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# Gene Modulation by Peptide Nucleic Acids (PNAs) Targeting microRNAs (miRs)

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## 1. Introduction

Since non-viral gene therapy was developed and employed in different *in vitro* and *in vivo* experimental systems as an effective way to control and modify gene expression, RNA has been considered as a molecular target of great relevance (Li & Huang, 2008, López-Fraga et al., 2008). In combination with standard chemotherapy, the siRNA therapy can reduce the chemoresistance of certain cancers, demonstrating its potential for treating many malignant diseases. Examples of RNA sequences to be targeted for therapeutic applications are mRNAs coding oncoproteins or RNA coding anti-apoptotic proteins for the development of anti-cancer therapy.

In the last years, progresses in molecular biology have allowed to identify many genes Coding for small non coding RNA molecules, microRNA (miRNAs or miRs), able to regulate gene expression at the translation level (Huang et al., 2008, Shrivastava & Shrivastava, 2008, Sahu et al. 2007, Orlacchio et al., 2007, Williams et al., 2008, Papagiannakopoulos & Kosik, 2008). Accordingly, an increasing number of reports associate the changed expression with specific phenotypes and even with pathological conditions (Garzon & Croce, 2008, Mascellani et al., 2008, Sontheimer & Carthew, 2005, Filipowicz et al., 2005, Alvarez-Garcia & Miska, 2005). Interestingly, microRNAs play a double role in cancer, behaving both as oncogenes or tumor suppressor genes. In general, miRs promoting cancer targets mRNA coding for tumor-suppression proteins, while microRNAs exhibiting tumor-suppression properties usually target mRNAs coding oncoproteins. MicroRNAs which have been demonstrated to play a crucial role in the initiation and progression of human cancer are defined as oncogenic miRNAs (oncomiRs) (Cho, 2007). The oncomiR expression profiling of human malignancies has also identified a number of diagnostic and prognostic cancer signals (Cho, 2007, Lowery et al., 2008). Moreover, microRNAs have been firmly demonstrated to be involved in cancer metastasis (metastamiRs).

Examples of metastasis-promoting microRNAs are, miR-10b (Calin et al., 2006), miR-373 and -520c (Woods et al., 2007), miR-21, -143 and -182 (Hayashita et al., 2005; Si et al., 2007; Zhu et al.,

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2007). Reviews on metastamiR has been recently published Hurst et al. (Hurst et al. 2009, Edmonds et al. 2009). Reviews on metastamiRs has been recently published by Hurst et al. Table 1 shows examples of microRNAs involved in cancer onset and progression.

MicroRNAs	Tumor	Target mRNA	Reference(s)
miR-17-92	Lung cancer, lymphoma	E2F1	Woods et al., 2007
miR-21	Breast cancer, cholangiocarcinoma, head & neck cancer, leukemia, cervical cancer	tropomyosin 1	Iorio et al., 2005; Zhu et al., 2007
miR-155	Breast cancer, leukemia, pancreatic cancer, B-cell lymphoma	FOXO3a; SHIP1	Costinean et al., 2006; Kong et al., 2010; Pedersen et al., 2009
miR-221	Glioblastoma	PUMA	Ciafre et al., 2005; Zhang et al., 2010
miR-222	Thyroid carcinoma	P27 <sup>Kip1</sup>	Visone et al., 2007
miR-31	Lung cancer	LATS2	Liu et al., 2010

Table 1. Examples of microRNAs involved in cancer onset and their putative targets.

Thus, therapeutic strategies involving miRNA silencing could be proposed based on the roles of these small non-coding RNAs as oncogenes. For these reasons, the development of molecules able to specifically recognize microRNA target sequences is of particular interest, from both a diagnostic and a therapeutic point of view. Indeed, miRNAs can be antagonized *in vivo* by highly-affine oligonucleotides (Lowery et al., 2008, Stenvang & Kauppinen, 2008). Up to now synthetic oligonucleotides have been used for targeting microRNAs, although with several problems, including delivery and stability. However, the use of oligonucleotide analogues has recently been proposed to be effective for the inhibition of miR expression and, accordingly, as a potent tool for the regulation of gene expression (Kota & Balasubramanian, 2010). Peptide nucleic acids (PNAs) (figure 1) are DNA analogues in which the sugar-phosphate backbone is replaced by *N*-(2-aminoethyl)glycine units (Nielsen et al., 1991, Nielsen and Egholm, 1999, Lundin et al., 2006).

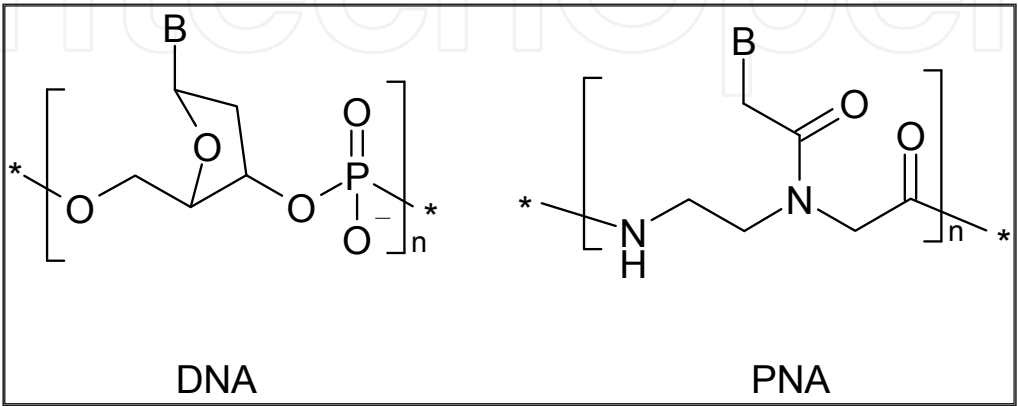


Fig. 1. Structure of DNA and PNA

These molecules efficiently hybridize with complementary DNA and RNA, forming Watson-Crick double helices. In addition, they can generate triple helix structures with double stranded DNA and perform strand invasion. Accordingly, they have been proposed for antisense and anti-gene therapy in a great number of studies (Larsen and Nielsen, 1996, Gambari, 2004, Nielsen 2005, 2006, Yin et al. 2008). PNAs are very promising for RNA recognition, since they have a higher affinity for RNA than for DNA, are more specific and are resistant to DNases and proteases (Demidov et al 1994).

PNAs can be modified in order to achieve better performances in terms of cellular permeation, higher affinity, and specificity for the target DNA and RNA sequences (Corradini et al., 2004, 2007, Sforza et al., 2000, 2007, 2010, Tedeschi et al., 2005 a,b,c, Wojciechowski and Hudson, 2007, Dragulescu-Andrasi 2005, Rapireddy 2007).

In this chapter we will describe the recent results reported in the literature by using PNAs as anti-miR agents and the perspectives of this technology for future development.

## 2. MiR targeting: therapeutic significance

MicroRNAs are a family of small (19 to 25 nucleotide in length) noncoding RNAs that regulate gene expression by sequence-selective targeting of mRNAs, leading to a translational repression or mRNA degradation, depending on the degree of complementarity between miRNAs and the target sequences (Krol et al., 2010).

The expression pathway of these molecules consists in several steps as depicted in figure 2.

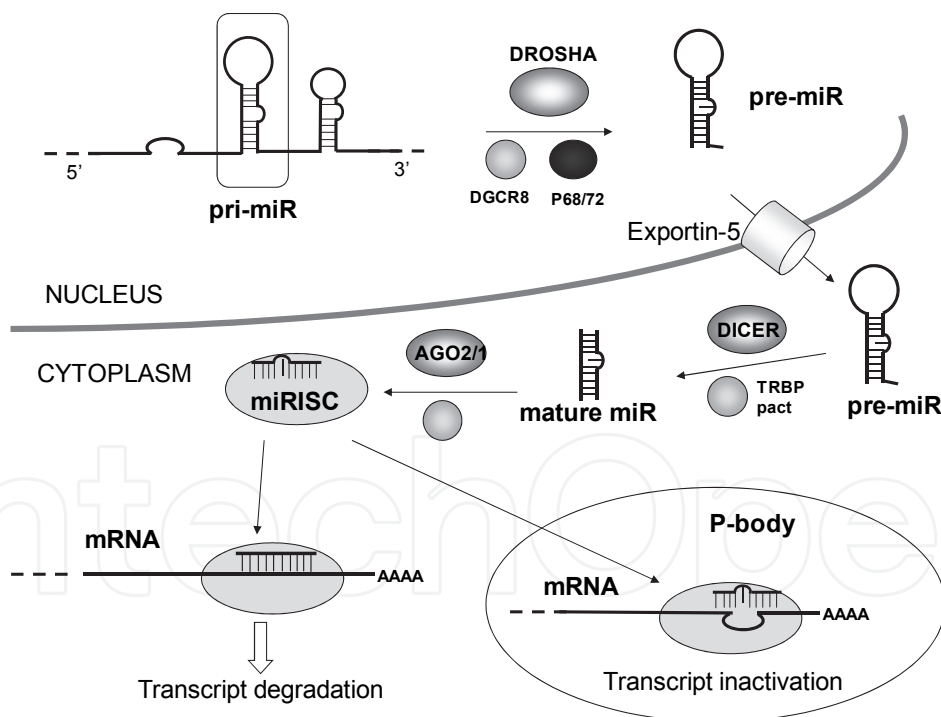


Fig. 2. Pathways of miR production and action. The primary transcript microRNA (pri-miR) is processed by DROSHA in combination with other factors such as DGCR8 to yield an hairpin structure called pre-miR, which is then exported by exportin 5 to the cytoplasm where it is cleaved by DICER in combination with other factors. The mature 21-23nt long dsRNA is processed by incorporation of the guiding strand into the miRISC complex, which act as inhibitor of translation, by either degradation of the mRNA bearing the target sequence or by incorporation of the miRISC-mRNA complex into the P-body, leading to inactivation.

Since their discovery and first characterization, the number of microRNA sequences deposited in the miRBase databases is significantly growing (Kozomara & Griffiths-Jones, 2010). On the other hand, considering that a single miRNA can target several mRNAs and a single mRNA might contain in the 3'UTR sequence several signals for miRNA recognition, it can be calculated that at least 10-40% of human mRNAs are targets for microRNAs (He & Hannon, 2004). Therefore, a great interest is concentrated on the identification of validated targets of microRNAs. This specific field of research has confirmed that the complex networks constituted by miRNAs and RNA targets coding for structural and regulatory proteins leads to the control of highly regulated biological functions, such as differentiation (Masaki et al., 2007), cell cycle (Wang & Blelloch, 2009) and apoptosis (Subramanian & Steer, 2010).

More in detail, and considering the role of microRNAs, a low expression of a given miR is expected to be linked with a potential accumulation of targets mRNAs; conversely, a high expression of miRNAs is expected to be responsible for a low expression of the target mRNAs.

However, since a single 3'UTR of a given mRNA contains signal sequences for several microRNAs, which microRNA should be targeted in order to achieve alteration of the expression of the gene should be experimentally evaluated. With respect to the possible effects of the expression of other mRNA targets, it should be clearly stated that alteration of a single microRNA might retain multiple effects. Finally, multiple targeting of several microRNAs might be considered for achieving strong biological effects. Whatever strategy is considered, several examples of biological effects of targeting microRNA involved in human pathologies are already available in the literature.

### 3. MiR targets relevant in gene therapy

The number of known microRNAs which regulate gene expression is continuously growing, with 1048 sequences present to date in the miRbase for humans (as available on march 30th, 2011 at [http://www.mirbase.org/cgi-bin/mirna\\_summary.pl?org=hsa](http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=hsa)).

Although the discovery of new miRNA is presently carried out using massive sequencing technologies (Kozomara & Griffiths-Jones, 2010), the miR targets important in biochemical processes rely on comparison schemes. As a general rule, selection of the target miRNA can be achieved using the microarray approach by quantitative analysis of the miR profile in a particular cellular state compared to a control lineage. MiR specific RT-PCR protocols can be used, followed by hybridization to specialized miR microarrays. This analysis has been used to identify miR targets which are over- or, more frequently, under-expressed under pathological conditions. Moreover, several very important miR targets have been addressed by many studies on account of the fact that they were able to act as critical points in the regulation of pathological states.

Chemically engineered oligonucleotides, termed 'antagomirs', are efficient and specific silencers of endogenous miRNAs in mice. Silencing of microRNAs in vivo with 'antagomirs' is a very interesting strategy, supporting studies on the involvement of miRs in gene expression and providing new tools for non-viral gene therapy (Czech, 2006).

MiR-155 is one of the first known miR to be overexpressed in cancers, especially in those occurring in B-cells; high level of this miR have been associated to several neoplastic states and to autoimmune diseases, since it leads to the suppression of a large number of genes involved in the control of cellular proliferation. Therefore miR-155 has been identified as



possible target for gene therapy associated to lymphomas and chronic lymphocytic leukemias and colorectal cancer (Zhang et al., 2007, Kota & Balasubrasmanian, 2010). Furthermore, miR-155 was shown to control inflammatory response to microbes (Ceppia et al., 2009).

MiR-21 overexpression was shown to be associated to several solid tumours (lung, breast, colon, gastric and prostate carcinomas and endocrine pancreatic tumours) as well as to cholangiocarcinoma and glioblastoma, since it is correlated to inhibition of apoptosis (Calin & Croce, 2006, Papagiannakopoulos et al., 2008). Knock down of this miR by locked nucleic acids (LNA) oligonucleotides, associated with neural precursor cells (NPC) expressing a secretable variant of the cytotoxic agent tumor necrosis factor-related apoptosis inducing ligand (S-TRAIL), was shown to have high antitumor activity (Corsten et al., 2007).

MiR-122 is another very important target: it is a well characterized liver-specific microRNA exhibiting particular therapeutic interest, since it is related to cholesterol levels in plasma, and it has been shown not only to facilitate Hepatitis C RNA replication (Jopling et al., 2005) but also to be up-regulated in HIV-1 infected cells (Triboulet et al., 2007). Krützfeldt and coworkers (Krützfeldt et al., 2005) demonstrated that intravenous administration of antagomirs against several miRs, including miR-122, resulted in a marked reduction of the corresponding miRNA levels in liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries and adrenals. The silencing of endogenous miRNAs by this novel method is specific, efficient and long-lasting. The biological significance of silencing miRNAs with the use of antagomirs was studied with miR-122. Gene expression and bioinformatic analysis of messenger RNA from antagomir-treated animals revealed that the 3' untranslated regions of upregulated genes are strongly enriched in miR-122 recognition motifs, whereas down-regulated genes are depleted of these motifs. These findings show that antagomirs are powerful tools to silence specific miRNAs in vivo and may represent a therapeutic strategy for silencing miRNAs in disease. This was confirmed by Elmén et al. (Elmén et al., 2008a), who demonstrated that antagonism of microRNA-122 in mice, systemically treated with LNA-antimiR, leads to up-regulation of a large set of predicted target mRNAs in the liver. These results were also confirmed in non-human primates (Elmén et al., 2008b), showing that lowering of plasma cholesterol could be achieved without signs of toxicity.

That interest on this target has been recently boosted by a report which showed increased resistance to chronic hepatitis C virus (HCV) in primates was achieved by targeting miR122 with LNA, with long-lasting suppression of HCV viremia, with no evidence of viral resistance or side effects in the treated animals (Landfrod et al., 2010).

MiR-221 and mir-222 have been shown to be associated to the suppression of p27<sup>Kip1</sup>, a cell cycle inhibitor and tumor suppressor; high levels of these miRs are present in glioblastoma (Le Sage et al 2007) and have been proposed as very important therapeutic targets.

More recently, miR-210 was identified as an highly expressed miR in the erythroid precursor cells from a patient exhibiting hereditary persistence of fetal haemoglobin (HPFH). When RT-PCR was performed on mithramycin-induced K562 cells and erythroid precursor cells, miR-210 was found to be induced in time-dependent and dose-dependent fashion, together with increased expression of the fetal  $\gamma$ -globin genes (Bianchi et al., 2009). Thus miR-210 plays a crucial role in the erythroid differentiation pathway, by limiting the expression of genes whose down-modulation might be associated with the progression of erythroid differentiation.

Other highly expressed miRs (such as miR-142-3p) are very important tools in gene therapy protocols since specific targets can be inserted in gene constructs in order to suppress toxicity associated to viral vectors or to inhibit immune response against a transgene, but they are not easily used as targets for specific inhibition of a pathological state (Brown & Naldini, 2009).

4. MiR targeting by PNAs

PNAs are very promising tools for RNA recognition, since they have a higher affinity for RNA than for DNA (Nielsen, 2004), are more specific and are resistant to DNases and proteases (Demidov et al 1994). As far as their role in targeting mRNAs in the antisense strategy, it should be underlined that, unlike oligonucleotide (ON) molecules, PNAs do not activate the RNase H mediated degradation (Bonham et al., 2005). However, since the RNase H degradation was shown not to be effective in the inhibition of miR by oligonucleotides, the steric block mechanism, i.e. the base pairing of the therapeutic ON with one of the strands of the miR target, should be one of the possible mechanisms, although a degradation of the miR target by a still unknown mechanism has been proposed for some ON derivatives (Krutzfeldt et al., 2007). The steric block mechanism is highly efficient when using PNAs, due to their high affinity for RNA, and the high stability to both chemical and enzymatic degradation (Demidov et al., 1994). However, very few works have been reported so far concerning the use of PNAs as anti-miR agents, showing good performances (table 2). One of the reasons is the lack of cellular permeation by simple unmodified PNA, or segregation in lysosomes of some PNA-peptide conjugates, which can prevent the access to the target miRNA. However, these problems can be easily circumvented by using appropriate carriers, as shown by our own experience and by other examples reported in the next paragraph.

Ref.	targets	PNA modification	Cellular /animal system	Effect
Fabani et al., 2008	miR-122	K-PNA-K <sub>3</sub>	human hepatocellular carcinoma cells primary rat hepatocytes	Decrease in miR-122 and mRNA of its target genes (Aldolase A)
Oh et al ., 2009	miR-16 miR-21 miR-24	Different cell penetrating peptides (CPP) Most effective: Tat-modified	HeLa cells	Upregulation of mRNA targets measured by luciferase assay
Fabani et al., 2010	miR-155	K-PNA-K <sub>3</sub>	LPS-activated primary B cells and mice	Up-regulation of 724 transcripts
Fabbri et al., 2011	miR-210	R <sub>8</sub> -PNA	K562 chronic myelogenous leukemia cells	Alteration of erythroid differentiation

Table 2. Works reporting PNA induced miR suppression.

The first example of targeting microRNAs using PNA-based molecules is provided by miR-122. Fabani and Gait demonstrated, using PNAs and PNA-peptide conjugates, that these oligonucleotide analogs, evaluated for the first time in microRNA inhibition, are more effective than standard 2'-O-methyl oligonucleotides in binding and inhibiting microRNA action (Fabani & Gait, 2009). In their experiments, PNAs were delivered by electroporation. Inhibition of miR-122 was evaluated by Northern blot and by the up-regulation effect upon both chemical and enzymatic degradation.

Interestingly, these authors showed that microRNA inhibition can be achieved without the need for transfection or electroporation, by conjugating the PNA to the cell-penetrating peptide R6-Penetratin, or merely by linkage to four Lys residues, highlighting the potential of PNAs for future therapeutic applications as well as for studying microRNA function. Both LNA/OMe and PNA oligomers were found to be much more effective than 2'-O-methyl RNA oligonucleotides usually used as anti-miR agents. The target miR disappeared from the Northern-blot analysis of the PNA-treated sample, suggesting a still unknown mechanism of degradation or segregation induced by PNA.

In a parallel work, Oh et al. described the effectiveness of miR targeting by PNA-peptide conjugates, using a series of cell penetrating peptides (CPP) as carriers, including R6 pen, Tat, a four Lys sequence, and transportan (Oh, et al., 2009). The best conditions were obtained with cationic peptides, and in particular with the Tat-modified peptide RRRQRRKKRR. In this study, cells were transfected with a plasmid containing a luciferase gene carrying a target site for each miR tested. Inhibition of the miR activity was monitored by expression of the luciferase gene. Inhibition of miR-16, which regulates Bcl-2 expression, and of miR-21 activity could be monitored in this way. PNAs were found to be more effective than LNAs and 2'-OMe oligonucleotides (Figure 3 A). Furthermore, PNAs showed no cytotoxicity at the concentration used, unlike LNAs which showed a reduction in cell viability (Figure 3 B). Furthermore PNAs were found to be more resistant to degradation than LNAs, even if stored at room temperature, suggesting better performances of the former class as candidate drugs.

More recently, a PNA targeted against miR-155 has been used in cellular systems and in mice (Fabani et al., 2010). In this study, the induction of miR-155 by bacterial lipopolysaccharide (LPS) was reduced by using a PNA matching the miR target and linked to four lysine residues. Mice challenged with sub-lethal dose of LPS were treated with 50 mg PNA/kg/day for 2 days and 24 h after the last injection (At which time the miR-155 expression is maximal) they were sacrificed and their spleen tissue was analysed. Complete suppression of miR-155 induction was observed. Genome-wide analysis of gene expression revealed a profile of normal mice treated with LPS and then with anti-miR PNA similar to transgenic miR-155-deficient animals receiving control PBS buffer.

This study revealed important clues on the miR-155 regulation of B-cells and suggested a possible use of anti-miR PNA in the treatment of diffuse large B-cell lymphoma (DLBCL).

In a recent study we evaluated the activity of a PNA targeting microRNA-210, which is firmly associated to hypoxia and is modulated during erythroid differentiation, in leukemic K562 cells (Fabbri et al., 2010). The major conclusions of our study were that a PNA against miR-210 conjugated with a polyarginine peptide (R-pep-PNA-a210): (a) is efficiently internalized within the target cells; (b) strongly inhibits miR-210 activity; (c) deeply alters the expression of raptor and  $\gamma$ -globin genes. Unlike commercially available antagomiRs, which need continuous administrations, a single administration of R-pep-PNA-a210 was sufficient to obtain the biological effects.



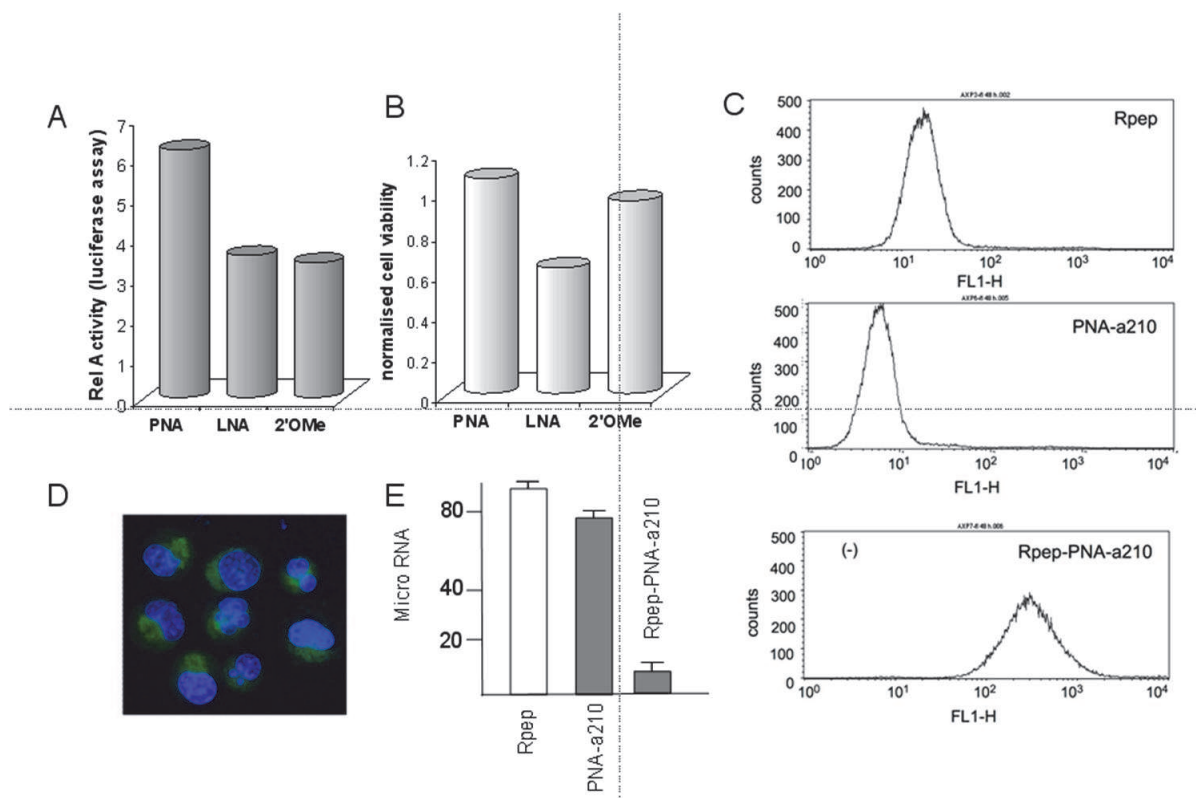


Fig. 3. A, B: Comparison of the PNA-based anti-miR activity and cellular toxicity with other oligonucleotide mimics as reported by Oh and coworkers (data from Oh et al., 2009). A) Effect of anti-miR on HeLa cells transfected with 200 nM of PNA, LNA-modified oligonucleotide, and a 2'-OMe-modified oligonucleotide (2'-OMe) specific for miR-24; a luciferase assay was performed to evaluate the effect anti-miR oligonucleotide mimics. B) Cell viability of HeLa cells after incubation with 200nm of PNA, LNA and 2'OMe oligonucleotides. (C-E) Cellular delivery and anti-miR210 activity of fluoresceinated R<sub>8</sub>-PNA. C. FACS analysis showing the uptake of fluoresceinated R<sub>8</sub> peptide (R-pep), anti-miR-210 PNA (PNA-a210), and R<sub>8</sub>-PNA (R-pep-PNA-a210) after 48 hours incubation of K562 cells at a 2 μM concentration. D. Intracellular distribution of K562 cells cultured for 48 hours with 2 μM of Fluoresceinated R-pep-PNA-a210 and then analyzed using a fluorescence microscope. The picture is the merged analysis of the fluorescence and of the staining of the same cell population with Hoechst 33258 (selectively staining nuclei). E. Effects of the treatment with R-pep, PNA-a210, R-pep-PNA-a210 on the miR-210 content in K562 cells.

Interestingly, cellular uptake was found to be crucial in order to obtain biological activity, since the PNA lacking of the polyarginine tail (PNA-a210), despite being able to hybridize to target nucleotide sequences, displayed very low activity on cells (Figure 3 C-E).

## 5. Modified PNAs can improve miR targeting

The major limit in the use of PNAs for the alteration of gene expression is the low uptake by eukaryotic cells (Rasmussen et al., 2006). In order to remove this drawback, several approaches have been considered, including the delivery of PNA analogues with liposomes and microspheres (Nastruzzi et al., 2000, Cortesi et al., 2004, Borgatti, 2002). One of the possible strategy is to link PNAs to polylysine (K) or a polyarginine (R) tails, based on the

observation that this cell-membrane penetrating oligopeptides are able to facilitate uptake of conjugated molecules (Abes et al., 2008).

Since their discovery, many modifications of the original PNA backbones have been proposed in order to improve performances in term of affinity and specificity.

Modification of the PNA backbone with positively charged groups (figure 4) has also been demonstrated to enhance cellular uptake and consequently PNA efficiency (Corradini et al., 2007, Zhou et al., 2003, 2006).

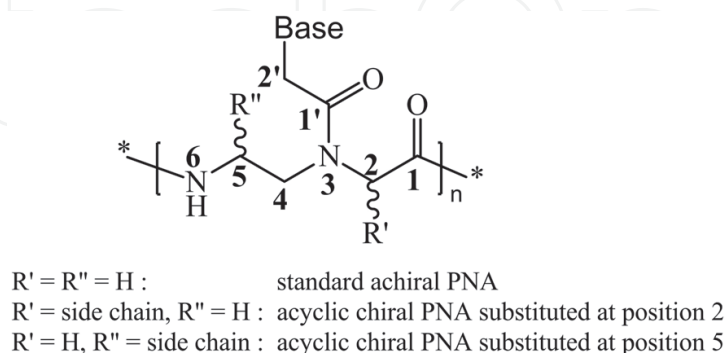


Fig. 4. Structure of backbone modified PNA.

Although the steric requirements for binding RNA have not been extensively studied so far, the availability of different chemical strategies to design and synthesize PNA analogues is the basis for the development of new peptide nucleic acids (PNAs) specifically aimed at targeting RNA, to be used for miR targeting.

In the last few years several research groups have been involved in the synthesis and in the studies of the binding properties of PNAs with a chiral constrained backbone obtained by insertion of stereogenic centers either at the C2 (alpha) or C5 (gamma) position of the monomer.

The insertion of one chiral monomeric unit in a PNA strand has resulted in increased DNA binding affinity, when the side chain was positively charged (e.g. lysine or arginine). The PNA:DNA duplex stability was found to be dependent on stereochemistry: PNAs carrying a monomer with a stereocenter derived from a D-amino acid at the C2 position bound complementary antiparallel DNA strands with higher affinity than the corresponding PNA carrying a monomer with a stereocenter derived from an L-amino acid at the same position. Therefore, the affinity of chiral PNAs for complementary DNA emerged to be a contribution of different factors: electrostatic interactions, steric hindrance and, most interestingly, enantioselectivity with a preference for the D-configuration at the 2 position of the monomer. A PNA:DNA duplex, in which three adjacent chiral monomers based on 2D-lysine ("chiral box") were present in the middle of the PNA strand, was characterized by X-ray diffraction, and the results showed that the D-lysine-based chiral PNA-DNA heteroduplex adopts the so-called P-helix conformation, with helical parameters significantly different from those of the canonical DNA helical forms (Menchise et al., 2003). The P-helix is characterized by a small twist angle, a large x-displacement and a wide, deep major groove. The 2D-lysine "chiral box" PNA showed also an increased sequence selectivity, both in terms of direction control and of recognition of a single base mismatch (Sforza et al., 2000). Therefore, this type of structures was found ideal for targeting point mutations in genes of diagnostic interest (Corradini et al., 2004, Tedeschi et al., 2005a,b). Recently chiral PNAs with L- or D-stereocenters either at the 2- or the 5-positions of the monomer or with both stereocenters simultaneously present have also been synthesized and

studied (Sforza et al., 2007, Manicardi et al., 2010).<sup>76</sup> The strongest directing factor was found to be the L-stereogenic center at position 5 derived from L-lysine. This preference (L-configuration at position 5) and the former (D-configuration at position 2) are related to the ability to form a preferred right-handed helicity of PNA and therefore a preferential preorganization for binding right-handed DNA.

More recently, three consecutive different chiral monomers, respectively modified with 2D-Arg, with 5L,2D-Arg and with 5L-Arg, were used by our group in order to build an "extended chiral box" PNA. Such PNA, analogously to the above mentioned "chiral box" PNA, showed very good mismatch discrimination towards DNA, was even more specific in RNA recognition, showing that PNA modifications can also be used in order to tailor PNA recognition towards RNA (Calabretta et al., 2011).

Recently, Ly and co-workers reported the synthesis and uptake properties of  $\gamma$ GPNA, in which the PNA backbone had a homo-arginine side chain at the 5-position (or  $\gamma$  position) (Sahu et al., 2009), showing an excellent cellular uptake.

Substitution at both C2 and C5 carbons of the PNA backbone with amino acid side chains leads to ambivalent structures having properties of DNA or RNA mimic on one side and peptide mimics on the other side, thus allowing recognition by specific receptors, as shown very recently by a short PNA mimicking the function of a nuclear localization peptide (NLS) (Sforza et al., 2010). Thus, and to obtain PNAs with both peptide properties and RNA binding ability. This strategy can be used to further improve the efficiency of PNAs for miR targeting. In fact, the use of peptides as carriers represents a "Achille's heel" of the potential PNA-based drug candidates, since the peptide part might be subjected to enzymatic degradation, whereas the incorporation of the peptide signal into the PNA backbone does not lead to enzymatic degradation, even in the presence of highly active proteases.

PNAs bearing modified nucleobases able to induce additional interactions providing high improvement in RNA and DNA binding affinities have also been described (Wojciechowski et al., 2009). Combination of modified nucleobases and backbone modification with C2 or C5 modified residues was found to be the best approach in order to achieve strand invasion into mixed DNA sequences (Ishizuka et al., 2008, 2009, Chenna et al., 2008), a strategy which could also be very fruitful in challenging double-stranded miRs.

## 6. Conclusions and perspectives

PNAs are very promising tools for the inhibition of miR activities, and this effect can be very important for obtaining gene modulation in a relatively simple way, with very important applications in gene therapy and in drug development.

The issue of the correct delivery of PNAs to their targets is still open, although efficient strategies have already been described, including conjugation with carrier peptides and backbone modification.

The very high affinity of PNAs for RNA and the very strong chemical and enzymatic stability of these compounds (especially the backbone-modified version) make them ideal candidates as miR inhibitors with long-lasting effect.

The first data available already indicate that this technology is likely to succeed, despite the limited number of targets studied so far. Apart from model systems, PNAs have the potentiality to perform like (and eventually outperform) other anti-miR agents such as 2'-OMe oligonucleotides and LNAs.

Furthermore, the possibility to introduce functional groups along the chain of the PNA strand by chemical synthesis allows to envisage strategies in which the PNA can be endowed of catalytic sites, thus leading to molecules not only capable of binding, but also of cleaving, leading to miR specific nuclease models.

## 7. Acknowledgment

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## **Targets in Gene Therapy**

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This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

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