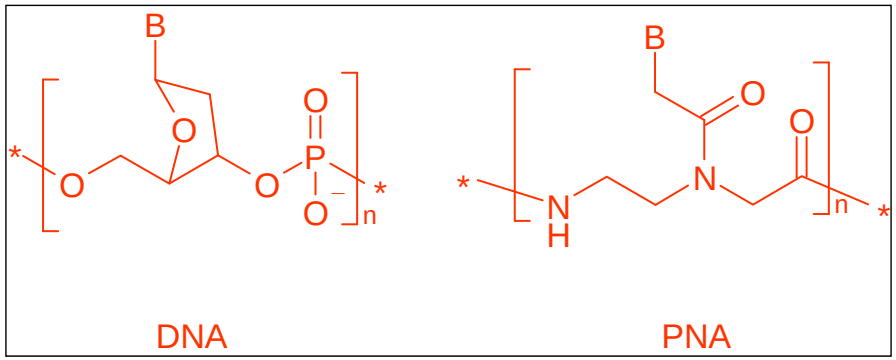
OUTLINE

- Fundamentals of PNA
- Modified PNA –

  in the backbone (chiral)
- Overview of PNA applications

  as diagnostic tools

  as drug candidates
- Advantages and limitations



Nielsen PE, Egholm M, Berg RH, Buchardt O. Science 1991; 254; 1497-1500

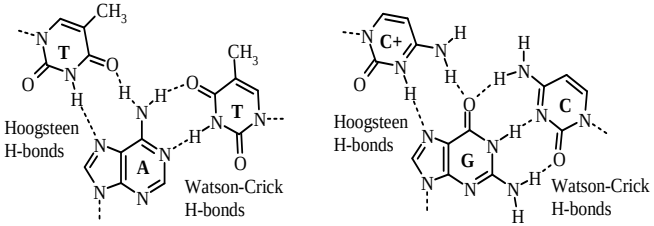
PROPERTIES OF PNA

DUPLEXES WITH COMPLEMENTARY DNA and RNA

Watson-Crick base pairing
High stability
High selectivity

FORMATION OF TRIPLEXES PNA:DNA:PNA

Watson-Crick+ Hoogsteen base pairing
Very High stability
Strand invasion of ds DNA



FORMATION OF PNA :PNA DUPLEXES

DUPLEX STABILITY

Melting temperatures

Target sequence: TGTACGTCACAACTA 15 mer

Duplex	Tm(C°)
PNA:DNA	69.5
PNA:RNA	72.3
DNA:DNA	53.3
DNA:RNA	50.6

Measured at 260 nm in 10 mM phosphate buffer, 0.1M NaCl and 0.1 mM EDTA. Strand Concentration: 4 µM.

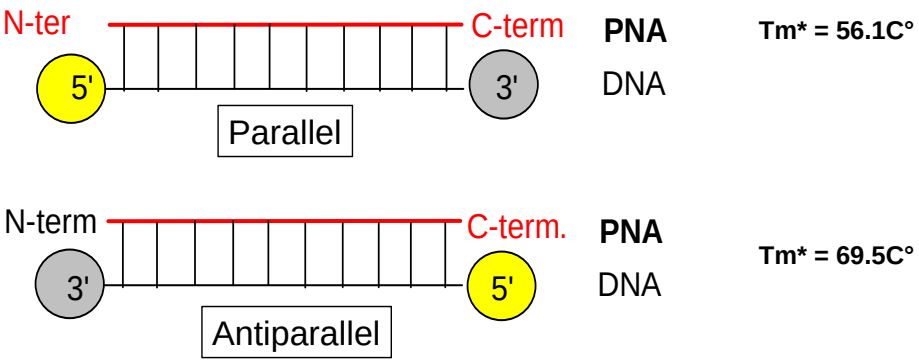
PNA:PNA > PNA:RNA > PNA:DNA > RNA:DNA > DNA:DNA

MISMATCH RECOGNITION (POINT MUTATIONS)

TGTACGTCACAACTA DNA
ACATGCCGGGTTGAT Tm 53° → 49°C

TGTACGTCACAACTA PNA
ACATGCCGGGTTGAT Tm 69° → 56°C

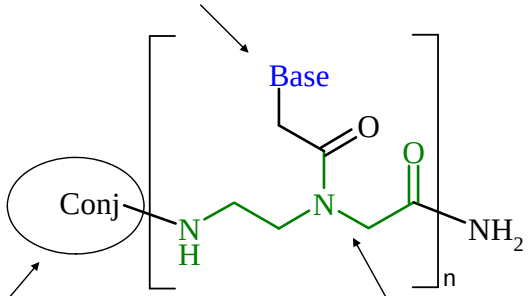
ORIENTATION OF PNA:DNA DUPLEXES



* For the target sequence: **TGTACGTCACAACTA**

Modified PNAs

MODIFIED BASES

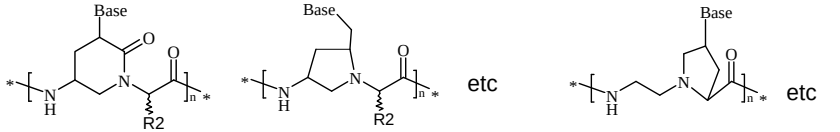


MODIFIED BACKBONES

CONJUGATION WITH FUNCTIONAL GROUPS

Backbone modifications

Preorganization through rigid structure

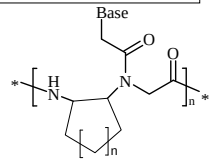


Ring closure on the aminoethyl residue

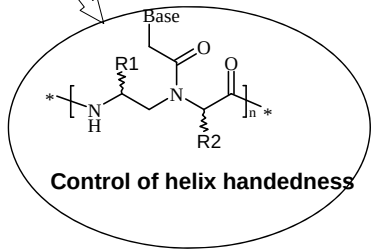
Ring closure on the glycine residue

Rigidification of the aminoethyl residue

Functional groups insertion (2' or 5')



Preorganization through conformational constraints

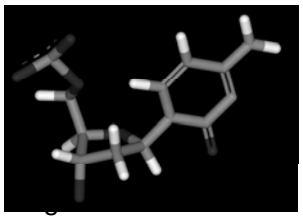


Control of helix handedness

R. Corradini, S. Sforza, T. Tedeschi, F. Totsingan, R. Marchelli *Curr. Top. Med. Chem.* 2007, *7*, 681-694.

DNA chirality

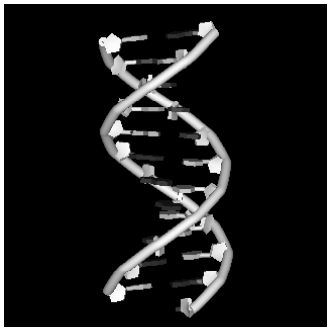
Double helix handedness
Supramolecular chirality

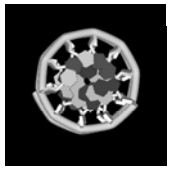


Nucleobase

Chiral monomers (D-deoxyribose))

Molecular Chirality

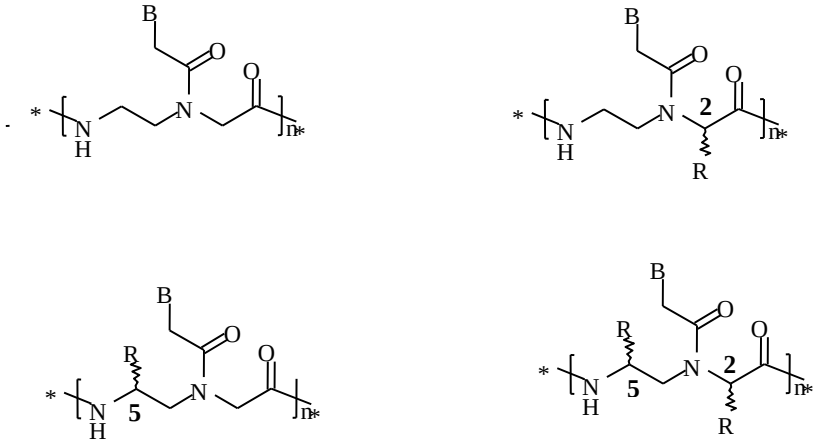




B-DNA
Right-handed

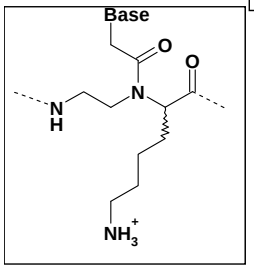
Z-DNA
Left-handed

Chiral PNAs



Both configurations are available for both positions from the amino acid chiral pool
Amino acids used were mostly **lysine** and **arginine** (electrostatic contributions to DNA binding) and water solubility

PNA with a stereogenic center at C-2



DNA showed enantioselectivity in binding to Chiral PNAs and seemed to be mostly due to the monomer placed in the middle of the sequence

N-GTAGATCACT-$_L$Lys-C
3'-CATCTAGTGA-5'

T_m PNA/DNA duplexes
55°C (2D-Lys)
52°C (achiral)
49°C (2L-Lys)

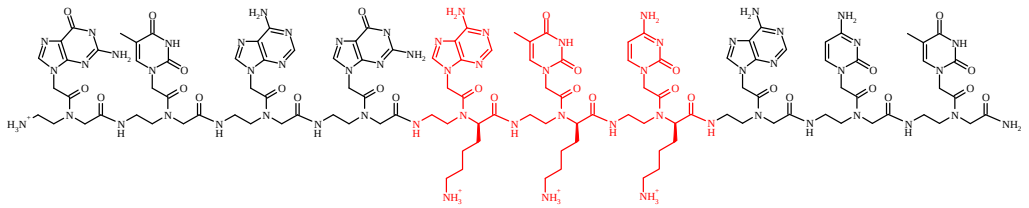
"…D-amino acids appear to be better accomodated in the backbone of a PNA-DNA duplex…"

2D-PNA also exerts a better mismatch discrimination

	T_m of mismatched antiparallel PNA/DNA duplexes	**Stability loss due to mismatch insertion**
N-GTAGATCACT-$_L$Lys-C 3'-CATCT**G**GTGA-5'	**36°C (2D-)** **35°C (2L-)** **37°C (achiral)**	**-19°C (2D-)** **-14°C (2L-)** **-15°C (achiral)**

Haaima et al., *Angew. Chemie Int. Ed.,* 1996, 35, 1939-1942.

A boost in the chirality effects: "chiral box" PNAs



Three 2D- or 2L-Lys-based monomers were placed in the middle of a PNA strand:
2D-Lys "chiral box" PNA and **2L-Lys"chiral box" PNA**

PNA	**Tm (°C)**
Chiral box D- **H-GTAGA$_{D\text{-Lys}}$T$_{D\text{-Lys}}$C$_{D\text{-Lys}}$ACT-NH$_2$**	**43**
Chiral box L- **H-GTAGA$_{L\text{-Lys}}$T$_{L\text{-Lys}}$C$_{L\text{-Lys}}$ACT-NH$_2$**	32

Sforza S., Ghirardi S., Corradini R., Dossena A., Marchelli R., *Eur. J. Org. Chem.*, 2000, 2905-2913.

Enhanced mismatch recognition

Complementary DNA: **5'-AGTGATCTAC-3'**
Mismatched DNA: **5'-AGTG*G*TCTAC-3'**

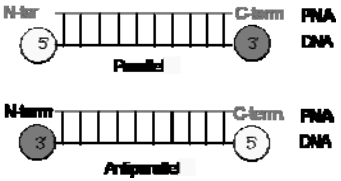
PNA	Tm(°C) Match	Tm (°C) Mism.	**Selectivity** ΔTm (°C)
H-GTAGATCACT-***L*-Lys**-NH_2	52	37	**15**
H-GT$_{L\text{-Lys}}$AGAT$_{L\text{-Lys}}$CACT$_{L\text{-Lys}}$-***L*-Lys**-NH_2	49	35	**14**
H-GT$_{D\text{-Lys}}$AGAT$_{D\text{-Lys}}$CACT$_{D\text{-Lys}}$-***L*-Lys**-NH_2	55	36	**19**
H-GTAG**A** $_{D\text{-Lys}}$**T**$_{D\text{-Lys}}$**C**$_{D\text{-Lys}}$ACT-NH_2	43	<15	**>28**

S. Sforza, R. Corradini, S. Ghirardi, A. Dossena, R. Marchelli *Eur. J. Org. Chem.*, 2000, 2905-2913.

PREFERRED ORIENTATION

Melting temperatures of achiral PNA , D-Lysine "chiral box" PNA and L-Lysine "chiral box" PNA bound to the the complementary antiparallel and complementary parallel DNA

PNA	**PNA- full matched DNA antiparallel Tm (°C)**	**PNA- full matched DNA parallel Tm (°C)**
achiral	50	40
L-Lys "chiral box"	30	(40)
D-Lys "chiral box"	(43)	<15

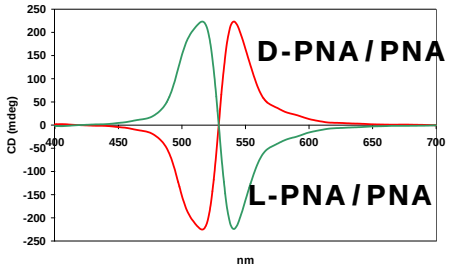


Chirality can be used for controlling orientation of the PNA sequence

Tedeschi, S. Sforza, A. Dossena, R. Corradini, R. Marchelli *Chirality* 2005, *17*, S196-S204

D- and L-"Chiral box" PNAs: helical preference in the antiparallel mode

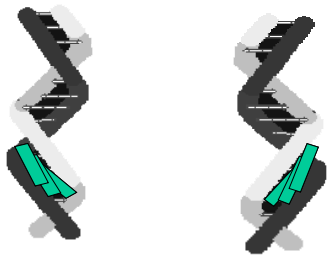
Handedness of the PNA-PNA duplexes with D- and L-chiral box PNAs were investigated by circular dichroism by addition of the cyanine DISC$_2$(5) dye

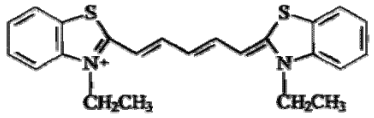


PNA-PNA antiparallel:

N-GTAG**ATC**ACT-C
C-CATCTAGTGA-N

2D-PNA right-handed
2L-PNA left-handed"





DiSC$_2$(5)

Smith et al., *JACS*, 1999, 121, 2686-2695

Tedeschi T., Sforza S., Dossena A., Corradini R., Marchelli R., *Chirality*, 2005, S1, S196-S204

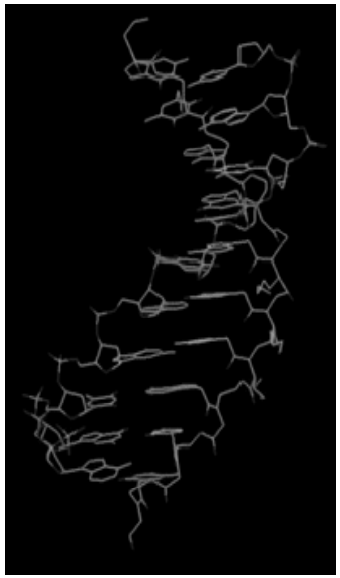
Duplex chiral PNA:DNA structure (X-rays)

First structure obtained
from X-ray diffraction

Twist: 23.2°
Helix pitch: 15.5 bp "P-HELIX"
Rise : 3.5 Å
PNA conformation conserved
(rms = 0.92-1.48 Å with other PNA structures)

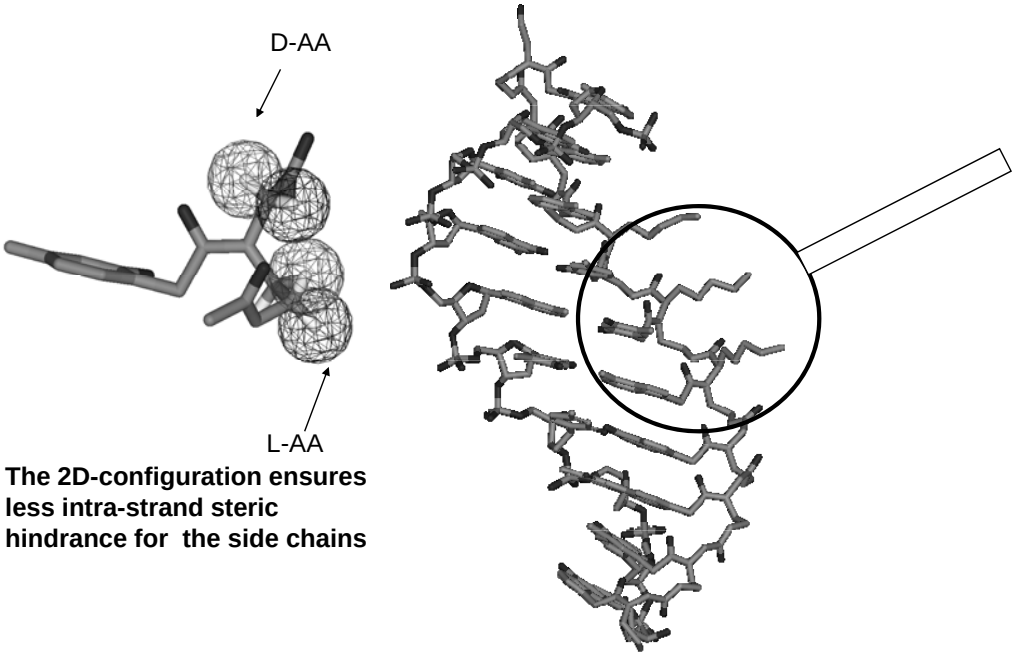
DNA is distorted
(partly in A and partly in B form)

The PNA is acting as a more rigid strand than DNA



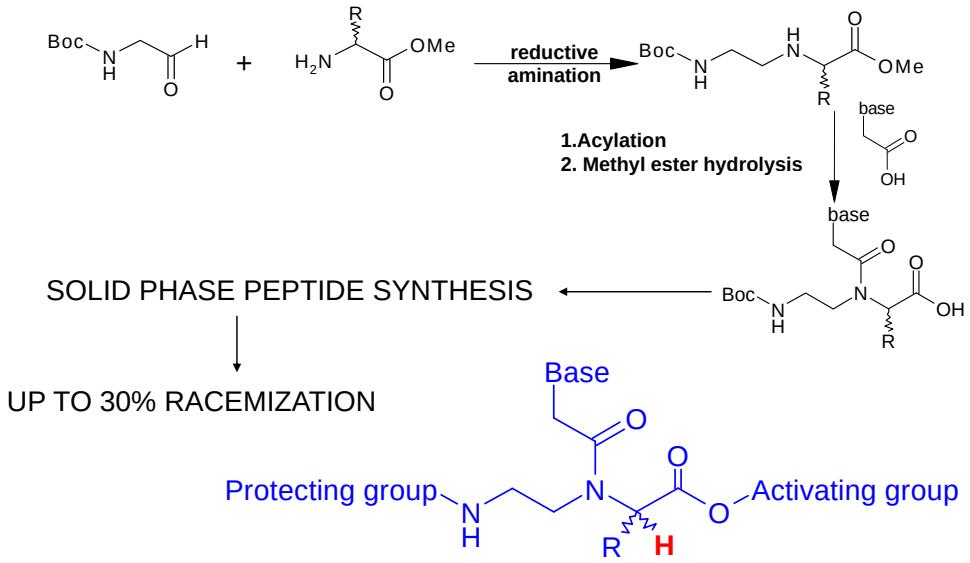
V. Menchise, G. De Simone, T. Tedeschi, R. Corradini, S. Sforza, R. Marchelli, D. Capasso, M. Saviano, C. Pedone *Proc. Natl. Acad. Sci. USA* 2003, *100*, 12021-12026.

Explaining the effect of chirality at the molecular level



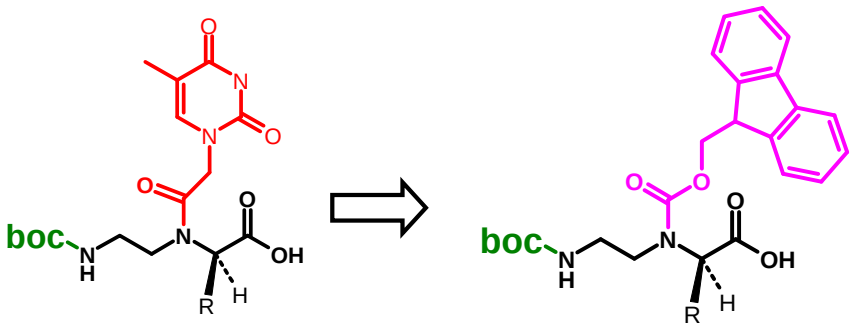
The 2D-configuration ensures less intra-strand steric hindrance for the side chains

The problem of racemization during solid phase synthesis



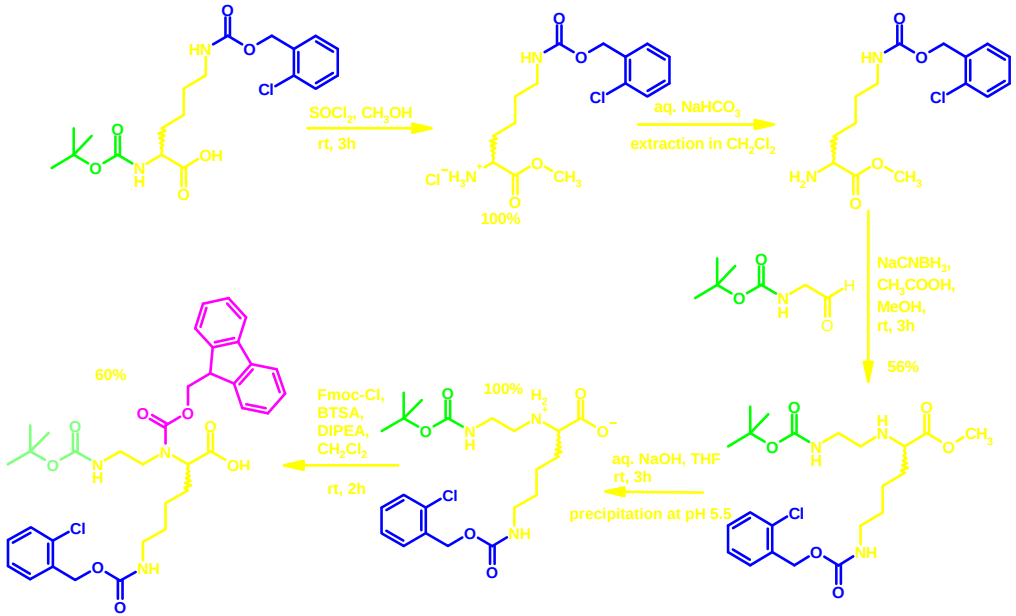
Corradini R., Di Silvestro G., Sforza S., Palla G., Dossena A., Nielsen P.E., Marchelli R.
***Tetrahedron Asymm.*, 1999, 10, 2063-2066.**

Chiral PNA synthesis: the submonomeric approach

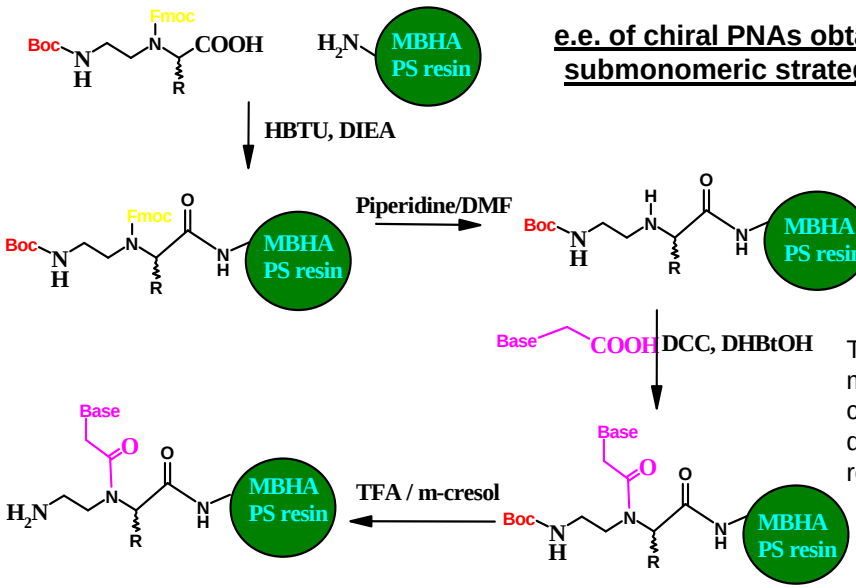


S. Sforza, T. Tedeschi, R. Corradini, D. Ciavardelli, A. Dossena, R. Marchelli
European Journal of Organic Chemistry, **2003**, 1056-1063.

Synthesis of the chiral submonomers with one stereogenic center at C-2



Submonomeric approach: a new synthethic strategy for optically pure chiral PNAs



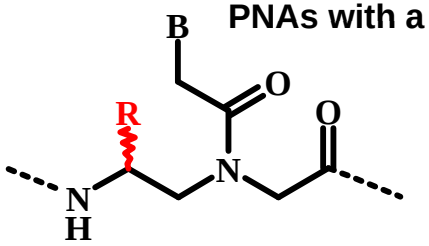
e.e. of chiral PNAs obtained by submonomeric strategy: 98%

The full monomer is completed directly on the resin

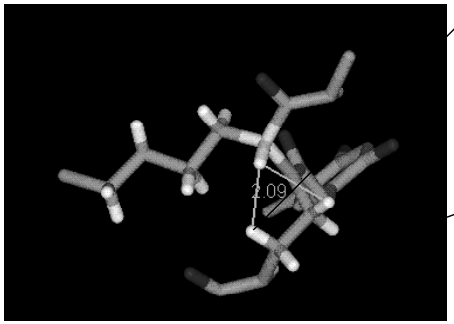
Sforza S., Tedeschi T., Corradini R., Ciavardelli D., Dossena A., Marchelli R., *Eur. J. Org. Chem.*, 2003, 1056-1063

A stereogenic center in a different position

PNAs with a stereogenic center at position C-5



From the PNA-DNA crystal structure it could be inferred that:

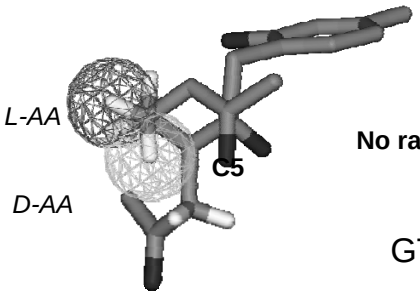


5-pro-D

More steric hindrance in the right-handed helix (much more hindered than in position 2)

5-pro-L

Less steric hindrance in the right-handed helix

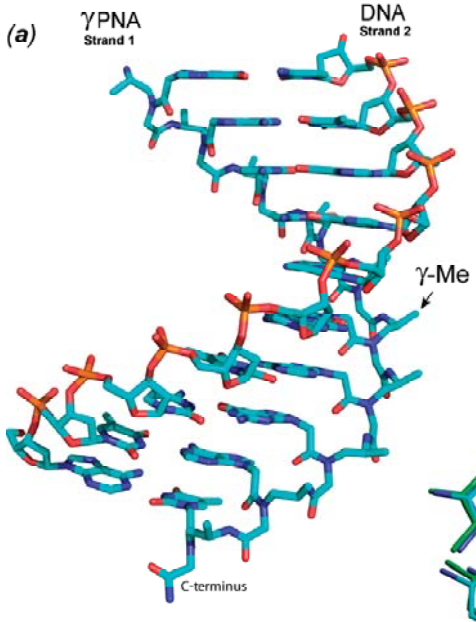


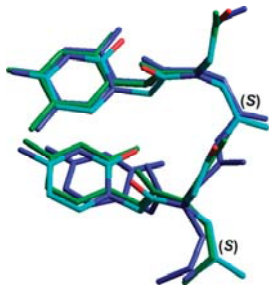
No racemization is involved in the synthesis

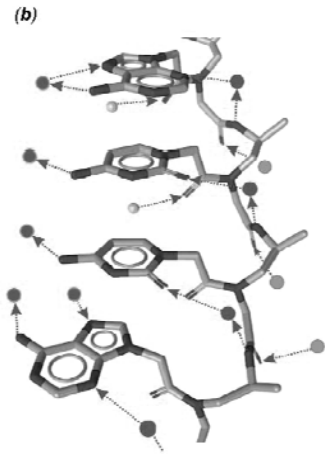
$GTAGAT_{5\text{-}Lys}CACT$

Stereochemistry	PNA:PNA	Tm (PNA:DNA) °C
(5-L)	Right-handed	56
(5-D)	Left-handed	32
Achiral	-	50

Crystal structure (X-rays) of chiral γ-PNA (C5) with DNA







Superposition of PNA strands from the 5L-PNA-DNA (cyan) and 2D-PNA-DNA duplex, both determined by X-ray crystallography (green) and single-strand CT 5-PNA dimer determined by NMR

Yeh, J.I. et al., *JACS*, **2010**, 132, 10717-10727

PNAs with two stereogenic centers

H-GTAGA T$_{(5L\text{-}Lys,2L\text{-}Lys)}$ CACT-NH$_2$

H-GTAGA T$_{(5D\text{-}Lys,2D\text{-}Lys)}$ CACT-NH$_2$

H-GTAGA T$_{(5D\text{-}Lys,2L\text{-}Lys)}$ CACT-NH$_2$

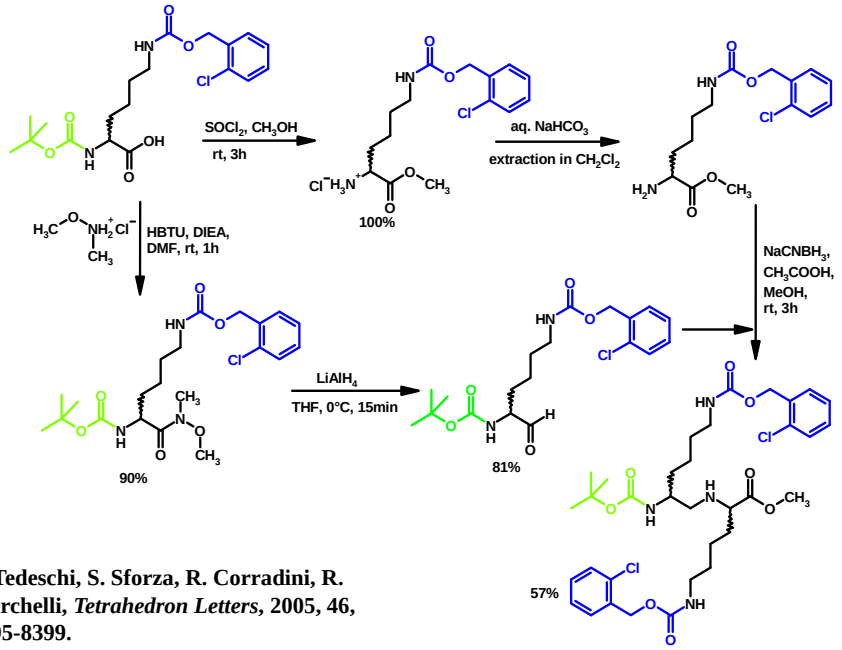
H-GTAGA T$_{(5L\text{-}Lys,2D\text{-}Lys)}$ CACT-NH$_2$

5-Lys

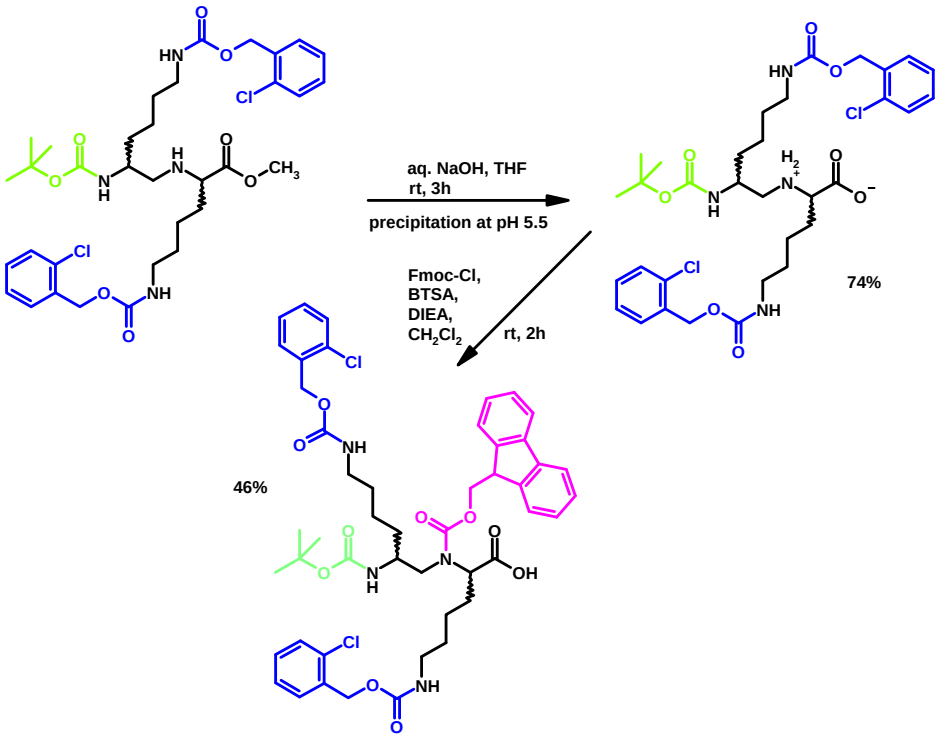
2-Lys

T. Tedeschi, S. Sforza, R. Corradini, R. Marchelli, *Tetrahedron Letters*, 2005, 46, 8395-8399.

Synthesis of the chiral submonomers with two stereogenic centers



T. Tedeschi, S. Sforza, R. Corradini, R. Marchelli, *Tetrahedron Letters*, 2005, 46, 8395-8399.

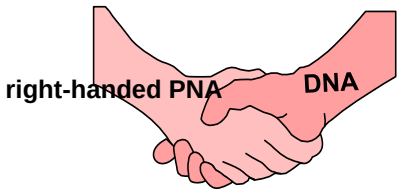


Chiral cooperativity and chiral conflict tune the PNA binding properties

PNA	Influence on the affinity for DNA			Tm
Achiral				50°C
5L,2D	strongly favours+slightly favours	r.h.	accordance	57°C
5L,2L	strongly favours+slightly disfavours	r.h.	conflict	52°C
5D,2D	strongly disfavours+slightly favours	l.h.	conflict	31°C
5D,2L	strongly disfavours+slightly disfavours	l.h.	accordance	<15°C

The PNA-DNA duplex stability is driven by the preferential handedness induced by the stereogenic centers

DNA not only recognizes a specific sequence, but also a specific helix handedness

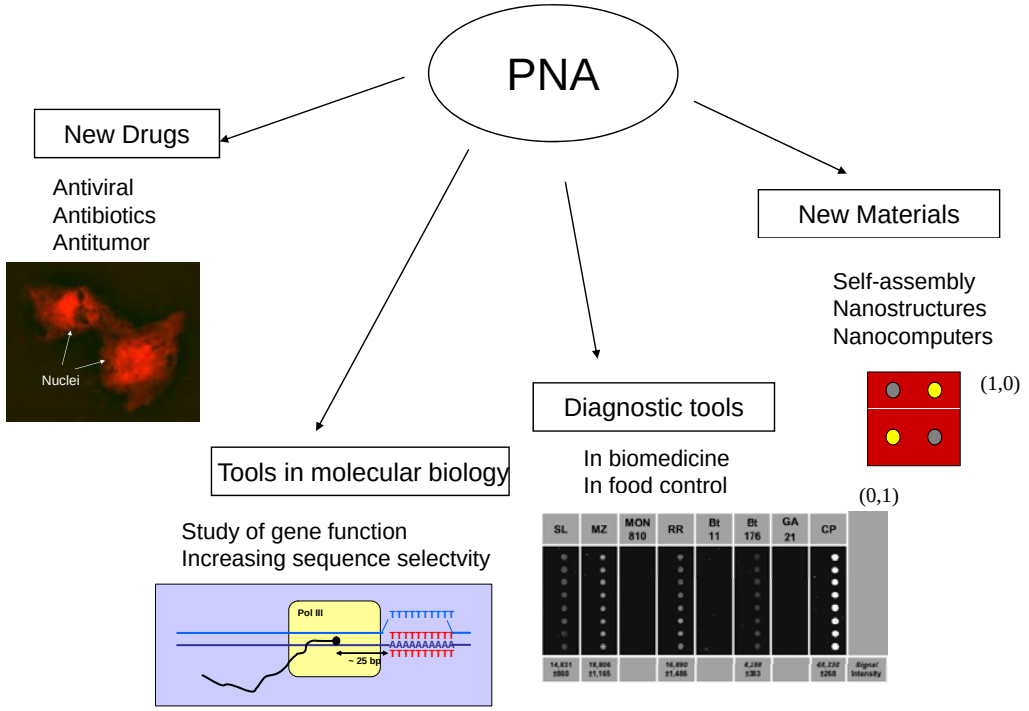


Sforza S., Tedeschi T., Corradini R., Marchelli R. *Eur. J. Org. Chem.*, 2007, 5879-5885.

RNA targeting PNA

T monomer	Tm PNA-DNA (°C)	Tm PNA-RNA (°C)	ΔTm (RNA vs DNA)	ΔTm (chiral vs achiral)
2D,5L	**57**	**67**	+ 10	+ 8
2L,5L	52	64	+ 12	+ 5
2D,5D	33	49	+ 16	- 10
2L,5D	**< 20**	**41**	**> 21**	- 18
5L	56	65	+ 9	+ 6
5D	32	49	+ 17	- 10
2D	52	60	+ 8	+ 1
2L	47	57	+ 10	- 2
achiral	50	59	+9	-

PNA sequence: H-GTAGATCACT-NH_2

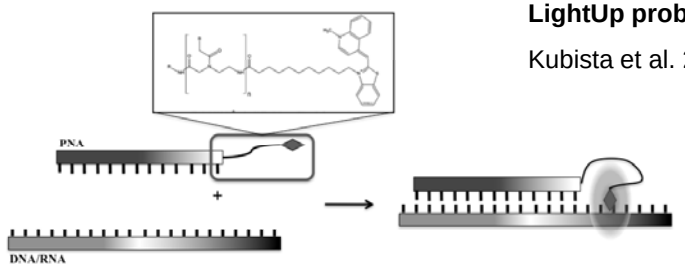


PNA IN DIAGNOSTICS

- **Fluorescence *in situ* hybridization (FISH)**
- **PCR clamping**
- **Real-time PCR**
- **Light Up and FIT probes**
- **Capillary electrophoresis**
- **MALDI-TOF mass spectrometry**
- **Electrochemical biosensors**
- **Quartz crystal microbalance (QCM)**
- **Microarrays**
- **Surface-plasmon resonance biosensors**

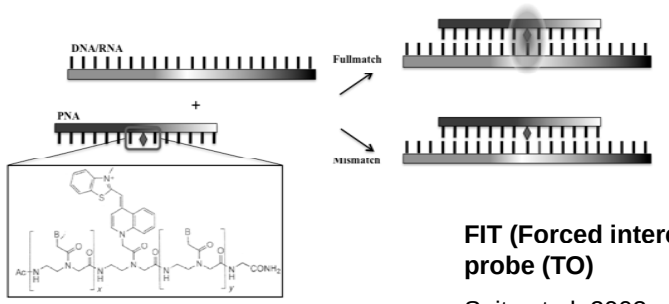
Food **Biomedicine**

Florescence switch-on probes (Thiazole Orange)



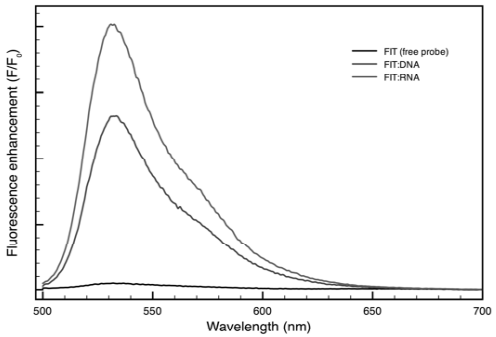
LightUp probe (TO)

Kubista et al. 2000

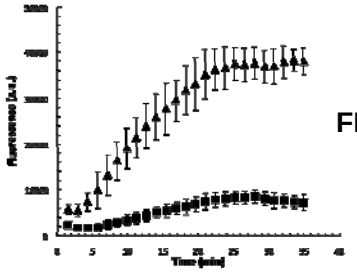


FIT (Forced intercalation) probe (TO)

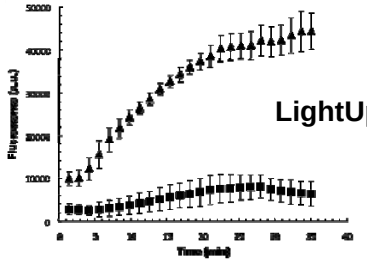
Seitz et al. 2003



Fluorescence emission spectra of FIT-NorovirusGII-PNA in the presence of complementary DNA (blue line) and of complementary RNA (red line)



FIT



LightUp

Real time detection of **RNA transcription**: the fluorescence signals of **FIT_NoV_GII** and **LightUp_NoV_GII** in the presence of ▲=Norovirus and ■= Rotavirus.

Authenticity:

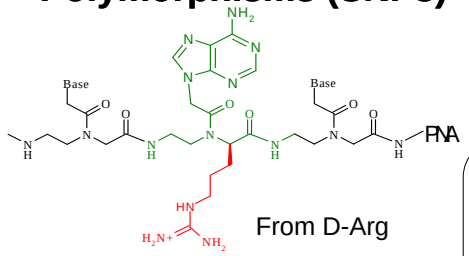
Olive oil cultivars

Cultivar	60	120	183	198	345
Biancolilla	A	A	G	C	G
Canino	A	R	R	S	G
Carolea	A	A	G	C	G
Coratina	A	A	G	C	G
Frantoio	A	A	G	C	G
Leccino	A	A	G	C	R
Nocellara belice	A	A	G	C	R
Ogliarola leccese	T	A	G	C	G
Moraiolo	A	R	R	C	G
Bosana	A	R	R	C	G
Nocellara etnea	A	A	G	C	R
Arbequina	A	A	G	C	G

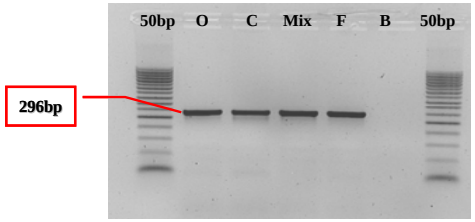
SNPs in the Actin gene

R = T o C
S = G o C

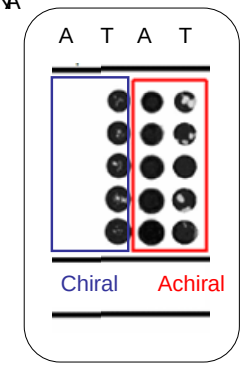
Olive cultivar identification by Single Nucleotide Polymorphisms (SNPs)



PCR amplified DNA



PNA microarray



Ogliarola (T)

Canino Frantoio(A)

Double stranded DNAs were obtained by amplification of different genomic DNA, extracted from olive leaves. These PCR products were used as templates for an unbalanced PCR in order to obtain a single stranded DNA labeled with Cy5. O: Ogliarola leccese; C: Canino; Mix: 50% Ogliarola, 50% Canino; F: Frantoio; B: Blank; 50bp: marker.

In collaboration with S. Arcioni and L. Baldoni (CNR-Perugia)

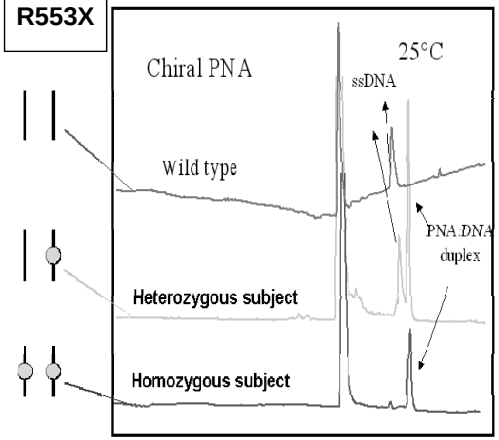
Detection of Cystic Fibrosis of R553X and of W1282X in the CFTR gene with "CHIRAL-BOX"D- Lys- PNA

No mutation (healthy subject)

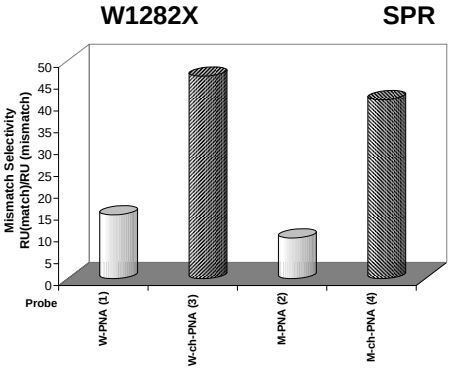
Heterozygous (healthy carrier) 1:25

Homozygous Disease 1: 25000

CE R553X



W1282X **SPR**



M. Chiari, G. Galaverna, S. Sforza, M. Cretich, R. Corradini, R. Marchelli *Electrophoresis* 2005, *26,* 4310–4316

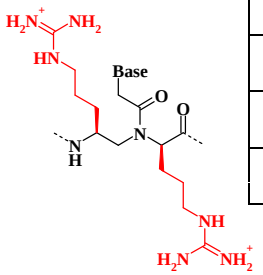
R. Corradini, G. Feriotto S. Sforza, R. Marchelli, R. Gambari *J. Mol. Rec.* 2004 *17, 76-84* .

Arginine-based PNA microarrays for the genomic analysis of SNPs linked to the Alzeheimer disease

Oligonucleotides simulating the SNPs normally found in the APOE gene

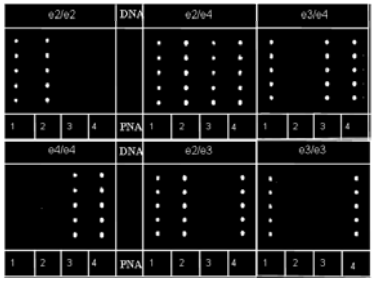
1': 5'-ACGTG**T**GCGGC-3'
2': 5'-AGAAG**T**GCCTG-3'
3': 5'-ACGTG**C**GCGGC-3'
4': 5'-AGAAG**C**GCCTG-3

PNAs with a modified monomer in the middle used to build up a microarray



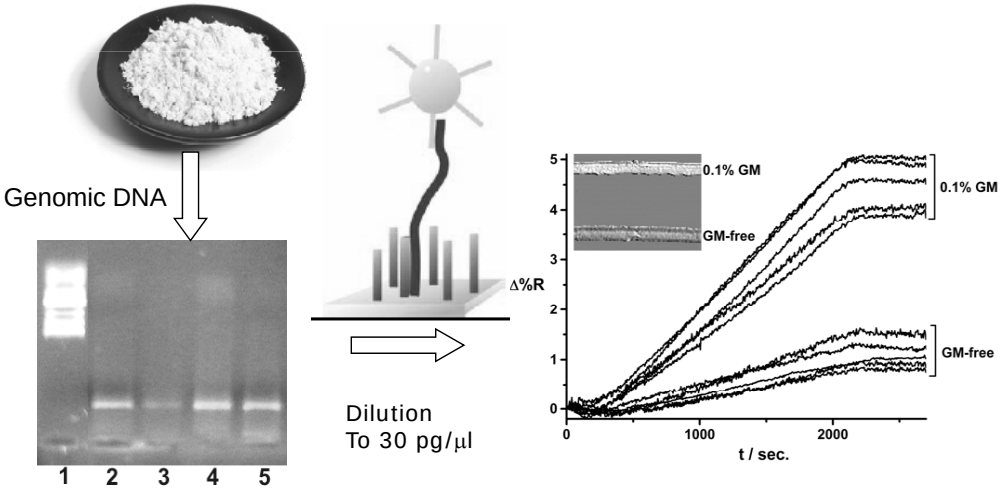
H-(AEEA)$_2$GCCGC**A**CACGT-NH$_2$
H-(AEEA)$_2$CAGGC**A**CTTCT-NH$_2$
H-(AEEA)$_2$GCCGC**G**CACGT- NH$_2$
H-(AEEA)$_2$CAGGC**G**CTTCT-NH$_2$

Genotypes related to ApoE mutations	
Genotype	Oligonucleotide simulation[a]
ε2/ε2	**1' + 2'**
ε2/ε4	**1' + 2' + 3' + 4'**
ε3/ε4	**1' + 3' + 4'**
ε4/ε4	**3' + 4'**
ε2/ε3	**1' + 2' + 4'**
ε3/ε3	**1' + 4'**



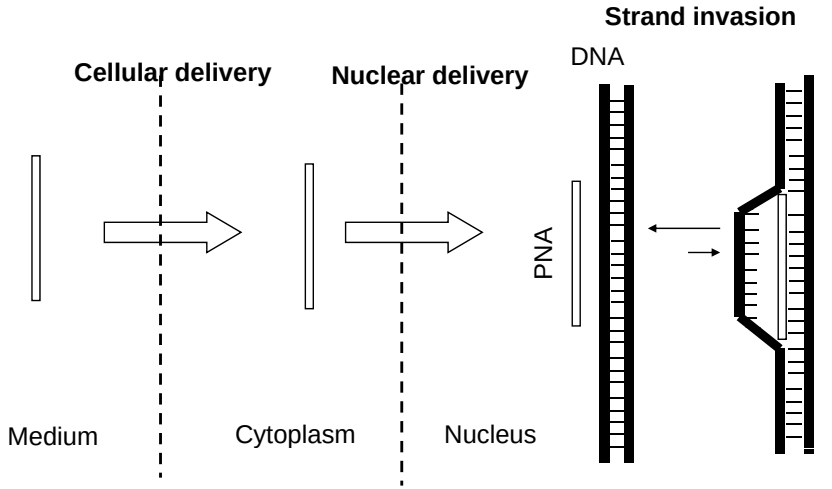
Calabretta A., Tedeschi T., Di Cola G., Corradini R., Sforza S., Marchelli R. *Molecular Biosystems*, 2009, 2009, 5, 1323–1330

PCR-free detection of genomic DNA (10pg/μl) of GM Soy by Nanoparticle-enhanced Surface Plasmon Resonance Imaging



It is possible to detect non-amplified genomic DNA down to a 41 zM concentration (4.1 x 10^{-20} M) corresponding to 18 yoctomoles (about 11 copies of the target)

PNA as drugs: Anti-gene PNA?

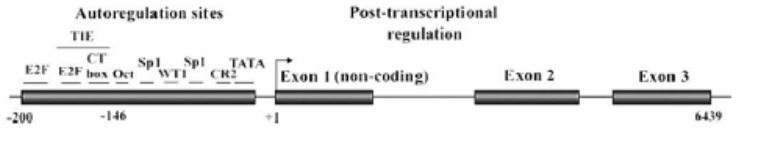


Difficult to perform with mixed sequences

Increasing uptake

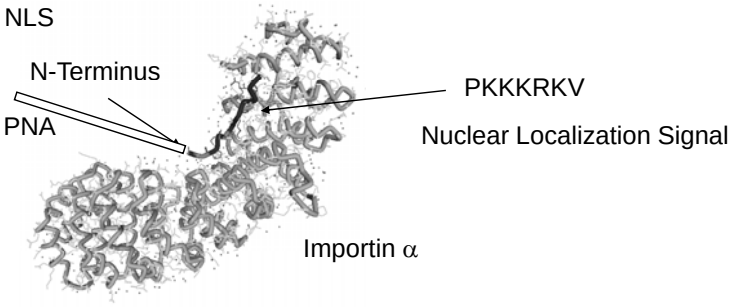
- Conjugation with peptides
- Conjugation with cellular receptor ligands
- Co-transfection with oligonucleotides
- Use of peptidic transfecting agents
- Use of PNA with modified backbones (embedded peptides)

MYCN



- **The *MYCN* gene is a member of the Myc family**
- **MYC proteins are transcription factors. Unlike MYC, MYCN is expressed only in early stages of embryonic development**
- **In Neuroblastoma, MYCN overexpression is associated with rapid progression and poor prognosis.**

Antigene PNA with NLS

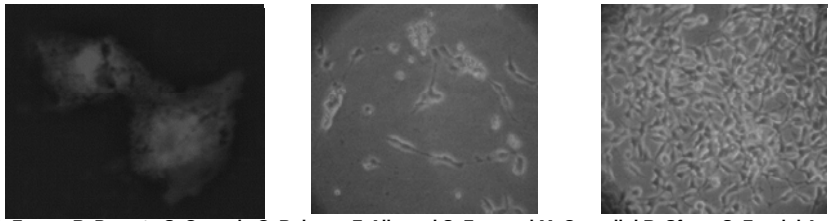


ANTITUMOR ANTI-GENE PNA

Neuroblastoma

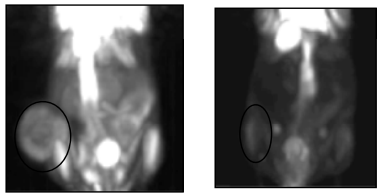
(Nuclear Localization Signal)

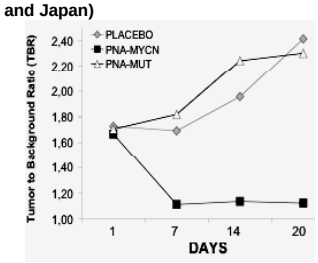
Nuclear uptake | PNAs-NLS | CTRL



Tonelli R, Fronza R, Purgato S, Camerin C, Bologna F, Alboresi S, Franzoni M, Corradini R, Sforza S, Faccini A, Shohet JM, Marchelli R, Pession A. *Molecular Cancer Therapeutics*, 2005, *4*, 779-86.
Pession A., Tonelli R., Fronza R., Marchelli R., Corradini R., Sforza S.
European Patent nr. 04730318.5-2406-IB2004001297 (also extended to USA, Canada and Japan)

Rhabdomyosarcoma

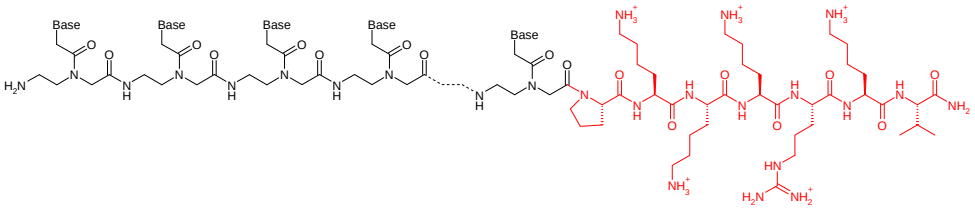




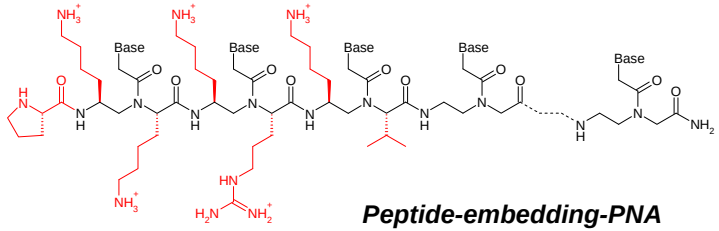
The PNA has no effect on cells not overexpressing MYCN

PNA simultaneously mimicking nucleic acids and peptides

Insertion of the peptide sequence INTO the backbone will make this sequence resistant to degradation (if the pseudopeptidic backbone can mimic the natural peptide)



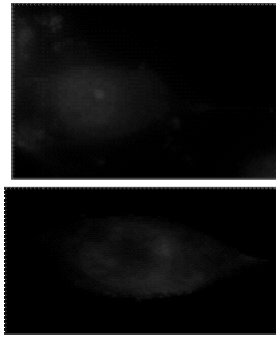
PNA-NLS



Peptide-embedding-PNA

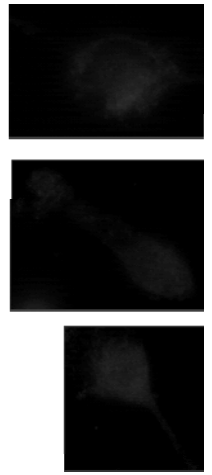
A new perspective on chiral PNAs: nucleic acid mimic AND peptide mimic

Standard NLS peptide is able to enter the cell nuclei….



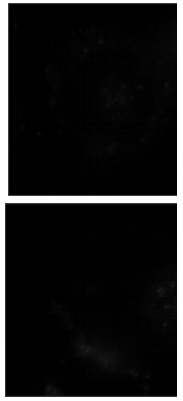
Standard NLS peptide

… and so does the modified PNA embedding the NLS sequence….



PNA embedding into the backbone a pseudopeptide mimic of NLS

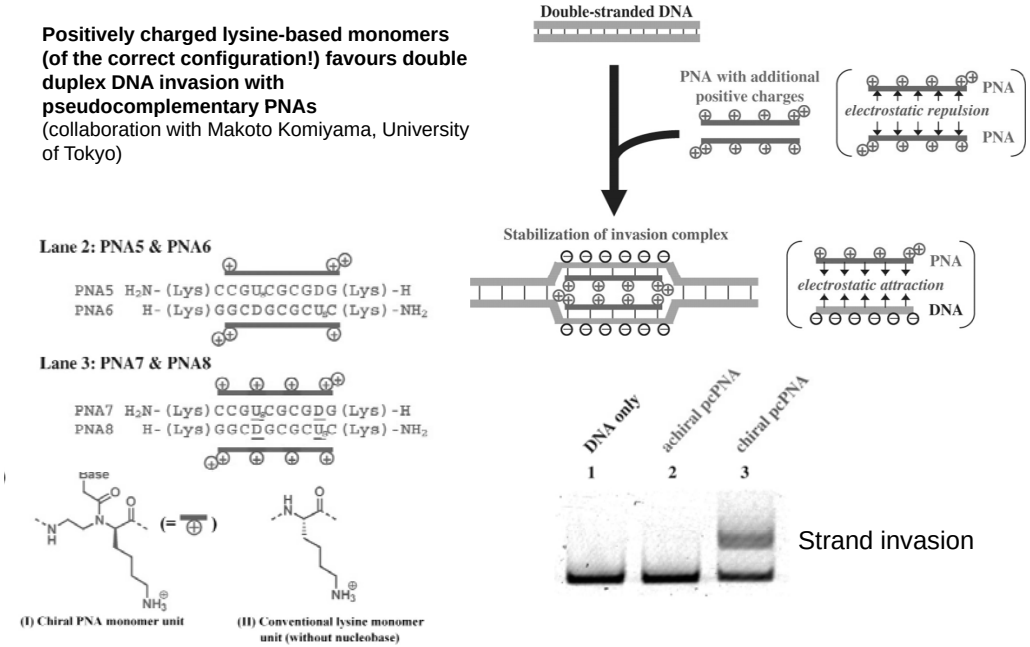
… but other chiral PNAs without the NLS sequence into the backbone can not.



PNA embedding into the backbone an **incomplete** pseudopeptide mimic of NLS

Strand invasion of duplex DNA

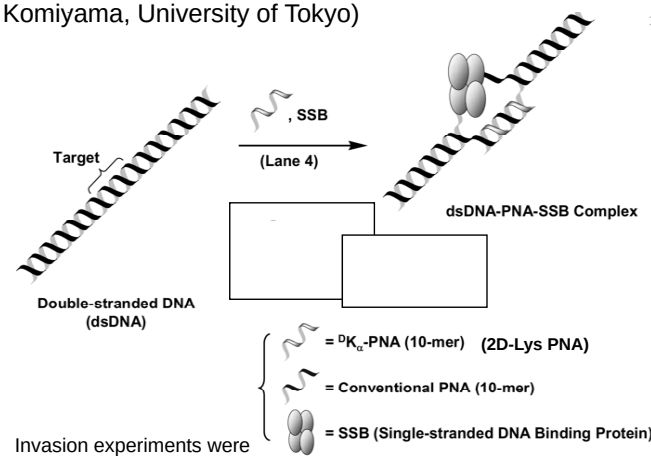
Positively charged lysine-based monomers (of the correct configuration!) favours double duplex DNA invasion with pseudocomplementary PNAs
(collaboration with Makoto Komiyama, University of Tokyo)



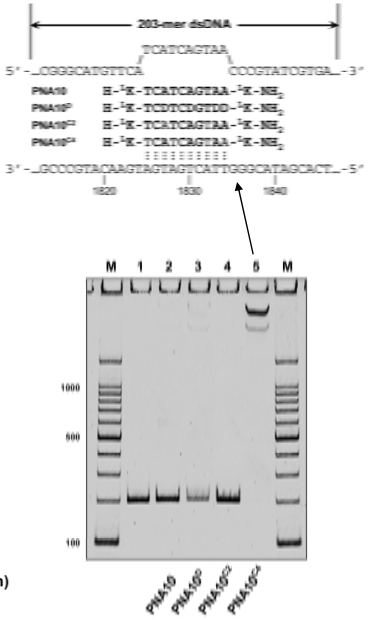
Ishizuka T.,Yoshida J., Yamamoto Y., Sumaoka J., Tedeschi T., Corradini R., Sforza S., Komiyama M. *Nucleic Acids Res.*, 2008, 36, 1464-147

Exploiting the chirality effects: strand invasion of duplex DNA

A different approach: invasion assisted by Single Strand Binding protein (SSB)
(collaboration with Makoto Komiyama, University of Tokyo)



Invasion experiments were made for 2 hr at 37°C



Ishizuka T., Tedeschi T., Corradini R., Komiyama M., Sforza S., Marchelli R. *ChemBioChem*, 2009, *10*, 2607 – 2612

Advantages offered by PNA

- Possibility to target dsDNA through invasion
- Possibility to block transcription
- Possibility to discriminate RNA from DNA
- High sequence selectivity
- High chemical stability
- High enzymatic stability
- Direct permeation on several cell types (e.g. neurons)
- Easy coupling with peptide moieties
- Large variety of conjugated
- Possibility to act as nucleic acid and peptide mimics

Limitations

- Uptake is low in many cell lines
- PNA is not able to activate enzymatic processes
- Cost is still high
- Only few pharmacokinetic studies are reported
- Very few toxicity studies have been performed