U, Hällbrink M, Valkna A, Saar K, Rezaei K, Kahl U, Hao J.X, Xu X.J, Wiesenfeld-Hallin Z, Hokfelt T, Bartfai T and Langel U (**1998**) Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission *in vivo*. **Nat Biotechnol** 16, 857-861

Rasmussen H, Kastrup JS, Nielsen J.N, Nielsen J.M and Nielsen P.E (**1997**) Crystal structure of a peptide nucleic acid (PNA) duplex at 1.7 Å resolution. **Nat Struct Biol** 4, 98–101.

Scarfi' S, Gaspsarini A, Damonte G and Benatti U.(**1997**) Synthesis,uptake and intracellular metabolism of a hydrophobic tetra-peptide nucleic acid(PNA)-biotin molecule. **Biochem Biophys Res Commun** 236,323-326

Scarfi' S, Giovine M, Gaspsarini A, Damonte G, Millo E, Pozzolino M and Benatti U (**1999**) Modified peptide nucleic acids are internalized in mouse macrophages RAW 264.7 and inhibit inducible nitric oxide synthase. **FEBS letters** 451,264-268.

Simmons C.G, Pitts A.E, Mayfield L.D, Shay J.W and Corey D.R (**1997**) Synthesis and membrane permeability of PNA-peptide conjugates **Bioorg Med Chem Lett** 7, 3001–3006.

Taylor R.W, Chinnery P.F, Turnbull D.M and Lightowlers R.N (**1997**) Selective inhibition of mutant human mitochondrial DNA replication *in vitro* by peptide nucleic acids. **Nat Genet** 15, 212–215.

Tyler B.M, Jansen K, McCormick D.J, Douglas C.L, Boules M, Stewart J.A, Zhao L, Lacy B, Cusak B, Fauq A and Richelson E. (**1999**). Peptide nucleic acids targeted to the neurotensin receptor and administered i.p. cross the blood barrier and specifically reduce gene expression. **Proc Natl Acad Sci USA** 96, 7053-7058

Tyler B.M, McCormick D.J, Hoshall C.V, Douglas C.L, Jansen K, Lacy B.W, Cusack B and Richelson E (**1998**) Specific gene blockade shows that peptide nucleic acids readily enter neuronal cells *in vivo*. .**FEBS Lett** 421, 280–284

Villa R, FoliniM, Lualdi S, Veronese S, Daidone M.R and Zaffaroni N. (**2000**) Inhibition of telomerase activity by a cell-penetrating peptide nucleic acid construct in human melanoma cells. **FEBS Letters** 473, 241-248.