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**Mimicking the action of ribonucleases:  
studies on RNase A and design of PNA  
based artificial enzymes**

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Mimicking the action of ribonucleases:  
studies on RNase A and design of PNA based artificial  
enzymes  
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*With love to my family,  
lontani ma sempre vicini...*



## ABSTRACT

A 3'-deoxy-3'-C-methylenephosphonate modified diribonucleotide is highly resistant to degradation by spleen phosphodiesterase and not cleaved at all by snake venom phosphodiesterase. Despite that both the vicinal 2-hydroxy nucleophile and the 5'-oxyanion leaving group are intact, the 3'-methylenephosphonate RNA modification is also highly resistant towards the action of RNase A.

Several different approaches were explored for conjugation of oligoethers to PNA with internally or N-terminal placed diaminopropionic acid residues. Using a post PNA-assembly procedures oligoether attachment to both N-terminal and sidechain amino groups was achieved. Use of a new oligoether functionalized amino acid allows inclusion of oligoether conjugates during on-line machine assisted synthesis, allowing combination of methods for attachment of different oligoethers and co-conjugation of neocuproine cleaver.

We have previously shown that PNA-neocuproine conjugates can act as artificial RNA restriction enzymes (PNAzyme). In the present study we have additionally conjugated the PNA with different entities and also constructed systems where the PNA is designed to clamp the target RNA forming a triplex. Some conjugations are detrimental for the activity while most are silent which means that conjugation can be done to alter physical properties without losing activity. Conjugation with a single oligoether close to the neocuproine does enhance the rate almost two folds compared to the system without the oligoether. The systems designed to clamp the RNA target by forming a triplex are effective if the clamping part is not too long. Changing the direction of a closing base pair, from a GC to a CG pair, enhances the rate of cleavage with a clamping PNAzyme and without compromising the selectivity, leading to the so far most efficient artificial nuclease reported.

Tris(2-aminobenzimidazole) conjugates with antisense oligonucleotides are effective site-specific metal-free RNA cleavers. Here we investigate conjugates with peptide nucleic acids (PNA). In a first study we show that RNA degradation occurs with similar rates and substrate specificities as in experiments with DNA conjugates. In a second study we show that tris(2-aminobenzimidazole) based artificial nucleases cleave RNA substrates, which form a bulge upon binding to the PNA, with turnover of substrate and a cleavage rate that is also dependent on the bulge sequence. Two methods of analysis for the kinetics, based on IE-HPLC separation of oligonucleotide fragments and analysis of Cy5-labelled oligonucleotide fragments by denaturing PAGE on a DNA sequencer respectively are also compared.

To be able to target microRNAs also at stages where these are in a double stranded or hairpin form we have looked at BisPNA designed to clamp the target and give sufficient affinity to allow for strand invasion. We show that BisPNA complexes are more stable with RNA than with DNA. In addition, 24-mer BisPNA (AntimiR) constructs form complexes with a hairpin RNA that is a model of the microRNA miR-376b, suggesting that PNA-clamping may be an effective way of targeting microRNAs.

## LIST OF SCIENTIFIC PAPERS

- I. **Alice Ghidini**, Charlotte Ander, Anna Winqvist and Roger Strömberg;  
An RNA modification with remarkable resistance to RNase A.  
*Chem. Commun.*, 2013, 49, 9036
- II. **Alice Ghidini**, Peter Steunenberg, Merita Murtola and Roger Strömberg;  
Synthesis of PNA Oligoether Conjugates.  
*Molecules*, 2014, 19, 3135-3148
- III. Friederike Danneberg, **Alice Ghidini**, Plamena Dogandzhiyski, Elisabeth Kalden, Roger Strömberg and Michael W. Göbel;  
Sequence-specific RNA cleavage by PNA conjugates of the metal-free artificial ribonuclease tris(2-aminobenzimidazole).  
*Beilstein J. Org. Chem.* 2015, 11, 493–498
- IV. **Alice Ghidini**, Helen Bergquist, Tanel Punga, Rula Zain and Roger Strömberg;  
Clamping of RNA with PNA enables targeting of microRNA.  
*Manuscript*
- V. **Alice Ghidini**, Merita Murtola and Roger Strömberg;  
Influence of conjugation and other structural changes on the activity of Cu<sup>2+</sup> based PNAzymes.  
*Manuscript*
- VI. Plamena Dogandzhiyski<sup>§</sup>, **Alice Ghidini**<sup>§</sup>, Friederike Danneberg, Roger Strömberg, Michael W. Göbel;  
Studies on tris(2-aminobenzimidazole)-PNA based artificial nucleases and comparison of two analytical techniques.  
*Manuscript*

<sup>§</sup> Equal contribution.



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## LIST OF ABBREVIATIONS

2'-OMe	2'- <i>O</i> -methyl
A	Adenosine
Ac	Acetyl
AEEP	9-Amino-4,7-Dioxanonanoic acid
AS-ON	Antisense oligonucleotide
Bz	Benzoyl
C	Cytidine
CDI	1,1'-Carbonyldiimidazole
Dap	Diaminopropionic acid
DCM	Dichloromethane
DIC	Diisopropyl carbodimide
DNA	Deoxyribonucleic acid
Fmoc	9-fluorenylmethoxycarbonyl
G	Guanosine
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HBTU	<i>O</i> -(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEPES	<i>N</i> -(2-hydroxyethyl)piperazine <i>N'</i> -(2-ethanesulphonic acid)
HPLC	High performance liquid chromatography
HOBt	Hydroxybenzotriazole
IE-HPLC	Ion exchange HPLC
LNA	Locked nucleic acid
M-BCR/ABL	Major brakepoint cluster region/Abelson tyrosine kinase
Me	Methyl
MES	2-( <i>N</i> -morpholino)ethanesulfonic acid
miRNA	micro RNA
MOE	Methoxy ethyl

mRNA	Messenger RNA
MS	Mass spectroscopy
Mtt	4-methyltrityl
NMP	<i>N</i> -methylpyrrolidone
OBAN	Oligonucleotide based artificial nuclease
PEG	Poly(ethylene glycol)
PNA	Peptide nucleic acid
PNAzyme	Peptide nucleic acid based enzyme
pre-mRNA	Precursor mRNA
PS	Phosphorothioate
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
RP-HPLC	Reverse phase HPLC
siRNA	small interfering RNA
T	Thymidine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIS	Triisopropylsilane
T <sub>m</sub>	Thermal melting temperature
U	Uridine
UCH <sub>2</sub> pG	guanosine 5'-(uridine 3'-deoxy-3'-C-methylenephosphonate
UpG	Uridyl-3', 5'-guanosine

# 1 Introduction

## 1.1 RNA: knowing the target

Deoxyribonucleic acids (DNA), Ribonucleic acids (RNA) and proteins are three major biological macromolecules essential for all forms of life. The genetic information in a cell is in general flowing from DNA through RNA to proteins, “DNA makes RNA makes protein”. Whereas DNA (deoxyribonucleic acid) is considered the first transcript of the cell, where all the genetic information are stored, RNA has the function to transfer the genetic code from the nucleus to the ribosome in order to produce the proteins. Therefore RNA represents an important control point for regulating protein production.

RNA and DNA are polymers where the nucleosides, in general, are linked by 3',5'-phosphodiester bonds. Even if their chemical structure is similar, the presence of the 2'-hydroxy function of the ribonucleic acid makes the phosphodiester bonds of RNA chemically and enzymatically less stable than in DNA. The hydroxyl group has a predisposition for intramolecular nucleophilic RNA cleavage.[1, 2] Phosphodiester linkages, for both RNA and DNA, are highly resistant to spontaneous hydrolysis. In 2005 Schroeder et al. determined the rate of spontaneous hydrolysis of dineopentyl phosphate (Np2P).[3] The dineopentyl phosphate anion undergoes hydrolysis in water with a  $t_{1/2}$  of 30,000,000 years at 25°C, furnishing an indication of the resistance of the internucleotide linkages of DNA to water attack at phosphorus. Whereas for RNA, the calculated half-life for the cleavage of a single phosphodiester linkage is around 100 years at pH 6.[4]

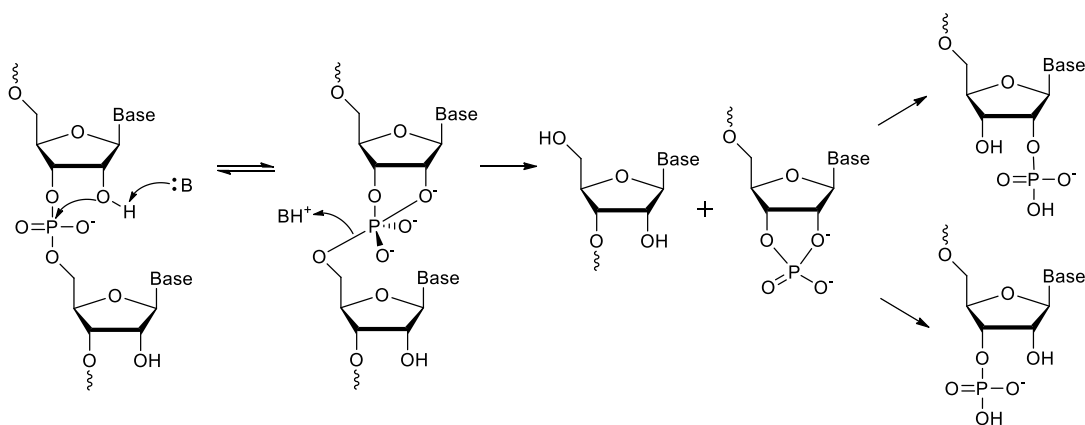


Figure 1: Cleavage of phosphodiester bond in RNA.

The cleavage of the phosphodiester bond in RNA is usually initiated by the 2'-hydroxy group which is acting as a nucleophile attacking the phosphate linkage.[5] The reaction of transesterification proceeds then through the formation of a phosphorane intermediate (or phosphorane-like transition state), characterized by a pentacoordinated phosphorus (Figure 1). The cyclic phosphate formed is then hydrolyzed. In studies of ribozymes and deoxyribozymes, it has been estimated that both the deprotonation of the attacking nucleophile (2'-OH) and protonation of departing nucleophile (5'-OR) can accelerate the reaction by a factor of up to 106.[6] Protein enzymes and ribozymes that cleave RNA via the transesterification reaction,

proceed via an in-line  $SN_2$ -like mechanism, accommodating an in-line conformation at the site of cleavage.[7] It has been established that this in-line orientation provides a minimum of 10- to 20-fold rate enhancement for the cleavage of RNA by an intramolecular transesterification mechanism.[8]

## 1.2 Enzymatic cleavage of RNA by nucleases: Phosphodiesterases (PDEs) and RNase A

Phosphodiesterases (PDEs) are a class of enzyme which catalyse the reaction of cleavage of the phosphodiester linkage in a RNA strand and are the major cause of the lability of the oligonucleotides *in vivo*. PDEs are divided into subclasses I, II, and III. [9] Phosphodiesterases can be classified also as exonucleases or endonucleases, and in both cases the product of the cleavage is the 3'-phosphomonoester or the 5'-phosphomonoester (Figure 2).

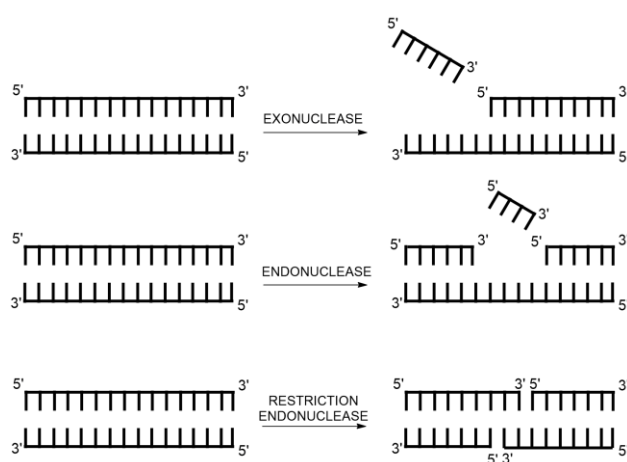


Figure 2: Exo and endo nucleases action on double stranded DNA.

The Snake Venom Phosphodiesterases (commonly known as SVPD) and Spleen Phosphodiesterase PDE II are two of the most commonly used exonucleases. The Snake Venom Phosphodiesterases are 3'-exonucleases of the class PDE I which cleaves the phosphate linkage from the 3' end of a nucleic acid strand with the 3'-O as leaving group and leaving a 5'-phosphate.[10] Its optimal pH is 9. It can attack both DNA and RNA. Spleen Phosphodiesterase PDE II is a membrane-bound glycoprotein that is used to catalyze the hydrolysis of various nucleotide polyphosphates, with an optimal pH at 4.8. It is a 5'-exonuclease that attacks both ribo- and deoxyribopolynucleotides by removal of 3'-mononucleotides in a stepwise and random fashion from the terminus bearing an unsubstituted 5'-hydroxyl group.

There are three major classes of intracellular RNA-degrading enzymes (ribonucleases or RNases): endonucleases that cut RNA internally, 5'-exonucleases that hydrolyze RNA from the 5'-end, and 3'-exonucleases that degrade RNA from the 3'-end. Endo and 3'-exonucleases have been characterized in all domains of life, whereas 5'-exonucleases were, until recently, believed to be absent from bacteria.[11]

### 1.3 Ribonucleases: RNase A and its RNA cleavage mechanism

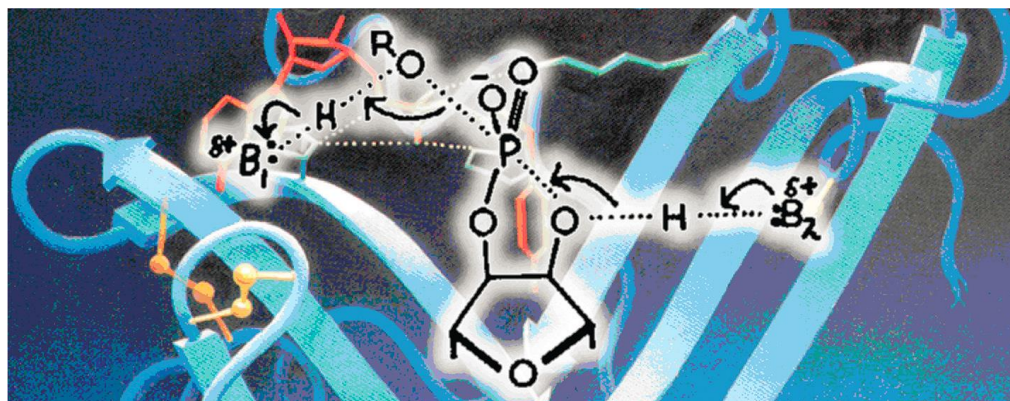


Figure 3: Raines's revisited representation of Rabin and Mathias RNase A mechanism drawn. Reprinted with permission from Biochemistry. Copyright (2011) American Chemical Society.[12]

Four years ago an historical review written from Ronald Raines et al. came out in the Biochemistry Journal.[12] 2011 was the year of the 50<sup>th</sup> anniversary of the first hypothesized mechanism for catalysis by a ribonuclease enzyme, RNase A.[13] RNase A was the first enzyme that could be purified in large amounts and crystallized by Kunitz in 1940 [14] and the first enzyme whose 124 amino acid sequence was determined.[15] This and the small molecular size (14 kDa) made the RNase A one of the most useful model for the studies in all protein research for many years.[14, 16] However, the nature of the RNase A substrate, RNA, has always been an obstacle, for the enzymologists aiming to rationalize the kinetic analysis, because of its excessive lability in presence of the enzyme.

In 1961 Mathias and Rabin from University College in London, revealed the suggestion for the still unknown catalytic mechanism of RNase A.[13] The first proposed mechanism of RNA cleavage was divided in 2 steps: a reversible transphosphorylation step in which a 2',3'-cyclic nucleotide intermediate is formed at the 3'-terminus of one product and a 5'-hydroxyl group appearing at the 5'-terminus of the other product, followed by an irreversible hydrolysis of the 2',3'-cyclic nucleotide to a 3'-nucleotide. One year after the Nature paper [13], Mathias and Rabin published a series of five papers in Biochemical Journal where they showed the experiments in support of the formulated mechanism.[17-21] This mechanism of RNA cleavage involves a general acid–base catalysis which is promoted by two histidine residues, (Figure 4).

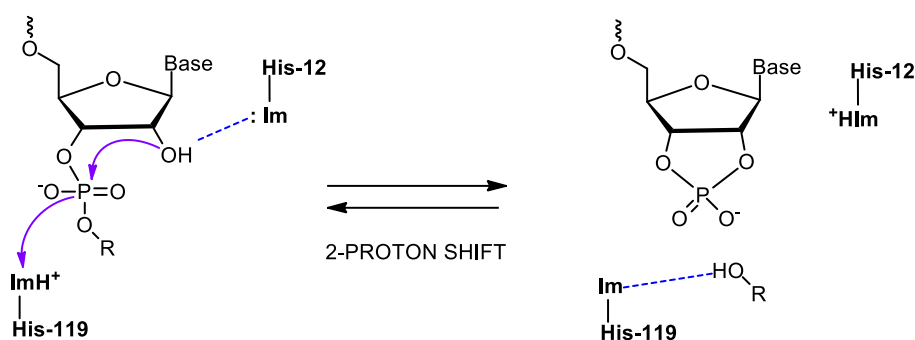


Figure 4: First mechanism hypothesized by Mathias and Rabin in 1961.

Five years later, Mathias and Rabin proposed a more refined structure of the transition state.[22] They showed for the first time a cationic acid, corresponding to the ammonium group of the side chain of a lysine, which has the function of stabilizing the charge on the nonbridging oxygens of the phosphoryl group by Coulombic interactions. This theory was confirmed 30 years later first by Benner's group and successively by Raines's group through site-directed mutagenesis.[23, 24] Messmore *et al.* demonstrated that the variant enzyme with the Lys41 replaced by an Arg shows in fact only a 2% of the activity of the wild-type enzyme.[23, 24]

Initially the hypothesized RNase A mechanism didn't convince all researchers in the field and this was partially due to the less advanced techniques of the time. Many secondary theories started to grow and among the alternative mechanisms suggested, those of Witzel,[25] Wang,[26] and Breslow[27, 28] were the most relevant. In the Witzel mechanism, only one histidine is acting in the cleavage mechanism, and the role of the second histidine is instead covered by the oxygen O2 of the pyrimidine nucleobase, whereas the Wang mechanism involves a charge-relay system which is similar to the one of chymotrypsin. The theory mooted by Breslow and his group is the formation of a pentacoordinated phosphorane intermediate and a second transition state on the path from substrate to product (Figure 5).

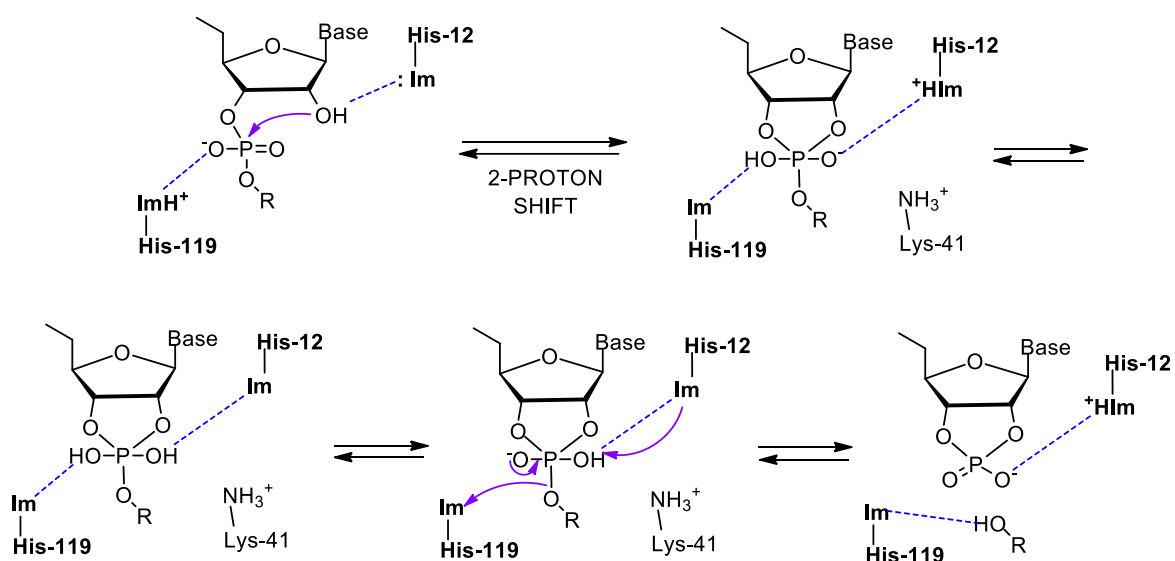


Figure 5: RNase A mechanism hypothesized by Breslow with the formation of a pentacoordinated intermediate.[28]



### *1.3.1 RNase A substrate specificity.*

RNase A is divided in three subsites: B1, B2, and B3.[29] The B1 subsite binds specifically to a pyrimidine nucleotide which, in turn, is bound to 5'-phosphate of the second nucleotide. The B1 subsite binds selectively only residues with a pyrimidine base. The hydroxyl group of the side chain and carbonyl group of the main chain of Thr45 present in B1 lead to the pyrimidine specificity of RNase A, creating sterical hindrance for a purine base. B2 and B3 subsites bind all residues. B2 shows a slight preference for nucleotide with an adenine base and B3 has some preferences for those with purine base. When comparing CpX substrates with the corresponding UpX, RNase A cleavage rate is 2 fold faster, whereas a poly(C) substrate is cleaved approximately 20 fold faster than poly(U). RNase A will also catalyze the cleavage of poly(A), but, due to sterical hindrance, the rate of reaction drops to values that are  $10^3$  less than for the cleavage of poly(U). Site-directed mutagenesis has been used to create variants that cleave efficiently at the 3'-end of purine containing residues.[30]

## **1.4 Cleavage of RNA promoted by antisense oligonucleotides and artificial ribonucleases**

### *1.4.1 Oligonucleotides as RNA targeting therapeutics.*

Therapeutic oligonucleotides usually regulate the function of a targeted RNA (or DNA) and typically include one or more chemical modifications at the phosphate, sugar or base level. Modifications are introduced in order to increase affinity and selectivity towards the RNA (or DNA) target, to increase resistance to degradation by exo- and endonucleases, as well as to change other properties that enhance the therapeutic action. Oligonucleotide therapeutics are 8 to 50 long polymers which are designed to bind to a specific target, usually by Watson-Crick base pairing.[31] The block of the function of the targeted RNA can be achieved through a translation block which can be or not followed by an endogenous enzyme degradation, RNase H, (antisense oligonucleotides) or degradation of the RNA using catalytic oligonucleotides (ribozymes, artificial ribonucleases, DNazymes).

Oligonucleotides that work through an RNA interference mechanism are another example of potentially therapeutics that induces cleavage of the target RNA through an endogenous enzyme (39). In this case, the enzyme responsible for cleavage of the RNA in human cells is Argonaute 2 (Ago 2), which cleaves the RNA through an RNase H-like enzyme mechanism (40, 41). Oligonucleotides that work through the RNA interference (siRNA) mechanism appear to mimic endogenous small RNAs present in cells, which naturally regulate expression of the targeted gene.

Even after more than 30 years of research in the field, the biggest challenges in the use of oligonucleotides as therapeutics remain (i) the intracellular enzymatic degradation, (ii) the difficult delivery through the membrane of the target cells, and (iii) the selectivity towards the

target.[32] In order to solve these major problems many different modified oligonucleotides were developed.[33] These chemically changed oligonucleotides are often classified in three generations: the ones with a modified phosphate linkage, belong to the first generation, the ones with modification in the 2' sugar position, belong to the second generation, whereas the oligonucleotides with more extensively modified or replaced phosphodiester linkages or change of the sugar to new or locked moieties, belong to the third generation. The first are designed to circumvent the intracellular enzymatic degradation. Phosphorothioates (PS) are the most widely used modification of this class for gene silencing.[34] They are more resistant to enzymatic degradation and are able to efficiently elicit RNase H cleavage of targeted RNA. The negative charge on the sulfur atom helps delivery through the cellular membrane. In order to improve uptake by cells and the stability to nucleases, Miller and Ts'O eliminated the negatively charge on the internucleotide phosphate bridge. In the methylphosphonate oligodeoxynucleotides, a negative charged oxygen is replaced by a neutral methyl group, but the modification does not support RNase H activity. [35, 36] The poor binding and specificity to the target RNA are the major weakness of the first generation of antisense OD, partially solved by the second generation of antisense oligonucleotides.[31, 37]

It has been shown that the substitution of the hydroxyl group on the sugar 2' position with an electronegative substituent, which organizes the sugar into a 3'-endo pucker, increase the binding affinity, a major improvement obtained with the design of the second generation of oligonucleotides. However, the exchange of the deoxyribose with a new unit, typically reduces the cleavage activity of RNase H.

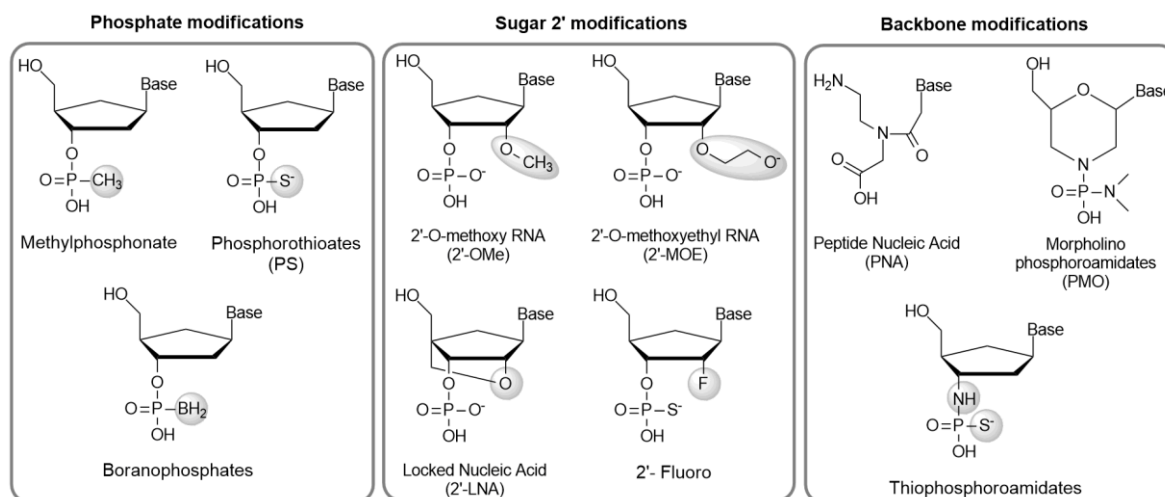


Figure 6: Examples of oligonucleotides modifications.

Peptide nucleic acids (PNA), morpholino phosphoramidates (PMO) [38] and locked nucleic acids (LNA) [39] belong to the third generation class of antisense oligonucleotides and nowadays are the most studied members of this generation. They are born from the need to improve the affinity to the targeted RNA and/or the resistance from degradation which afflict the previous two generations. Both PNA and morpholino oligonucleotides are neutral modifications completely stable to nuclease degradation. However, they do not activate RNase H.

Locked nucleic acid, LNA, is a modification with the higher binding affinity due to its sugar, having locked structure in a north conformation, which is the one adopted naturally by RNA in A-form. LNA has many attractive properties such as high binding affinity, excellent mismatch discrimination, and increase stability to nuclease digestion. [39-41] However no RNase H-mediated cleavage was observed with fully modified LNA, a gap of six DNA nucleotides is necessary to activate RNase H action.[42, 43]

Peptide nucleic acids, PNAs, were first described by Nielsen et al. in 1991 and are characterized by an non-charged N-(2-aminoethyl)-glycine backbone carrying the common nucleobases, and has shown to possess quite many interesting properties.[44, 45] It is able to hybridize with a higher affinity and specificity than natural oligonucleotides in a sequence-specific manner to both DNA and RNA targets. Because of the neutral backbone, PNA does not result in electrostatic repulsion of the target. Polypyrimidine PNAs can also form quite stable triplexes with single-stranded oligonucleotides. The malleability of PNA gives therefore many possibilities in the antisense field.

#### *1.4.2 Antisense oligonucleotides (ASO)*

Oligonucleotides that work through an RNase H dependent reduction of RNA expression are the best understood class of antisense oligonucleotides, accounting for the majority of drugs in development. RNase H recognizes an RNA-DNA heteroduplex, cleaving the RNA strand, resulting in a 5'-phosphate on the product and release of the intact DNA strand.

The effect of a synthetic antisense oligonucleotide (ASO) sequence for potential therapeutic purposes was shown by Zamecnik and Stephenson in 1978 when they demonstrated that an oligonucleotide complementary to the 3' end of the Rous sarcoma virus could block viral replication in chicken fibroblasts.[46] Oligonucleotides are attracting interest mostly because of the recent clinical use approval although they are not considered having done yet a big step in pharmacopeia.[47] Later data suggest that there is still a need to improve the efficacy, especially in terms of delivery. Only some of the recent oligonucleotides therapies were actually clinically tested in the last years but the field is rapidly booming due to the great results obtained in terms of selectivity and toxicity.

Most of the oligonucleotide modifications described in the previous chapter have a huge limitation: all the 2'-modifications greatly reduce or inhibit the ability of RNase H to cleave the targeted RNA strand opposite the modification.[48] Therefore turnover of the RNA target is not achieved lowering the efficiency of the therapy. A common way to solve this major problem is the use of a gapmer strategy, whereby regions of 2'-modified residues ("wings") flank a central oligodeoxyribonucleotide region ("gap", typically phosphorothioate modified) of the second generation ASOs (Figure 7).

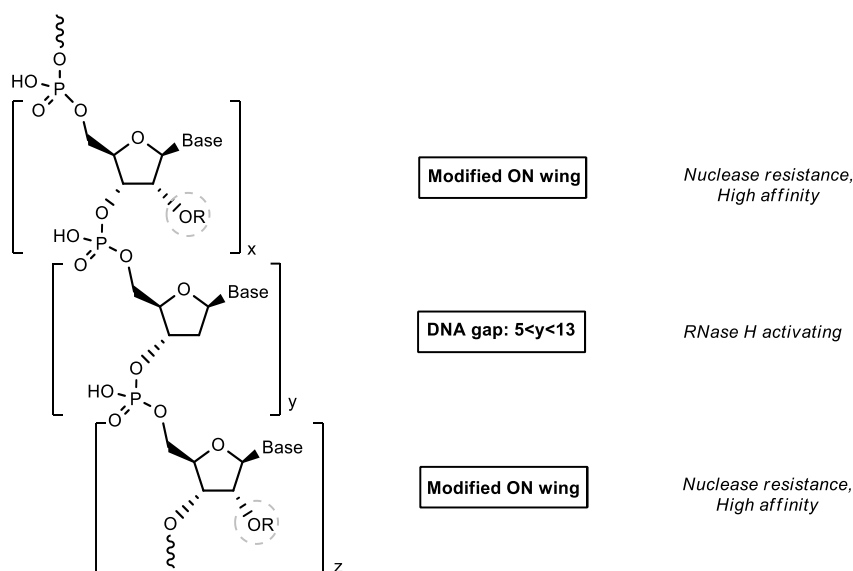


Figure 7: Chimeric gapmer generic structure.

It has been demonstrated that the optimal gap size is motif-dependent, and that a balance between gap size and affinity is required.[49] The chimeric gapmer ASOs usually contain gaps ranging from 5 to 13 non-modified phosphorothioate nucleotides.[49, 50]

## 1.5 Artificial ribonucleases

An alternative to the chimeric gapmers is the design of an antisense oligonucleotide which carries itself a group that cleaves the target RNA upon hybridization (oligonucleotide based artificial nuclease, OBAN), thereby achieving independence from native enzymes. Oligonucleotide based artificial ribonucleases consist of a ‘molecular scissor’, a catalytic group with the capacity of cleaving ribonucleic acids at the phosphodiester linkage, and an oligonucleotide strand for specific target recognition, which brings the catalytic moiety close to the targeted phosphodiester bond.[51-54] In this thesis we will describe examples of artificial ribonuclease developed during the last five years in the groups of Strömberg and Göbel.

Depending on the nature of the catalytic moiety, the most studied oligonucleotide based artificial ribonucleases can be divided in three different groups: (i) lanthanide ion chelates, (ii)  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  chelates, and (iii) metal ion-independent artificial ribonucleases.

### 1.5.1 Metal ion based artificial ribonucleases

The metals in natural metalloenzymes are known to be involved in the catalytic mechanism with different functions [55, 56]:

- delivery of a metal-bound hydroxide or alkoxide that serves as a nucleophile or a base,
- electrostatic stabilization of the anionic substrate, lowering the energy of the transition-state,

- stabilization of the leaving group through metal ion coordination or protonation of leaving group with a metal activated water molecule.

During the last decades many groups tried to mimic metalloenzymes in order to develop a synthetic metal based sequence recognizing artificial ribonuclease, most of them using lanthanides or  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  as catalytic metal.[53, 57-60]

Komiyama, one of the major pioneer in the field, developed systems based on lanthanide ion cleavers. The molecular scissors were usually at the termini and cleaved the RNA outside the sequence that is complementary to the oligo, giving no turnover of substrate. However, the introduction of the acridine group in the central part of the recognizing strand makes the RNA target more susceptible to cleavage by lanthanide ions in solution, which are near the acridine moieties, achieving high selectivity and turnover.[59-63] In the proposed mechanism the acridine pushes the unpaired ribonucleotide out of the heteroduplex changing the conformation of the RNA at the target site for the sequence-selective activation.[61]

The europium (EuTx) [58] and the dysprosium thexaphyrin (DyTx) [57] are artificial ribonuclease synthesized by Magda et al. which are quite active (Figure 8). DyTx derivative attached to an internally positioned glyceryl linker gave turnover of the substrate RNA. There was no turnover when the cleaver was attached to end of the oligonucleotide, presumably because the cleaved substrate was not released.[64]

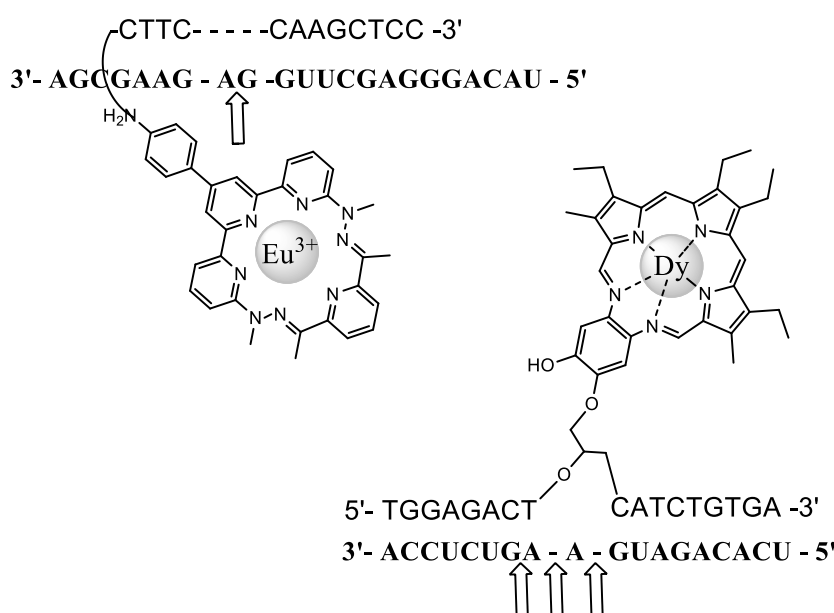


Figure 8: Lanthanide metal ion complexes and oligonucleotide based artificial nucleases by Magda et al.

This is explained by the fact that metal ion chelates are not efficient in the cleavage of double-helical DNA/RNA, characterized by a strong base-stacking interactions which is known to hamper the 5' nucleoside from taking an apical orientation. Hänens group also showed that RNA cleavage oligonucleotide lanthanide chelate based cleavers is enhanced at bulged-out sites.[65] For this reason, the sequence of the oligonucleotide based artificial nuclease must be planned so that a target bulge in form upon hybridization.

In 1994, Bashkin reported for the first time a  $\text{Cu}^{2+}$ -based artificial nuclease which is based on a 17-mer oligonucleotide with a C5 terpyridine conjugated uracil base. After some optimization, including the formation of a trinucleotide internal loop at the cleavage site and turnover in excess of the target, the half-life was reduced to 40h at pH 7.5 and 37 °C.[52, 66, 67]

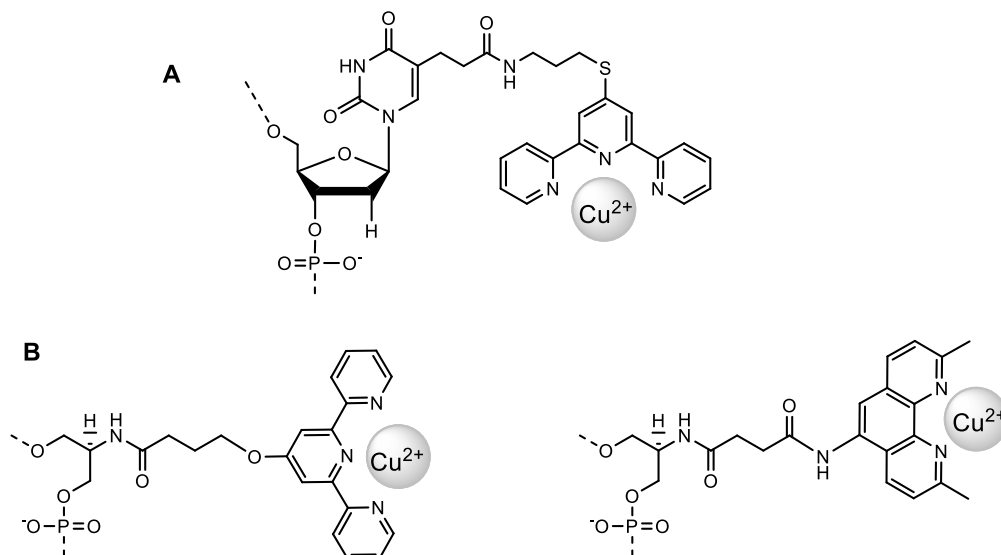


Figure 9: Examples of copper ion chelates of terpyridine and 5-amino-2,9-dimethyl-1,10-phenanthroline used by Bashkin group: A, first example of synthetic ribozyme mimic directed to the major-groove target; B, serinol derivatives.

Another chelate that has been used for construction of  $\text{Cu}^{2+}$  based cleaving oligonucleotide is the 2,9-dimethyl-5-aminophenanthroline. Attaching it to a serinol linker in a oligonucleotide chain, made the cleaving reaction five time faster than the one catalyzed by the corresponding terpyridine conjugate (Figure 9B).[68]

### 1.5.2 Metal-free artificial ribonucleases

The advantage of using non-metal ion based systems is that, for *in vivo* applications, there are no restrictions due to metal ion toxicity or dependence on the stability of the complex, i.e., keeping or finding the metal ion in the living cell.

The group of Vlassov has performed a number of studies with the imidazole as cleaving moiety.[69, 70] They synthesized different deoxyoligonucleotide derivatives conjugated with two histamine residues, which aim at mimicking the catalytic center of RNase A (His-12 and His-119). The best system was a 5'-linked bisimidazole constructs which ultimately was used to cleave, in a site-selective way, a yeast tRNA<sup>Phe</sup> short sequence (Figure 10).

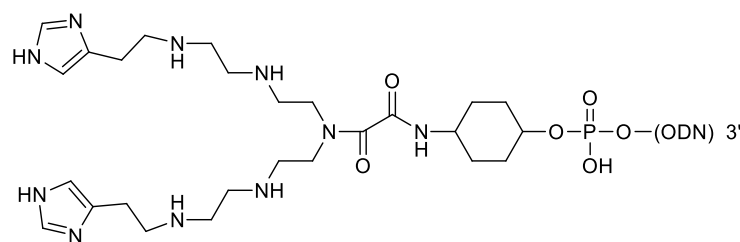


Figure 10: Vlassov's best bisimidazole cleaving system.

The cleaving groups were conjugated to the terminal phosphates of the oligonucleotides so that in the complexes they could reach two CA sequences in the tRNA, known to be susceptible to RNase A action and therefore to constructs with have imidazole moieties. The results obtained are controversial due to the natural predisposition of the target to enzymatic cleavage. Maximum cleavage (60%) of the target was obtained after 8 h at 37 °C and pH 7.0.

Another example of non-metal cleaver oligonucleotide is an oligoarginine-leucine peptide conjugates (G(RL)<sub>4</sub>) which show RNA cleavage in a catalytic but in a non-specific way.[71]

Göbel group is instead using trisbenzimidazole as catalytic moiety, which has been conjugated to the 5'-end of DNA oligonucleotides, cleaving complementary RNA sequences with target specificity, although turnover of the substrate was not shown. In this thesis I will describe a new trisbenzimidazoles PNAzyme (paper VI) and its development (paper III), which is born from our collaboration with Göbel group.

## 2 Development of PNA based artificial ribonucleases: PNAzymes

### 2.1 RNA bulges as target for artificial ribonucleases

It is well known that double-stranded RNAs are less prone to cleavage than single-stranded RNAs.[72] In the A-form RNA helix, the 2'-oxyanion group and the adjacent 5'-oxyanion leaving group are in fact precluded from adopting an in-line conformation, making the linkage more resistant to cleavage.[8] This was also identified by the Ciba-Geigy group of researchers as being important for the development of artificial nucleases and bulges in the target RNA should promote cleavage [73] and that a major reason for this would be due to in line positioning of the 2'-hydroxyl relative to the leaving 5-oxygen. Their modeling study of the RNA backbone suggested that this local conformation of the RNA backbone, when it is forming a bulge upon binding with oligo based artificial ribonucleases, promotes the metal-assisted catalytic cleavage (Figure 11).[73] Our reasoning has rather been that a major cause for the difference is that in a double stranded RNA one has to pay the penalty of breaking up base pairing and a part of the helix for cleavage to occur. In any case if a bulge is formed in RNA upon binding to another oligonucleotide, due to incomplete complementarity, cleavage should more readily occur in the unpaired region. In contrast, single stranded RNAs are more susceptible to spontaneous or metal-catalyzed transesterification because each linkage is free to explore conformational space and occasionally sample the reactive in-line structure. The pocket formed could also house the catalytic entity of an oligonucleotide based artificial nuclease.

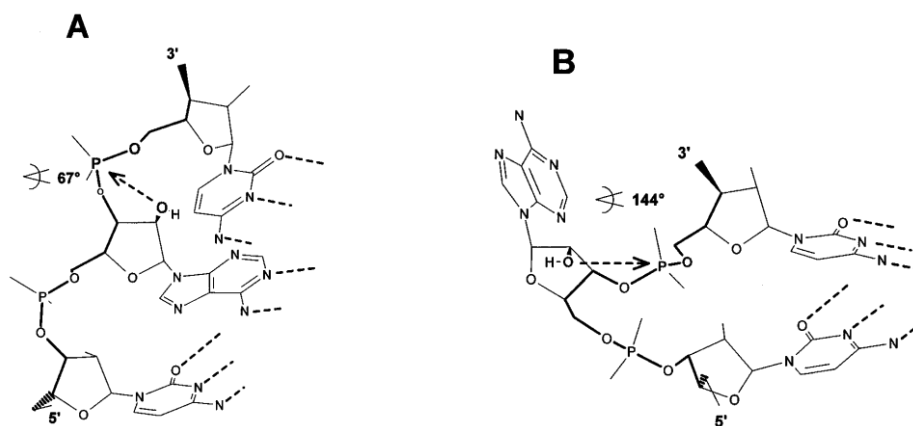


Figure 11: Comparison of the sugar-phosphate backbone of a ribotrinucleotide r(CAC) for regular duplex and in-line structures: (A) a normal A-type RNA duplex conformation, (B) a model for the in-line conformation of the looped-out base. Adapted with permission from Biochemistry. Copyright (1996) American Chemical Society.[73]

Tinoco *et al.* solved the 3D structure of a five nucleotide RNA bulge from the Group I ribozyme domain of *Tetrahymena thermophila*, through NMR experiments (Figure 12).[74] This RNA sequence represented the starting point of our OBAN and PNAzyme development.[75]



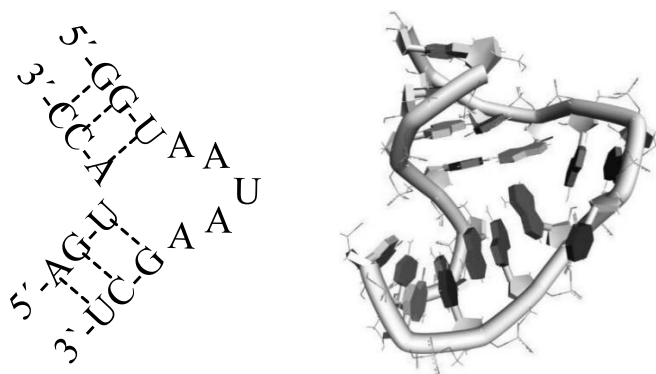


Figure 12: The five nucleotide (AAUAA) RNA bulge from the NMR structure of the bulge-loop in the Group I ribozyme domain.

## 2.2 The first OBANs systems

The early studies on oligonucleotide based artificial ribonucleases in our group included neocuproine (2,9-dimethylphenanthroline) as chelating agent and  $Zn^{2+}$  as the catalytic metal ion and 2'-*O*-methyloligoribonucleotide as recognition element.[75] The  $Zn^{2+}$ -neocuproine unit was attached at different positions in order to evaluate the rate of cleavage and the influence on the selectivity of the cleavage position. It was observed that the position is in fact crucial for the reaction rate.

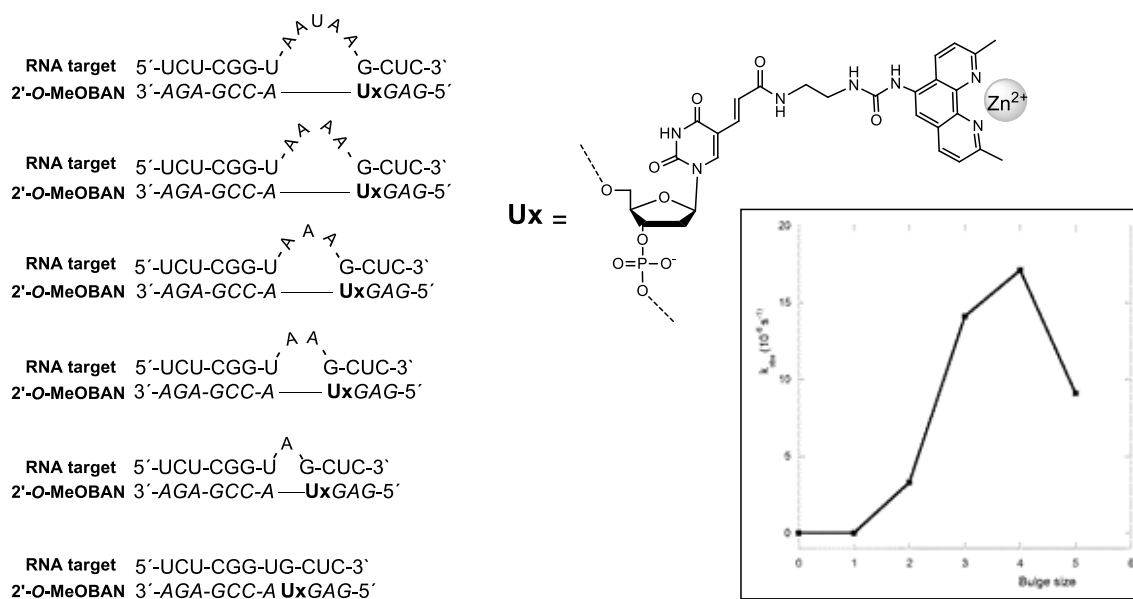


Figure 13: Bulge size dependence of rate of cleavage for a 2'-OMeOBAN

The RNA target bulge sizes formed upon hybridization were investigated as well (Figure 13). As expected the only cleavage obtained was in the bulged out RNA segment, with 70% selectivity for specifically one cleavage site and an optimal size of 3-4 nt bulges. A Michaelis-Menten type of kinetics and dependence on the metal ion was also shown.[75, 76]



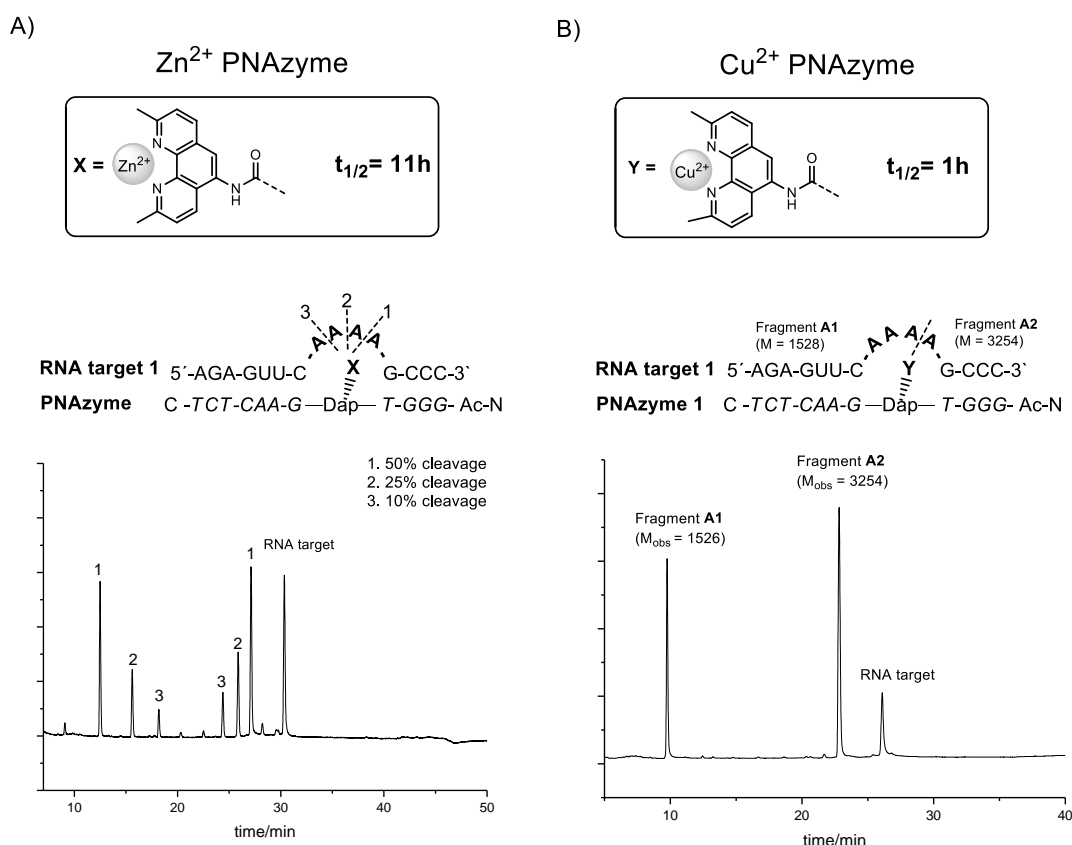


Figure 15: Zn<sup>2+</sup> (A) and Cu<sup>2+</sup> (B) PNAzymes systems and examples of IE-HPLC chromatograms of the cleavage of the M-BCR/ABL RNA model, at pH 7.4 and 37 °C. Cleavage takes place exclusively in the bulged out region.

The main remarkable improvement was that the metal ion used in the reaction was exchanged from Zn<sup>2+</sup> to Cu<sup>2+</sup> (Figure 15B). The new Cu<sup>2+</sup> based PNAzyme showed single site cleavage within the RNA bulge, turnover of RNA substrate even when used in 100 times excess of substrate *vs* enzyme, and excellent mismatch rejection.[86] The wobble base GT pair was shown to be crucial for the cleavage activity, simply replacing it with standard Watson-Crick pair GC, caused a substantial drop in the RNA cleavage rate.

### 2.3.1 Hypothesized PNAzyme mechanism

It is not known yet why changing the metal ion caused this remarkable difference in the rate and selectivity of PNAzymes, but a plausible reason can be the different geometry the two metals adopt. The cleavage mechanism of the metal based PNAzyme has not been disclosed yet, but from the results obtained, we hypothesized that the molecules of water bound to the copper ion, can have an effect in promoting the departure of the 5' leaving group (Figure 16). The mechanism of the metal ion promoted cleavage resemble the base-catalyzed reaction, where the metal ions act as intracomplex base catalysts. In this scenario, water acts as an acid catalyst, protonating the oxyanion leaving group.[87]

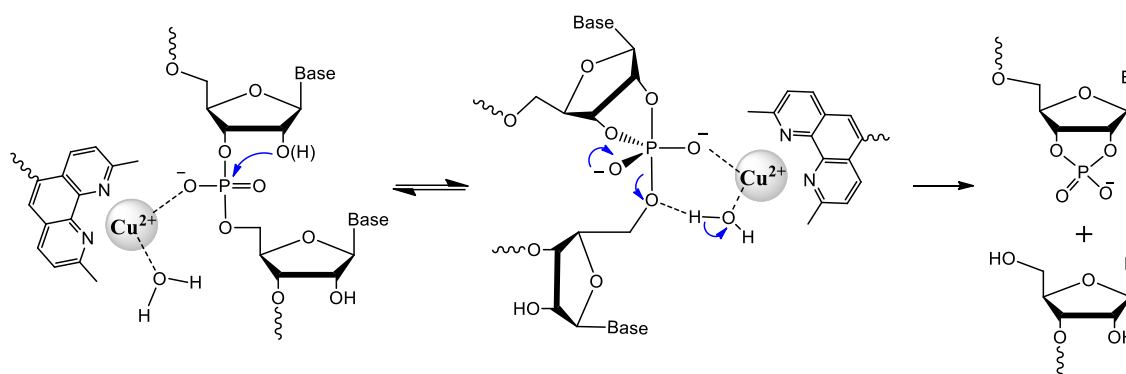


Figure 16: Proposed mechanism for the phosphate cleavage reaction catalyzed by the Cu<sup>2+</sup>-PNAzymes.

PNAzymes can be considered to be highly specific artificial RNA restriction endonucleases and are now usable tools for molecular biology and not far from reaching a stage when they can be used for gene silencing.

In order to improve further the efficiency of the PNAzymes there are for example two types of modifications that can be introduced in the system: one is to construct a PNA-conjugated which carries a Cu<sup>2+</sup> chelate that strongly hold the potentially toxic copper ion tighter and potentially increase the rate of cleavage, the other option is to make the active site of the PNAzyme/RNA construct more hydrophobic, decorating the PNA with PEGs moiety for example. These types of modification are subject for this thesis and will be discussed in the chapter 4.

### 3 RNase A: into the enzyme mechanism

During the last decades, the use of short oligonucleotides for different therapeutic approaches, like for example siRNA (short interfering RNA), antisense technologies, splice-switching or artificial ribonucleases has been an object of many research groups. Therefore there is a need to design oligonucleotide modifications which show resistance to enzymatic action and, at the same time retain, or even enhance the affinity and selectivity towards the target.

#### 3.1 An RNA modification with remarkable resistance to RNase A (*paper I*)

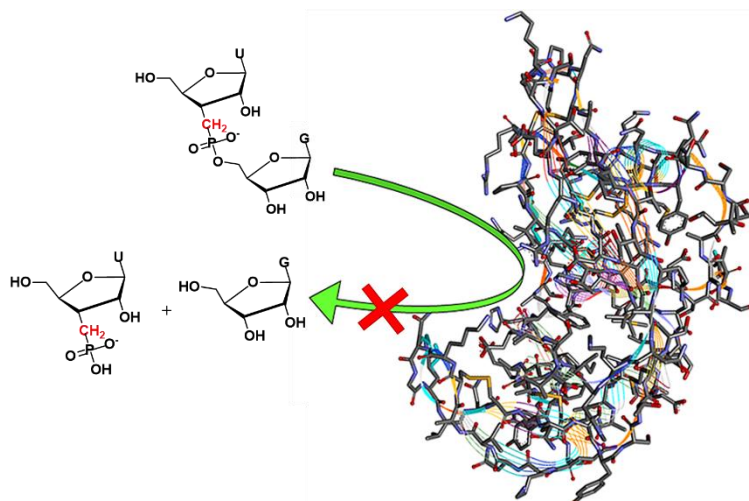


Figure 17: Schematic representation of the RNase resistance of the new methylene RNA dimer. [88]

As mentioned in the introduction, the major cause of the RNA lability is the presence of the 2'-hydroxyl group, consequently most of the non-natural oligonucleotides are missing this function or have it modified with different chemical groups like amides, acetal,[89] or O-alkyl groups. We started to be curious about the possibility of synthesizing an oligonucleotide which has the 2'-hydroxyl group preserved but still stable in presence of phosphodiesterases enzymes, which are the major cause of therapeutic oligonucleotides degradation in gene therapy. In 1970 Albrecht et al. synthesized an isosteric analogs of a nucleotides 3'-phosphate where the O in 3' was replaced by a methylene group, but no results about the enzymatic stability were reported.[90, 91] This analogue is characterized by having approximately the same size and shape as the natural RNA monomer. The synthesis procedure presented in the Albrecht paper was quite laborious and low yielding and not particularly suited to incorporation during oligonucleotide synthesis. However, the successful development of H-phosphonate chemistry [92-98] suggested an alternative route via methylene-H-phosphinates [99] that should be applicable also to solid phase synthesis.

The first compound synthesized in our lab was a UCH<sub>2</sub>pG dimer (Figure 18) where the methylene group in the linkage between a pyrimidine and a purine was the only modification, with respect to the natural RNA, present. The dinucleotide contains in fact all the groups that typically are involved in mechanisms for catalyzed cleavage of RNA (except when the 3'-

oxygen is the leaving group), in particular the 2'-hydroxy nucleophile, the 5'-oxyanion leaving group and a negatively charged phosphoryl functionality.

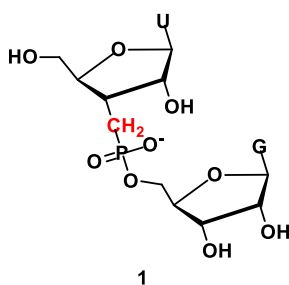


Figure 18: New RNA methylenephosphonate dimer.

### 3.1.1 Synthesis of the UCH<sub>2</sub>pG dimer

Between 2001 and 2008, a series of four papers were published in European Journal of Organic Chemistry by our group, in particular by Anna Winqvist where the key steps for the synthesis of the UCH<sub>2</sub>pG dimer were developed.[99-102] In the first paper, a new strategy to introduce of the hydroxymethyl group using a hydroboration reaction of 3'-deoxy-3'-C-(methylene)uridine was presented.[102] In the last three papers, the synthesis of methylenephosphinate building blocks was developed [100] and the oxidation of methylenephosphinate dimer[101] as well as the reaction for linking the methylenephosphinate to a second nucleoside was investigated.[99]

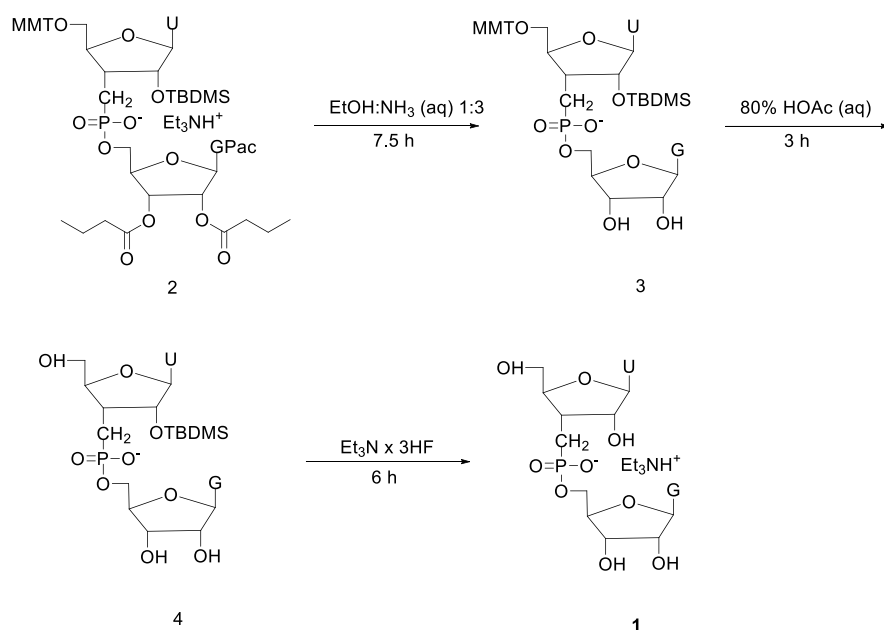


Figure 19: Deprotection scheme for preparation of UCH<sub>2</sub>pG

To obtain the UCH<sub>2</sub>pG dimer (**1**) used to evaluate the enzymatic stability, compound **2**, synthesized through the steps described in the previous papers, was fully deprotected with 32% aqueous ammonia, then with 80% aqueous acetic acid and last with triethylamine trihydrofluoride (Figure 19). The crude product was then purified by reversed-phase HPLC.

### 3.1.2 Stability of UCH<sub>2</sub>pG in presence of 3' and 5' exonucleases.

To evaluate the stability towards enzyme catalysed degradation of the newly synthesized dimer, UCH<sub>2</sub>pG was first subjected to snake venom phosphodiesterase (SVPD, PDE I) from *Crotalus adamanteus* and analysed by HPLC at different times. SVPD is a phosphodiesterase which cleaves phosphate esters giving a 3'-oxyanion as leaving group of the cleavage reaction. Due to the 3'-methylene modification, there is no leaving group and hence we didn't observe any cleavage even after days of incubation.

UCH<sub>2</sub>pG and the native diribonucleotide UpG were then subjected (at pH 6.5 and 37 °C) to a second class of PDE, the Spleen phosphodiesterase (PDE II) which is a 5'-exonuclease. The methylenephosphonate analogue proved to be considerably more stable than the native UpG (Figure 20). Under the same conditions, where UpG is more or less completely degraded (a few hours), the modified dimer is only 20-25% cleaved (Figure 20, left panel) displaying an approximate half-life of about 1-1.5 days (Figure 20, right panel).

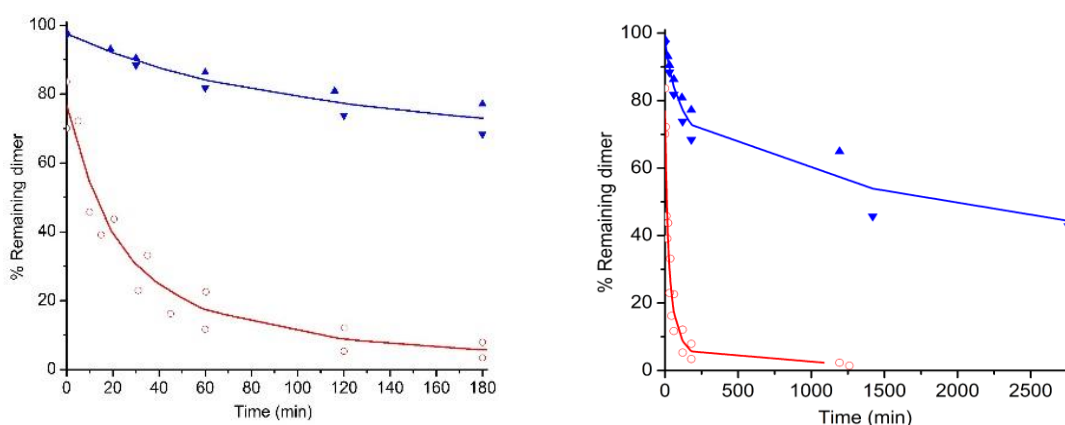


Figure 20: Graphs showing % remaining dinucleotide (UCH<sub>2</sub>pG in blue and UpG in red) at different times, when subjected to Spleen exonuclease, PDE II at 37 °C at different times. Quantification of dinucleotide and product was done by integration of the RP-HPLC analysis.

### 3.1.3 Stability of UCH<sub>2</sub>pG in presence of endonucleases (RNase A).

After determining the stability of the methylene RNA dimer towards SVPE and Spleen exonuclease treatment, the resistance of UCH<sub>2</sub>pG to Bovine pancreatic RNase A was tested (37 °C at pH 7). The difference in rate of cleavage between the natural dimer and our modified one is even higher than the one observed for the Spleen PDE II (Figure 21); even after seven days of incubation UCH<sub>2</sub>pG was only cleaved to a small percentage. The results were quite impressive and unexpected. The difference in rate of cleavage between the natural dimer and our modified one is even higher than the one observed for the Spleen PDE II; even after seven days of incubation UCH<sub>2</sub>pG was only cleaved for a small percentage (Figure 21).

The results were quite impressive and it was unexpected that the difference was so large, since the modified dimer maintains the functional groups of the natural RNA like the 2'-hydroxy nucleophile, the 5'-oxyanion leaving group and a negatively charged phosphoryl functionality.

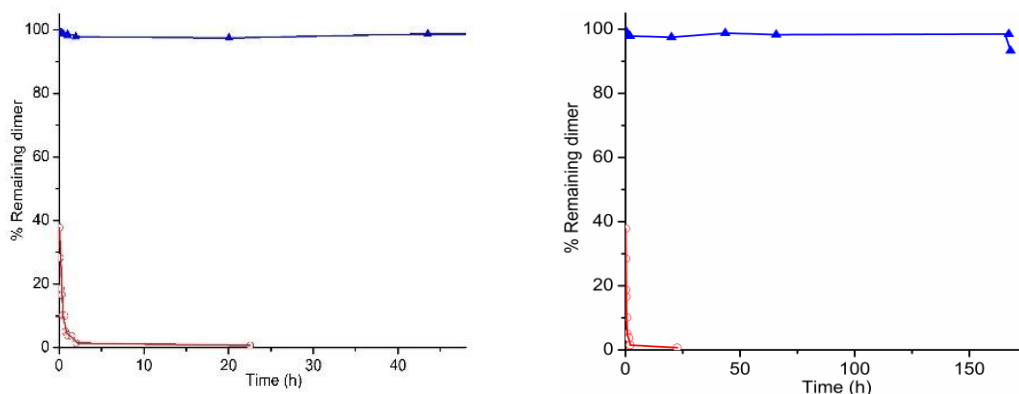


Figure 21: Graphs showing % remaining dinucleotide (UCH2pG in blue and UpG in red) at different times, when subjected to RNase A at 37 °C. Quantification of dinucleotide and product was done by integration of the RP-HPLC analysis.

To explain the results we formulated different hypotheses, one of them is that the presence of the methylene group in the cyclic phosphate gives a different intrinsic chemical reactivity upon cyclization. To exclude that, I subjected the natural dimer and the modified dimer to hydrolysis under alkaline conditions (Figure 22). The base catalyzed cleavage was studied at two different sodium hydroxide concentrations at room temperature.

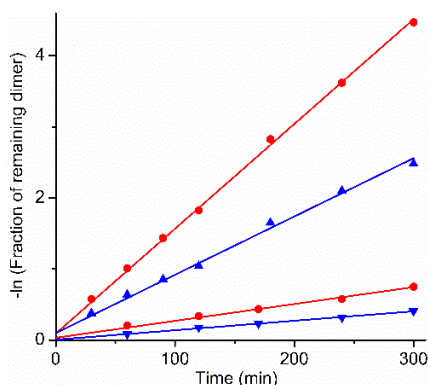


Figure 22: Graph showing the natural logarithm of the fraction of remaining dinucleotide (UpG in red and UCH2pG in blue) when subjected to hydrolysis in 0.01 M (lower two lines) and 0.05 M NaOH solutions (upper two lines) at 50 °C.

The natural RNA dimer UpG was cleaved somewhat faster than the modified UCH2pG but the rate difference in both solutions is only a factor of 1,7. This means that the results obtained from RNase A assay cannot be explained by the intrinsic chemical difference.

The second hypothesis was that, even if the size and chemical differences between UCH2pG and the native dimer are small, the methylene dimer cannot access or bind the active site of the enzyme. Stereoelectronic effects should enforce the methylene modification to adopt the 3'-endo/north conformation, which NMR studies also confirmed, whereas the RNA dimer has only a slight preference for north over south conformation in solution (Figure 23).



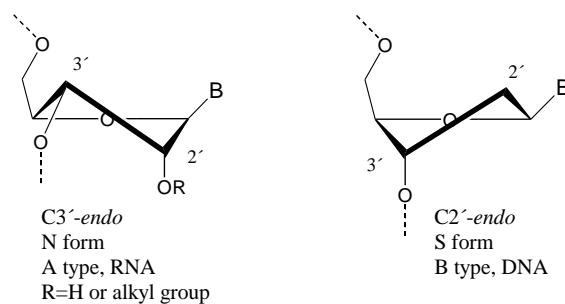


Figure 23: Sugar pucker conformations.

If RNase A catalyses reaction on oligo and dinucleotides which initially bind in the south conformation, UCH<sub>2</sub>pG, would not be able to compete with the native UpG substrate for RNase A. Competition experiments were performed under the same conditions as described for the cleavage assays above, but with both UpG and UCH<sub>2</sub>pG present in the same sample at different ratios: the concentration of the UpG was maintained constant as was the concentration of the RNase A, whereas the concentration of the modified dimer UCH<sub>2</sub>pG was increased depending on the experiment (Figure 24).

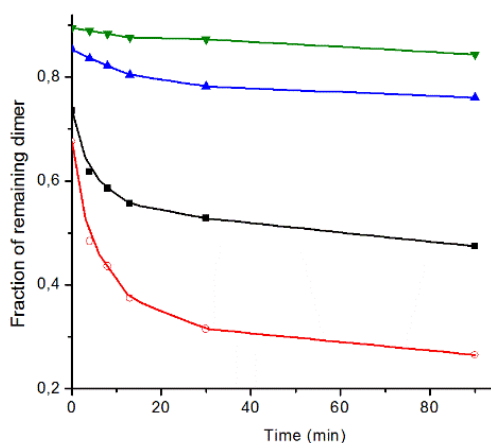


Figure 24: Graph showing % remaining UpG at different times, when subjected to RNase A, in the presence of different amounts of UCH<sub>2</sub>pG at 37 °C (Red line: only UpG, i.e., 1:0; Black line: equimolar amounts, i.e., 1:1; Blue line: 1:5; Green line: 1:10).

Already at equimolar amounts, UCH<sub>2</sub>pG competes strongly with UpG (Figure 24, black line) and the cleavage of UpG is even more retarded when increasing the amount of the methylene dimer until there is almost no cleavage of RNA with a ratio of 1:10 (Figure 24, green line). From the result obtained it seems that UCH<sub>2</sub>pG, not only competes with UpG, but it even binds tighter to the enzyme, which could be explained if RNase A binds the substrate in north conformation which is the preferred conformation for our modified dimer.

### 3.1.4 A couple of hypotheses to explain UCH<sub>2</sub>pG stability to RNase A degradation.

As mentioned in the RNase A chapter, Breslow propose a mechanism [28] which involves the formation of a phosphorane pentacoordinate intermediate, which has a bipyramidal geometry (Figure 25, left panel). If the 5'-oxygen, which is the site of breakage between the phosphate

and the leaving group, is not in an apical position after attack by the 2'-hydroxyl, pseudorotation will be necessary for completion of reaction (Figure 25, left panel).[103] The methylene group in the 3'-position of the UCH<sub>2</sub>pG dimer has a low apicophilicity [104], therefore the pseudorotation process would be severely retarded and could possibly explain the low rate with the modified substrate, i.e., why after 7 days of incubation we only see a small fraction of cleaved product (Figure 21). With an excess of enzyme we can more readily follow cleavage of UCH<sub>2</sub>pG but it is still at a very slow rate (Figure 25, right panel).

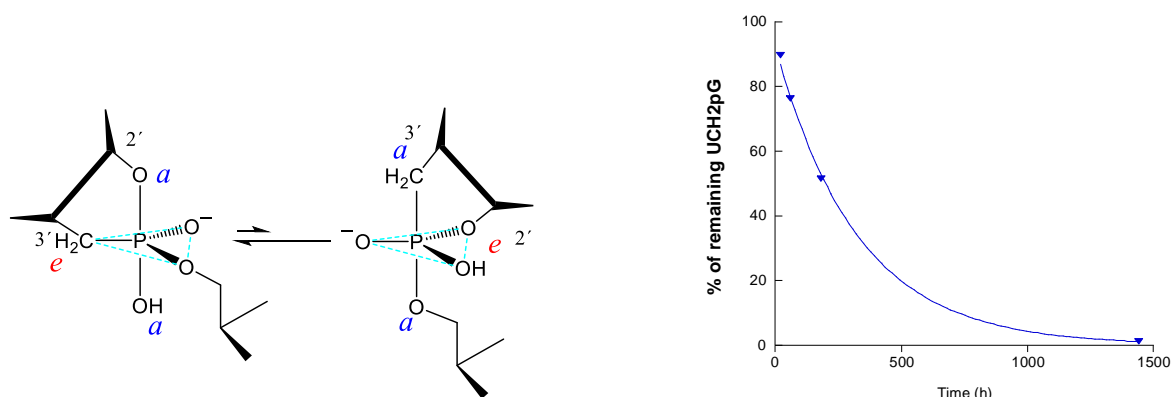


Figure 25: (left panel) Equilibrium between phosphoranes if, hypothetically, the attack of the 2'-hydroxyl when bound to the RNA initially places the 5'-oxygen of the leaving nucleoside in an equatorial position where the pentacoordinate intermediate has to and (right panel) graphs showing % remaining dinucleotide UCH<sub>2</sub>pG when subjected to high excess of RNase A at 37 °C.

A second hypothesis is that the 3'-endo (north) conformer initially bound has to flip to 2'-endo (south) before the attack on phosphorus can take place and that this is retarded by the conformational preference of the methylenephosphonate, perhaps accentuated when bound to the enzyme. It is also plausible that lysine-41, suggested to stabilize charge build up on the non-bridging phosphoryl oxygens, is somehow involved and interacts with the 3'-oxygen. This could also be connected to one of the conformational changes above.

### 3.2 Conclusion and future perspective

The complete resistance towards a 3' exonuclease and high resistance towards a 5' exonuclease as well as to RNase A suggests that the 3'-deoxy-3'-C-methylenephosphonate modification can be highly interesting for incorporation into therapeutic oligonucleotides, especially when 2'-hydroxyl functions are needed in certain positions, as in siRNA's. Interesting would be also the co-crystallization of RNase A with the dimer inside the active site. The structure of an RNase A crystal with an RNA dimer in the active site would help to disclose the still uncertain RNase A mechanism of cleavage. In collaboration with Raines group, we did attempt to find the conditions for the co-crystallization but unfortunately we were not successful.

## 4 Design of PNA based artificial ribonucleases: PNAzymes (*paper II and paper V*)

### 4.1 RNA target for studying the PNAzymes activity

Metal ion catalyzed cleavage of phosphate diesters can be up to ca  $10^{12}$  times faster in methanol than in aqueous solution.[55, 105] Neverov et al. synthesized a series of phosphate mono-, di-, and triesters with 2'-(2-phenoxy)-1,10-phenanthroline in order to study the kinetics of decomposition of the Cu(II) complexes in methanol at 25 °C (Figure 26).

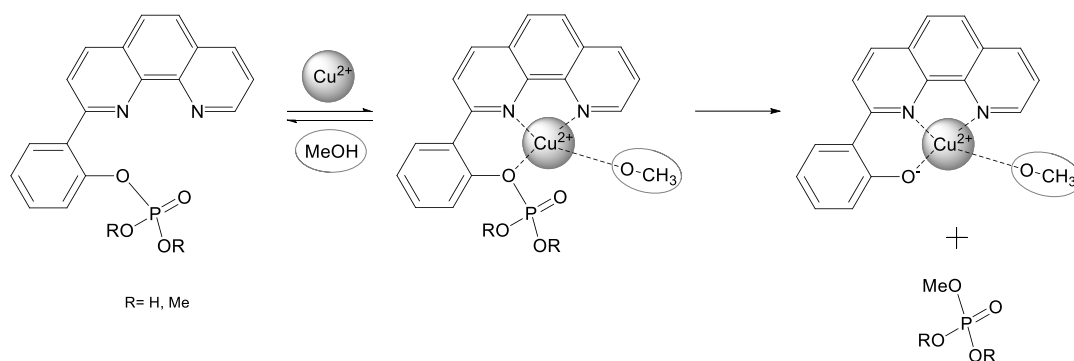


Figure 26: Neverov model for studying phosphate cleavage in MeOH.

They demonstrated that the media has an important role in the catalyzed reaction, in fact they show that Cu(II) catalyst cleaves the phosphate in methanol and ethanol media with a rate acceleration up to  $\sim 10^{14}$  fold compared to the uncatalyzed reaction, whereas in water there is no difference in rate.

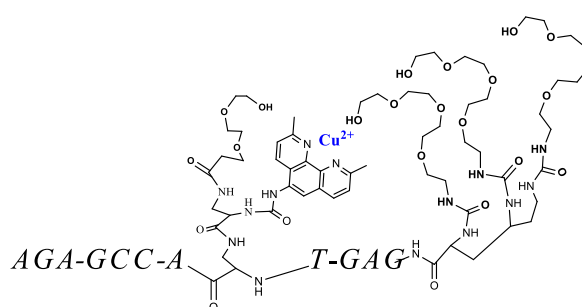
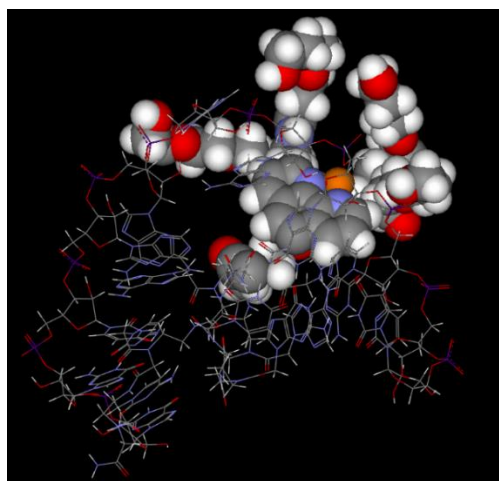


Figure 27: Structure and model of a potential construct showing a copper chelate surrounded by polyethers when the RNA substrate is bound.

Since it is not possible to have a methanolic environment *in vivo*, we aim to create a ‘local solvent effect’ in the proximity of the PNAzyme active site. The idea is to create, upon hybridization with the RNA target, a sort of cage around the cleavage site, thus making the environment less aqueous (Figure 27).

## 4.2 Synthesis of PNA Oligoether Conjugates: towards a ‘local solvent effect’ (paper II)

In the attempt to get even higher rates of RNA cleavage than with the reported Cu<sup>2+</sup>-PNAzyme[86], I synthesized different PNAs conjugated to oligoethers. The PNAzyme conjugation to polyethylene glycol (PEG) moieties can also enhance uptake, solubility, stability, pharmacokinetics *etc.* for enhanced drug delivery.[106, 107] We investigated different methods for the conjugation of oligoethers to PNA linked internally or at the N-terminal through diaminopropionic acid (Dap) residues. The presence of oligoethers could in fact affect the target binding and the solubility of the PNA, but most important was if they would affect positively or negatively the cleavage action of the PNAzymes.

### 4.2.1 Synthesis of oligoethers/carbohydrates PNA derivatives

Before moving to conjugated PNAzymes we wished to first evaluate some methods for oligoether conjugation. The first approach evaluated was the conjugation of oligoether amines through use of 1,1-carbonyldiimidazole (CDI). Since the reaction didn't give a good yield, a double coupling with CDI was performed, reducing the amount of unreacted PNA; unfortunately the result was not an increased in amount of PNA **2**, but the formation of the double substituted product PNA **3** (Figure 28).

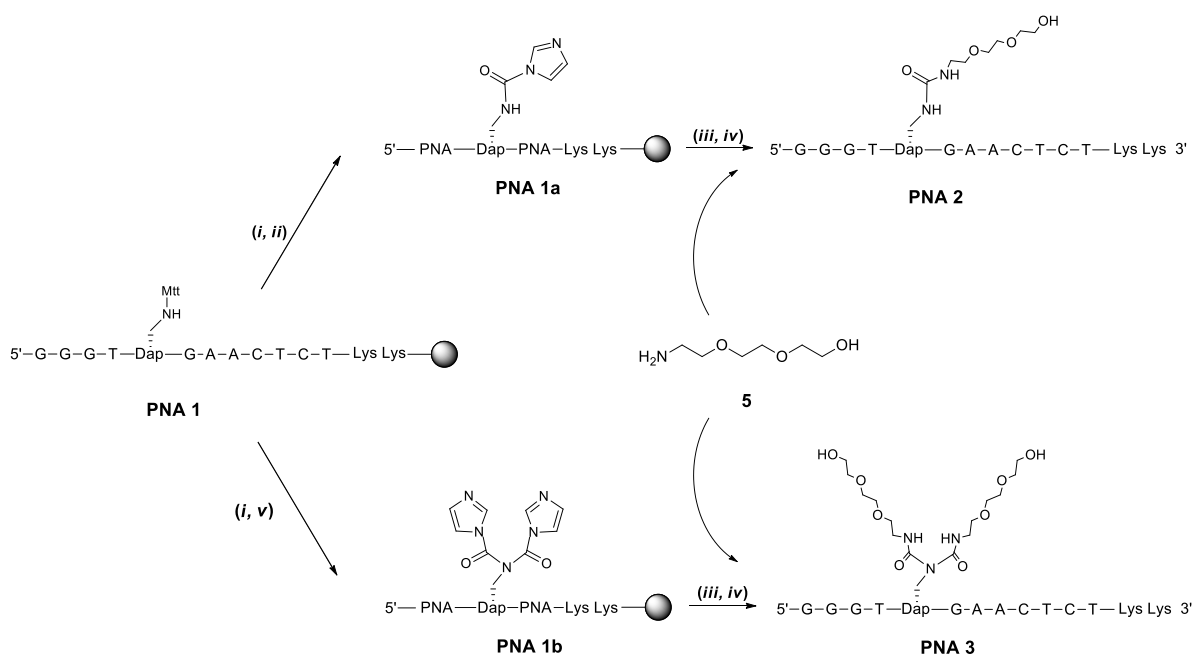


Figure 28: Scheme of the PNA **2** and PNA **3** synthesis. (i) 1% Trifluoroacetic acid (TFA) in DCM, 5 times for 1 min; (ii) CDI in N-Methyl-2-pyrrolidone (NMP), NMM, 1 h; (iii) oligoether **5**; (iv) TFA/Triisopropylsilane (TIS)/H<sub>2</sub>O (95/2.5/2.5) for 2 h; (v) CDI in NMP, NMM, 1 h, 2 times.

Because of the limited conversion and the demanding purification, we decided to utilize conditions similar to those for peptide synthesis HBTU/ HOBt and an oligoether carrying a carboxylate function that can be coupled to the  $\beta$ -amino group of Dapa and/or the N-terminal amine.

Compound **8** was synthesized protecting one terminal of triethylene glycol with a benzoyl group and oxidizing the other hydroxyl group to a carboxylic acid (Figure 29A). Compound **8** was then coupled to the PNA **4** using a procedure similar to the one used for attaching amino acids to the Dapa side chain (Figure 29B).[84] After removal from support, debenzoylation of the oligoether on the crude PNA was done with methanolic ammonia in solution (50 °C for 5 h) obtaining a complete conversion.

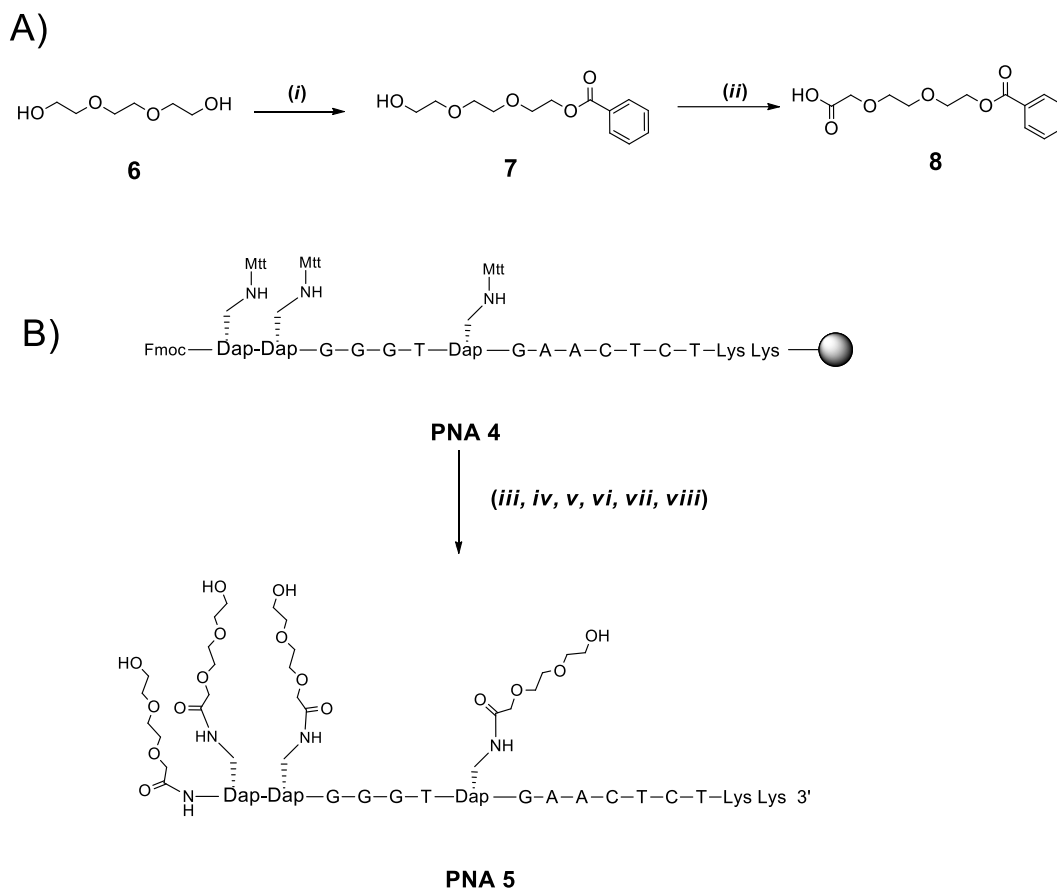


Figure 29: Scheme of compound **8** and PNA **5** synthesis. A) (i) Triethylene glycol: Benzoyl chloride 3:1, pyridine; (ii) Acetone, H<sub>2</sub>SO<sub>4</sub> (1.5 M), Cr<sub>2</sub>O<sub>3</sub>; B) (iii) 20% Piperidine in NMP; (iv) oligoether **8**, HBTU, HOBT, NMM; (v) 1% TFA in DCM, 5 times for 1 min; (vi) oligoether **8**, HBTU, HOBT, NMM; (vii) 20% NH<sub>3</sub>/MeOH, 5 h, 50 °C; (viii) TFA/TIS/H<sub>2</sub>O (95/2.5/2.5), 2 h.

Another class of compounds which we were interesting in, is sugars and aminosugars. Compound **9** was synthesized as described with initial protection of the amino group and successive acetylation of hydroxyl groups and addition a trichloroacetimidate group in the anomeric position (Figure 30A). Subsequent glycosidation with a protected glycolic acid then gave product **11**. Reacting solid supported PNA **6** with an excess of compound **11** (30 eq). **11** and HATU as condensing agent allowed us to obtain the aminosugar conjugate PNA **7** (Figure 30B).

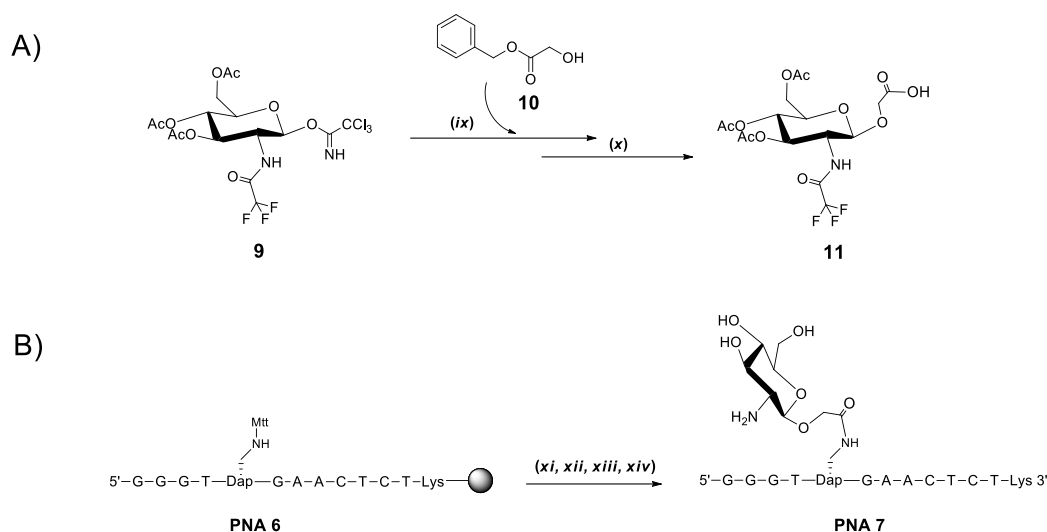
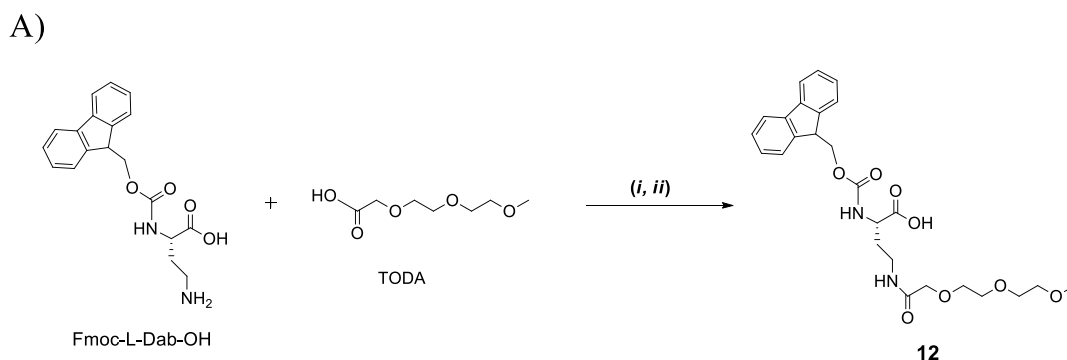


Figure 30: Scheme of compound **11** and PNA **7** synthesis. A) (ix) protected glycolic acid **9**, dry  $\text{CH}_2\text{Cl}_2$ , TMSOTf, from  $-20\text{ }^\circ\text{C}$  to room temperature, 3 h; (x) Pd-C catalyst in THF-MeOH (1:1), overnight; B) (xi) 1% TFA in DCM, 5 times for 1 min; (xii) Aminosugar **11**, HATU, DIPEA in NMP, 1h; (xiii) TFA/TIS/ $\text{H}_2\text{O}$  (95/2.5/2.5) for 2 h; (xiv) Ammonia, 5 h,  $50\text{ }^\circ\text{C}$ .

The possibility of an internal incorporation of an oligoether building block into PNA was the next step. The oligoether 2-[2-(2-methoxyethoxy)ethoxy]-acetic acid (TODA), which carries a terminal O-methyl group, was coupled with HATU-preactivated Fmoc-L-Dab-OH (N- $\alpha$ -Fmoc-L-2,4-diaminobutyric acid) to obtain building block **12**, used in the synthesis of PNA **9** (Figure 31). The synthesis could be achieved by a standard PNA synthesis protocol on an automated synthesizer followed by removal of the Mtt from the Dap unit and then postconjugation with 5-N(phenoxycarbonyl)-5-aminoneocuproine followed by removal of Fmoc, capping, cleavage from support and deprotection.

PNA **8** was converted to PNA **10** by reaction of the PNA with the reagent PEG **13** after removal of the Fmoc (Figure 31B). Contrary to what one could expect, PNA **10** solubility in water was problematic and we could not obtain a reliable thermal melting.



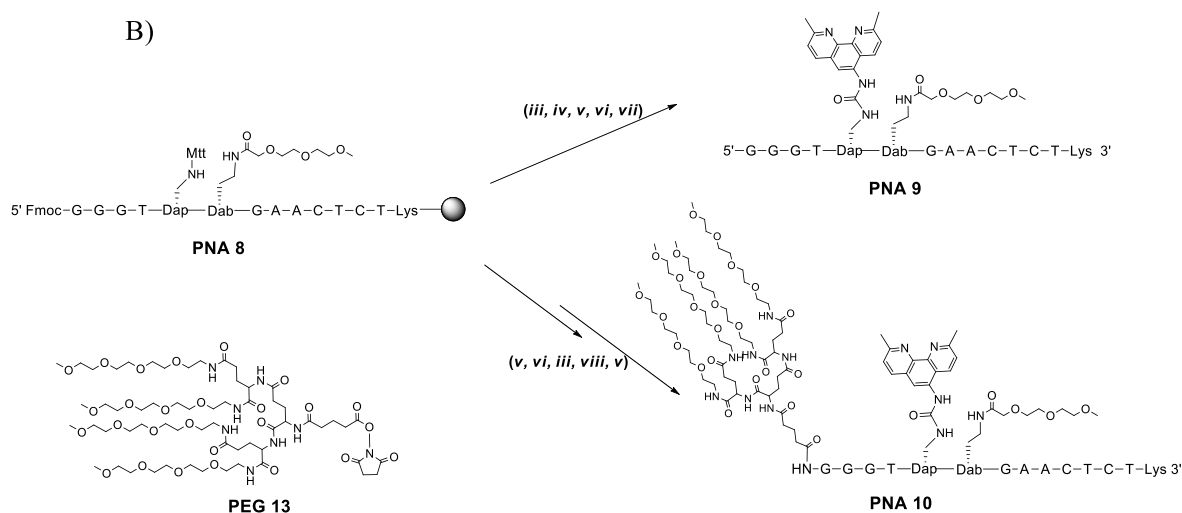


Figure 31: Scheme of compound **8** and PNA **10** synthesis A) (i) Preactivation of TODA with HATU, NMM in DMF (2 min); (ii) Fmoc-L-Dab-OH; B) Synthesis of PNA **9** and PNA **10**: (iii) 20% Piperidine in NMP, 30 min; (iv) acetic anhydride: lutidine: NMP 5:6:89 2x5 min; (v) 1% TFA in DCM, 5x1 min; (vi) 5-PhOC(O)NH-Neocuproine, NMM in NMP overnight reaction; (vii) TFA/TIS/H<sub>2</sub>O (95/2.5/2.5) for 2 h; (viii) PEG **13**, NMM, NMP.

#### 4.2.2 PNA derivatives polarity and thermal melting analysis.

The properties of all the PNAs synthesized, were then investigate to test if oligoether conjugation at the N-terminal would influence the stability of the complex. PNA-Dap-PNA **1** and PNA-Dap-PNA **6** were obtained by complete deprotection of PNA **1** and PNA **6**. These are similar to PNA **2** and PNA **3** or PNA **7** respectively, but with a nonconjugated Dap in the central position. In addition, PNA-Gly-PNA (PNA **11**) which is PNA-Dap-PNA **6** with a glycine instead of the Dap and PNA-GlyNeo-PNA which is PNA **9** with a glycine instead of the Dab-oligoether, all without oligoethers were also synthesized.

Table 1: T<sub>m</sub> for PNA conjugates.

Entry	PNA Construct	T <sub>m</sub>	Type and Number of Conjugated Entities
1	<b>PNA-Dapa-PNA 1</b>	54 °C	1 Dapa
2	<b>PNA 2</b>	49 °C	1 hydroxyoligoether
3	<b>PNA 3</b>	49 °C	2 hydroxyoligoethers
4	<b>PNA 5</b>	51 °C	1 central and 3 terminal hydroxyoligoethers
5	<b>PNA-Dapa-PNA 6</b>	-	1 Dapa
6	<b>PNA-Gly-PNA (PNA 11)</b>	-	1 glycine
7	<b>PNA 7</b>	51 °C	1 aminosugar
8	<b>PNA-GlyNeo-PNA</b>		1 glycine and 1 neocuproine
9	<b>PNA 9</b>	52 °C	1 methoxyoligoether and 1 neocuproine
10	<b>PNA 10</b>	Not measurable	1 methoxyoligoether and 1 neocuproine and 4 terminal methoxyoligoethers

From the thermal melting analysis with the target RNA sequence UGUGUUCAUAAGCCC, the oligoethers seem to be well accommodated in the PNA/RNA duplex. Comparing the values with the non-conjugated PNA ( $T_m = 54\text{ }^\circ\text{C}$ ) of the same sequence, it is clear that the all the PNA conjugates, whether they are oligoethers or a carbohydrate only slightly interfere with the stability. Thus, an oligoether in this position can be acceptable in future artificial nuclease designs without substantial compromise with regard to the stability of the PNA/RNA bulge complex.

### 4.3 Influence of conjugation of various groups on the activity of $\text{Cu}^{2+}$ based PNAzymes (*paper V - first part of the paper*)

After the results described in paper II, we started to investigate the cleavage activity of PNA neocuproine conjugates carrying additional oligoether moieties.

One of the most readily made PNAzyme constructs which can be used to test the effect of a polyether unit nearby the cleavage site, avoiding solubility problems, is PNA **9** (Figure 33), whose synthesis was described in paper II. Comparing the RNA cleavage of PNA **9** with a reference PNA without the oligoether, PNA **11**, we observed a significant difference (Table 1:  $T_m$  for PNA conjugates.): the half-time for PNA **9** cleavage is approximately two times lower than the half time for the reaction of PNA **11**. We adduce the difference to the presence of the polyether (PE) arm, since that is the main difference between the two compounds, but at this stage we cannot conclude that the effect is due to solvation. However, comparing PNA **9** with the PNAzyme without an additional central amino acid [86], the new system is a bit slower probably because of the structural difference imposed when creating a larger gap in the PNA-sequence.

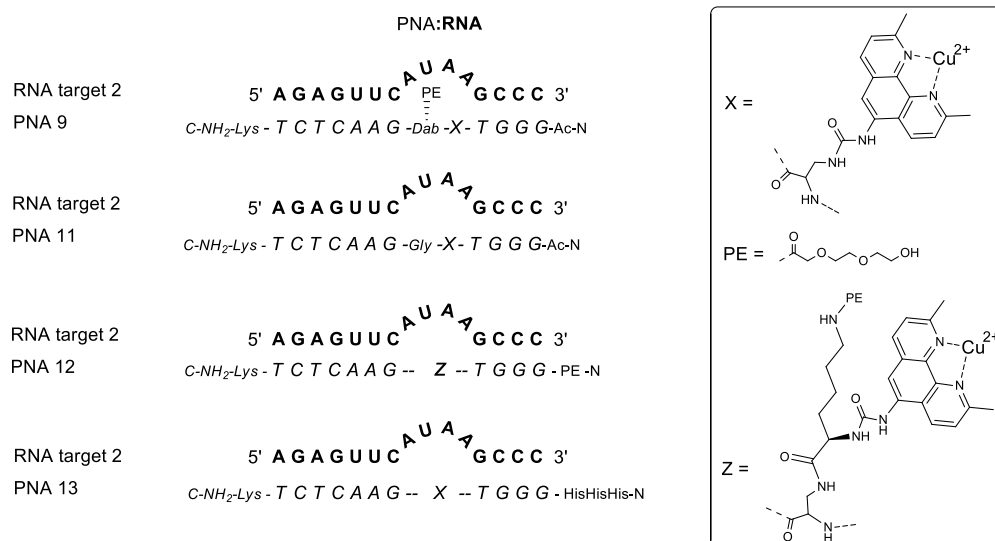


Figure 32: Complexes between RNA-target and PNA-neocuproine constructs, with further conjugation to oligoethers or a peptide.



Encouraged by the results obtained with a single oligoether conjugation, and knowing from previous study with  $Zn^{2+}$  PNAzymes, that elongating the neocuproine arm with one glycine [85], the cleavage rate is unchanged, I synthesized PNA **12** which has neocuproine and an oligoether branched out from the same central amino acid (unit Z, Figure 32), as well as an additional oligoether attached to the N-terminal. Unfortunately, PNA **12** was completely inactive as an artificial nuclease during the time investigated. The PNAzymes we create are apparently really sensitive to small structural changes as has also been shown when varying the RNA-sequence in the bulge region.

Table 2: Full length RNA remaining at different times after incubation of AGAGUUCAUAAGCCC (RNA target 2) with PNAs 9-13 (4  $\mu$ M of each).

PNA \ % full length RNA after	10 min	30 min	80 min
<b>PNA 9</b>	93,5	79,6	56,6
<b>PNA 11</b>	96,2	89,5	74,4
<b>PNA 12</b>	100	100	100
<b>PNA 13</b>	100	100	100

In an attempt to enhance the action of the  $Cu^{2+}$ -neocuproine by possible additional Brønsted general acid/base catalysis, PNA **13** was synthesized with three histidines conjugated at the N terminal (Figure 32). This PNA-neocuproine-peptide conjugate was also inactive (Table 2), and it is not unlikely that one or more histidines bound copper ion thereby making the metal ion inaccessible to the phosphodiester functions, which is similar to the effect found when two neocuproines were conjugated to the same PNA. [108]

#### 4.3.1 Conclusion and future perspective

In this chapter, several PNAzyme constructs have been described. All of them originated from modifications of PNAzyme **1**. In one of the PNA-RNA models done in collaboration with Lennart Nilssons group, the N terminal appeared to be close to the phosphate cleaved by PNAzyme 1. An oligoether and a short His-peptide were accordingly attached to the N terminal of the PNA and/or in the proximity of the active site. The cleavage rates obtained with oligoethers suggest that the flexibility of an oligoether is does not cover enough of the active site to give a substantial solvation effect and it is difficult to position functionalities that may have an effect on solvation without a reliable 3D-structure. Such a structure of the PNAzyme:RNA complex would be useful in modeling the positioning of water replacing groups, addressing them specifically to the vicinity of the cleaved phosphate.

In collaboration with Martin Egli's group, we are attempting to obtain a crystal structure of the PNAzyme1:RNA complex. To enable the metal ion to be present and still avoid cleavage of the RNA substrate, we exchanged the ribonucleoside containing the attacking 2'-hydroxyl, i.e.

vicinal to the cleaved phosphate, with a deoxyribonucleoside or 2'O-methylribonucleoside (Figure 33, left panel).

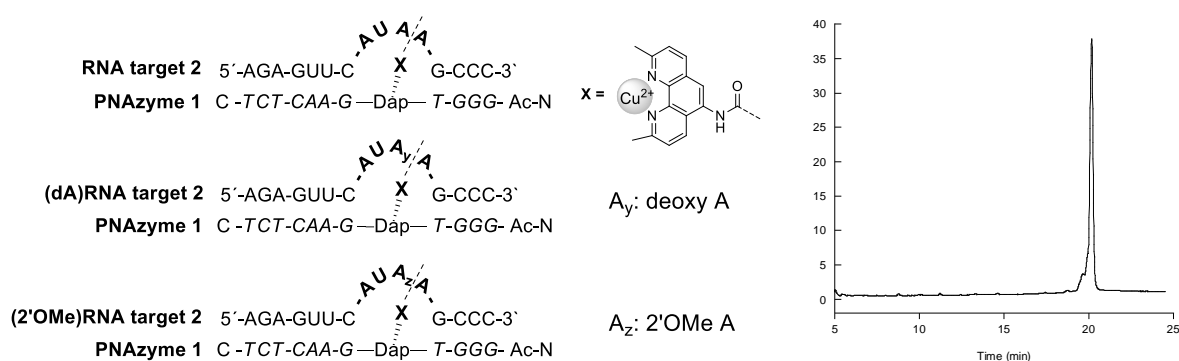


Figure 33: (Left panel) Complexes between RNA-target and PNAzyme 1 constructs; (right panel) IE-HPLC analysis of RNA target with bulge AU(dA)A cleavage by PNAzyme 1, using a ratio 1:1, pH 7 and 37°C, after 36 h of incubation.

The stability of the (dA)RNA and the (2'OMe A)RNA to PNAzyme 1 activity was tested. In both cases the mixed RNAs did not display any cleavage even after two days (Figure 33, right panel). The crystallization of both complexes is in progress in Egli's lab, and hopefully we will obtain some 3-D structures of either or both PNAzyme:RNA complexes which would be useful for further development of the project.

## 5 PNA based metal-free artificial ribonuclease tris(2-aminobenzimidazole), (*paper III and paper VI*)

One of the more efficient non-metal-ion based artificial ribonucleases reported is the tris(2-aminobenzimidazole), which was designed by Michael Göbel's group and also active when conjugated to an oligodeoxyribonucleotide.[109, 110]

The tris(2-aminobenzimidazole) design is the result of previous studies, where the influence of guanidinium ion on the phosphate had a central role (Figure 34, left panel).[110] The demonstrated cooperativity of proximal guanidinium ions [111] and the increase in rate due to the presence of heterocyclic analogues,[112] were the two main concepts combined in this new metal-free artificial ribonuclease.

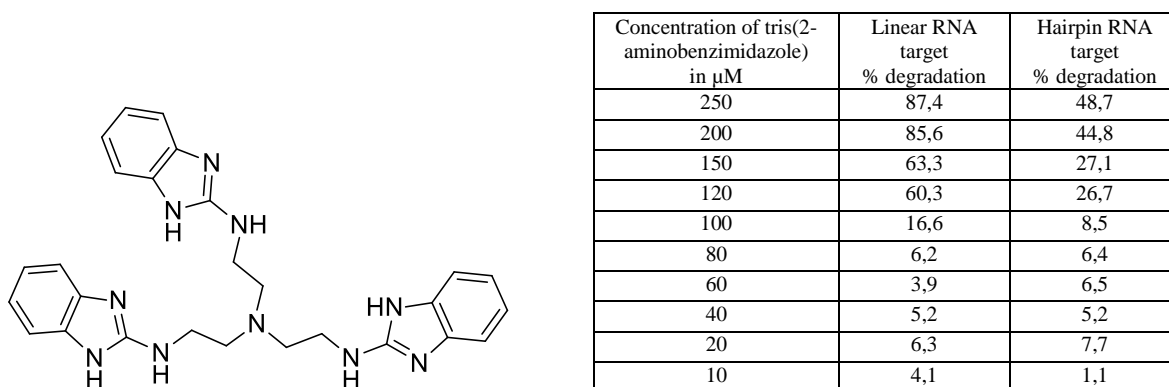


Figure 34: Tris(2-aminobenzimidazole) structure (left panel) and (right panel) RNA cleavage in the presence of 0.01% SDS, 120-140 nM RNA, 50 mM Tris-HCl, pH 6.0, 37 °C, 20 h.

Previous studies showed that moving from DMF to water retards the bisguanidinium ion (connected with a proper spacer) activated phosphate cleavage reaction dramatically, due to the high polarity of water, the concomitant strong solvation, and the complete protonation of the guanidine function in water.[110, 111] Both problems were partially overcome with the tris(2-aminobenzimidazole) which is characterized by a lower  $pK_a$  (from 14 for guanidines to 7 for tris(2-aminobenzimidazole)). To test cleavage activity of the new metal-free tris(2-aminobenzimidazole) artificial ribonuclease two substrates were chosen: a 31mer hairpin structure derived from HIV-1 TAR and a linear 29mer. The resulting half-lives are 120 min for the linear RNA and 200 min for the TAR analogue, in presence of approximately 9 times more catalyst, at pH 6,0 (Figure 34, right panel).

To study the properties of tris(2-aminobenzimidazole) as artificial ribonuclease, the next step was to conjugate the cleaver to an oligonucleotide. The oligonucleotide has the function of carrier, recognizing and cleaving a specific sequence and possibly also enhancing the cleavage selectivity and site-specificity.

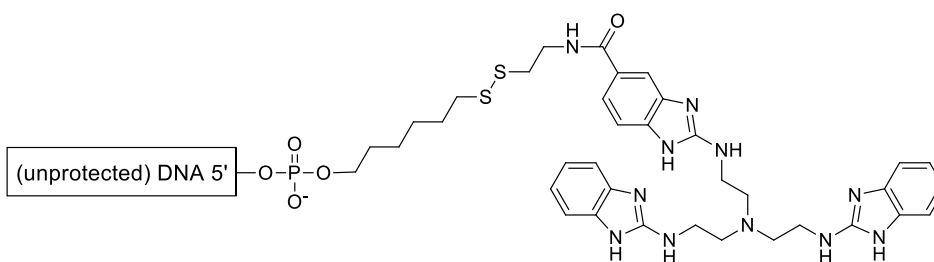


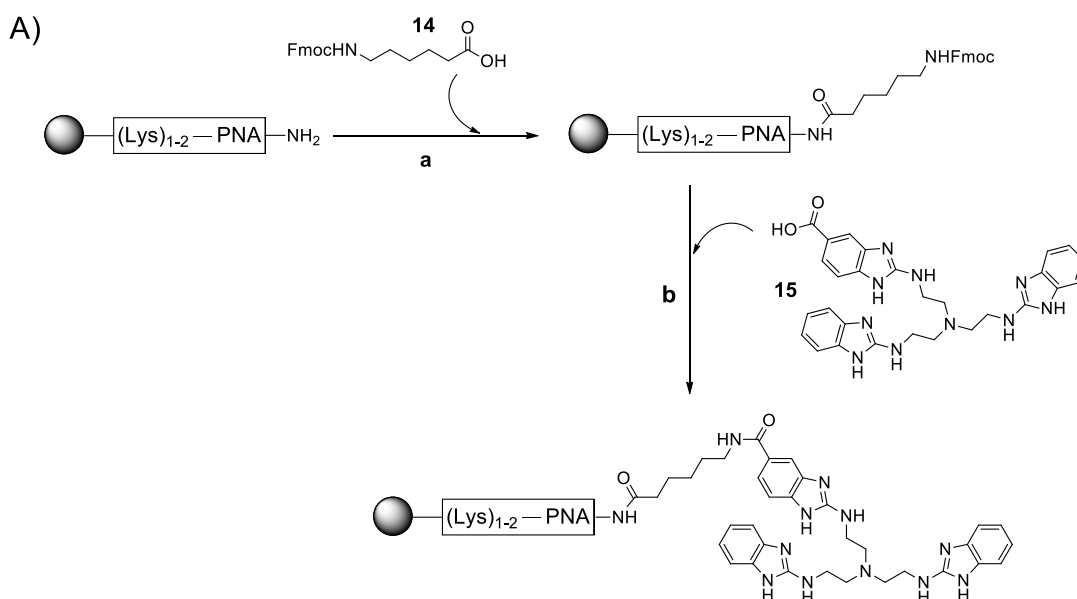
Figure 35: DNA conjugated with tris(2-aminobenzimidazole) cleaver unit.

Göbel et al. used DNA as oligonucleotide backbone for the first OBAN constructs and the effect of the linker on the rate of cleavage was studied (Figure 35).[109] It was observed that for all the conjugates the cleavage rate increased with pH, leveling off around pH 8. The cleaver activity seems to be associated to the form with at least one unprotonated guanidinium functionality. Most of the DNA based cleavers showed saturation kinetics, site and substrate specificity, giving half-lives of 12-17 h with excess of cleaver, but no turnover of the substrate.

### 5.1 Sequence-specific RNA cleavage by PNA conjugates of the metal-free artificial ribonuclease tris(2-aminobenzimidazole) (*paper III*)

When meeting each other regularly in a Marie Curie training network we decided join forces with the Göbel group and look at PNAzymes with tris(2-aminobenzimidazoles) as the cleaving entities. The use of PNA, instead of DNA, improves resistance against biodegradation and gives a higher affinity towards RNA.

The tris(2-aminobenzimidazole) unit (**15**) was conjugated to the PNA-oligomers on solid support (Figure 36A). To increase the solubility of the final PNA product, one to two lysine units were placed at the C-terminus. In order to have an attachment point for the cleaver, Fmoc-protected 6-aminohexanoic acid (**14**) was added to the PNA at the terminal amino group. DIC and HOBt were the reagents used for conjugation of both linker and cleaver.



B)

Conjugates with trisbenzimidazole:	RNA substrates and accessory oligonucleotides:
Lys- <b>AACAGTCCTC</b> - linker - cleaver <b>14</b>	Cy5-T10-ribo( <sup>5'</sup> <b>AUACCUUGUCAGGAG</b> AAGAGAGGCCGUUA)-T <sub>4</sub> <sup>3'</sup> <b>3</b>
Lys- <b>GCTGACGGCT</b> - linker - cleaver <b>15</b>	Cy5-T10-ribo( <sup>5'</sup> <b>CUAGCCGACUGCCGA</b> UCUCGCUGACUGAC)-T <sub>4</sub> <sup>3'</sup> <b>4</b>
LysLys- <b>TATGGAACAGTCCTC</b> - linker - cleaver <b>16</b>	Cy5-T10-ribo( <sup>5'</sup> <b>UAAAGUGCUUAUAGU</b> GCAGGUAG)-T <sub>4</sub> <sup>3'</sup> <b>5</b>
LysLys- <b>GATCGGCTGACGGCT</b> - linker - cleaver <b>17</b>	Cy5-TTTTTTTTTTTTTTTTTTTU <sup>3'</sup> <b>6</b>
LysLys- <b>ATTCACGAATATCA</b> - linker - cleaver <b>18</b>	<b>TGTGGAATTGTGAGCGGATA</b> <sup>3'</sup> <b>7</b>
	ribo( <sup>5'</sup> GGCG <b>CUAGCCGACUGCCGA</b> UCUCGCUGACUGAC) <sup>3'</sup> <b>8</b>

Figure 36: A) Scheme for the synthesis of the tris(2-aminobenzimidazole) PNA based artificial ribonucleases; B) lists of all the synthesized PNA constructs and respective RNAs used in the kinetic study.

All the cleavage experiments were run with Cy5-labeled RNA (fluorescent dye), allowing the quantification of RNA fragments after separation by gel electrophoresis in a DNA sequencer. Substrates **3** and **4** were used for comparison with previous results, described in the chapter introduction.[109, 110] RNA **5** is the sequence of miRNA 20a, a member of the oncogenic miRNA 17–92 cluster which is a target for site-specific RNA cleaver **18**. All the RNAs have a 3'-deoxythymidine stretch elongation which is used to help the separation and resolution of fragment peaks in the sequencer.

All the constructs were cleaved at all positions which are not protected by hybridization with the PNAs, with quite a broad pattern (

Figure 37). In the case of the 15mer-conjugate **17** and its cognate RNA **4**, there is no distinct cleavage pattern, probably due to stronger hybridization resulting from the G/C-rich sequence which makes the separation hard between PNA and RNA substrate.

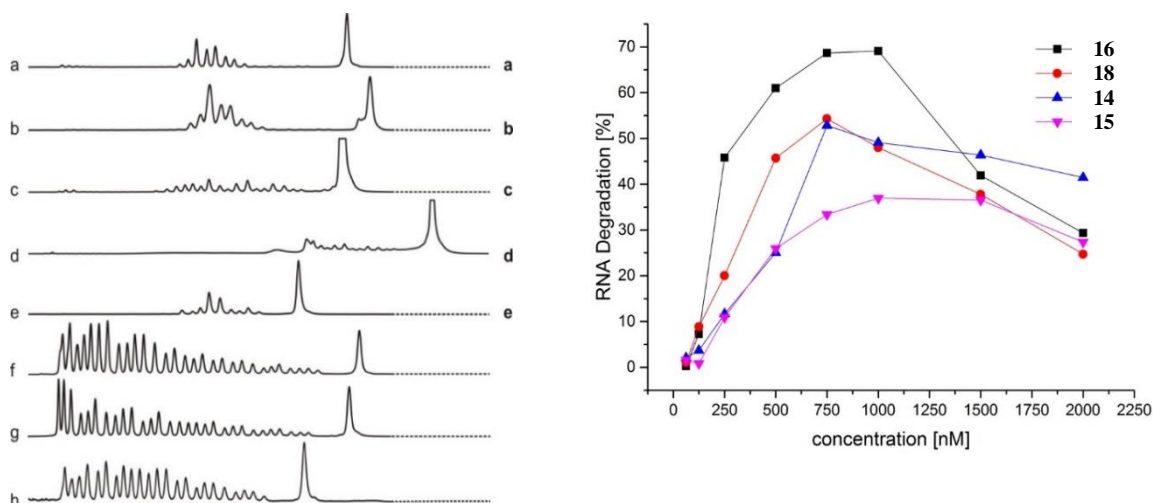


Figure 37: (Left panel) Cleavage of RNA by the corresponding PNA conjugates (150 nM substrate, 750 nM conjugate, 50 mM Tris-HCl, pH 8, 37 °C, 20 h). Lane a: conjugate **14** and substrate **3**. Lane b: conjugate **16** and substrate **3**. Lane c: conjugate **15** and substrate **4**. Lane d: conjugate **17** and substrate **4**. Lane e: conjugate **18** and substrate **5**. Lanes f, g and h: hydrolysis patterns of **3**, **4** and **5** (Na<sub>2</sub>CO<sub>3</sub>). (Right panel) Cleavage of RNA substrates **3**, **4**, and **5** by the matching conjugates as a function of conjugate concentration (150 nM substrate, 62.5–2000 nM conjugate, 50 mM Tris-HCl, pH 8, 37 °C, 20 h). Data points are connected by lines for the sake of clarity.

Because of the PNA's tendencies to form aggregates, which would interfere with the cleaving activity, the diffusion time of Cy5-labeled DNA **6** in absence and presence of PNA conjugates was studied by fluorescence correlation spectroscopy (FCS), but the results indicate no PNA aggregation. For the reaction of substrate **3** with conjugate **16**, cleavage kinetics were studied in detail. Almost complete degradation was achieved after 60 h. The data fitted to a first order rate equation and  $t_{1/2} = 11,2$  h. The rate comes close to the half-life of RNA **3** when cleaved by the analogous DNA conjugate ( $t_{1/2} = 12.4$  h).[109]

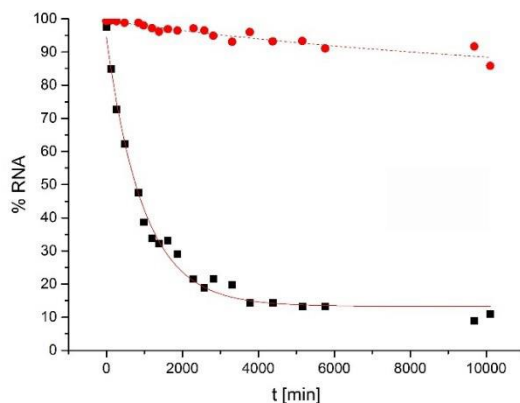


Figure 38: Cleavage kinetics of RNA **3** in the presence of conjugate **16** (black dots) or absence of PNA (control, red dots). Conditions: 150 nM substrate, 0 or 750 nM conjugate, 50 mM Tris-HCl, pH 8, 100 mM NaCl, 37 C. The solid curves are calculated assuming first order kinetics.

Tris(2-aminobenzimidazole) PNAs showed activity as hydrolytic cleavers with an efficiency similar to the corresponding DNA conjugates described above. The coupling of the Tris(2-aminobenzimidazole) unit to the PNA is faster and more convenient compared to the DNA 5' coupling. Further development is necessary to obtain turnover of substrate.

## 5.2 Studies on tris(2-aminobenzimidazole)-PNA based artificial nucleases and comparison of two analytical techniques (*paper VI*)

The fastest oligonucleotide based artificial ribonuclease at the time of this study was the peptide nucleic acid based artificial nuclease (PNAzyme **1**) carrying a Cu(II)-2,9-dimethylphenanthroline, designed in our laboratory. PNAzyme displays substrate half-lives as short as 30 minutes.[21] Metal-free synthetic nucleases are not at the risk of losing capacity and/or causing toxic effects by loss of the activating metal ions, but at present they are considerably less efficient in cleaving RNA as compared to the metal-ion systems.

### 5.2.1 Artificial ribonucleases kinetic studies: comparison between the two different analytical approaches.

As described in paper III, oligonucleotide conjugates of tris(2-aminobenzimidazole) cleave their RNA targets with half-lives ranging from 11 to 20h. [109, 113] These studies were based on completely different analytical approaches than we are using for evaluation of PNAzymes. The question posed was to what extent the results depend on the chosen method. In collaboration with Göbel's group, we decided to do a cross-check study of analytical methods

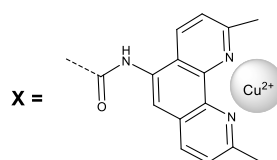
to identify their individual strengths. These results may also be advisory to other practitioners in the field.

Our method to qualify and quantify the RNA cleavage process is based on anion exchange HPLC (IE-HPLC). This technique is fast and proven to be accurate.[114, 115] In combination with mass spectrometry (ESI-TOF) it characterizes the RNA fragments which are products of the cleavage and defines precisely the positions of phosphodiester bond breakage.

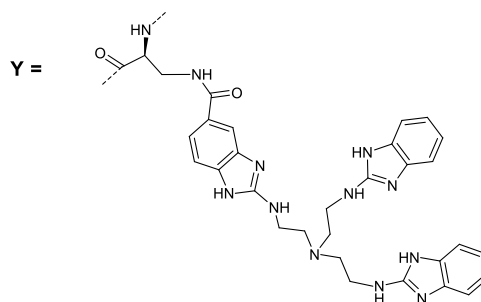
The other method, pioneered in the Frankfurt lab for the application of RNA cleavage characterization is done with a DNA sequencer. Cy5-labelled oligonucleotide fragments are separated by denaturing PAGE on the DNA sequencer and the readout, compared with the hydrolysis ladder of the substrate, defines the position where cleavage occurs.[116] Both techniques imply integration of peak areas to quantify the degraded RNA.

In order to compare the two techniques, PNAzyme **1** [86] cleavage of RNA (Figure 39), that has already been analyzed by our IE-HPLC method, was first used to evaluate the consistency relative to the DNA sequencer method (Figure 40).

**PNAzyme 1**



**PNAzyme 2**



**RNA substrates for gel electrophoresis:**



**RNA substrates for IE-HPLC:**

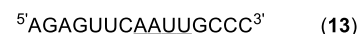


Figure 39: Sequences of PNAzymes (**1** and **2**) and oligonucleotides substrates. Underlined areas are non-complementary region where bulge formation is expected.

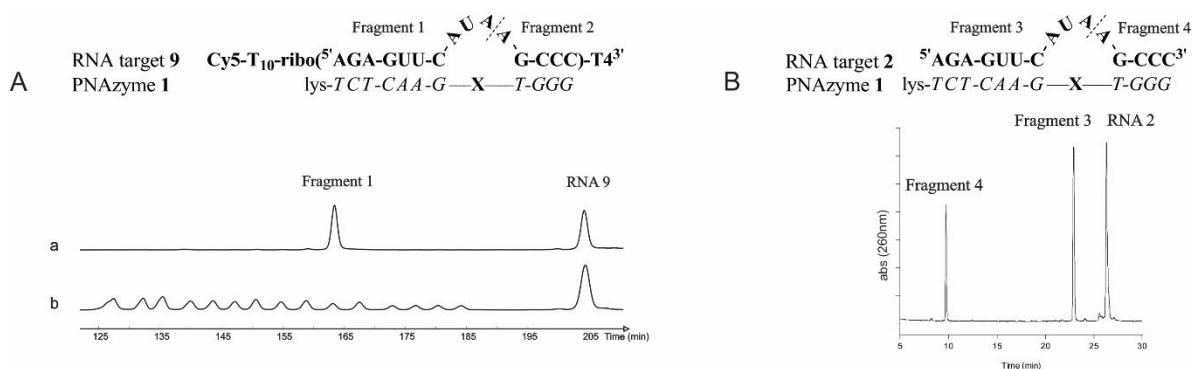


Figure 40: Comparison of two techniques showing site-specific cleavage of the target RNAs **9** and **7** as induced by PNAzyme 1. A: 4  $\mu\text{M}$  of RNA target **9**, 10  $\mu\text{M}$  PNAzyme 1, 10 mM HEPES buffer, pH 7.4, 0.1 M NaCl, 37°C, 20  $\mu\text{M}$   $\text{Cu}^{2+}$ , (a) 90 min incubation of PNAzyme **2** and RNA **9**, (b) Hydrolysis ladder of substrate **9**; B: 4  $\mu\text{M}$  of RNA target **7**, 4  $\mu\text{M}$  PNAzyme **1**, 10 mM HEPES buffer, pH 7.4, 0.1 M NaCl, 37°C, 10  $\mu\text{M}$   $\text{Cu}^{2+}$ , 30 min

The cleavage was obtained with single bond breakage in the bulge region between nucleotides A10-A11, as expected from the analysis with the IE-HPLC method shown in Figure 40B. With the HPLC-method both cleavage fragments are visible and were analyzed with MS in order to identify the cleavage position. The cleavage rates for RNA target **9** and RNA substrate **2** are in the same range, slightly lower for the Cy-5 substrate, maybe due to the substrate elongation with the terminal polyT which may soak up some copper ions as also indicated by the need to use a somewhat higher metal ion concentration. The electrophoresis/sequencer based technique has the advantage of assigning the cleavage position without the need for MS analysis, whereas the IE-HPLC/ESI-TOF based technique is giving an exact identification of the cleavage products and the ability to distinguish between 2',3'-cyclic phosphate and the hydrolysis of its products.

### 5.2.2 Tris(2-aminobenzimidazole) PNAzyme

We show for the first time RNA cleaving activity of a new PNA based tris(2-aminobenzimidazole) construct, which has the catalyst conjugated in the position usually occupied by the 2,9-dimethyl-phenanthroline in several of our PNAzyme systems.

PNAzyme **2** was synthesized by conjugating the trisaminobenzimidazole carboxylic acid **15** to PNA on solid support using the same procedure as in the preceding paper, paper IV. In order to evaluate the cleaving activity, PNAzyme **2** was incubated with four oligoribonucleotide RNA substrates **9**, **10**, **2** and **14**, which form tetranucleotide bulges upon hybridization. The formation of bulged RNAs upon hybridization can promote cleavage and turnover of target RNA compared to duplex structures, due to their conformational flexibility (see chapter 2.1).[73, 117]



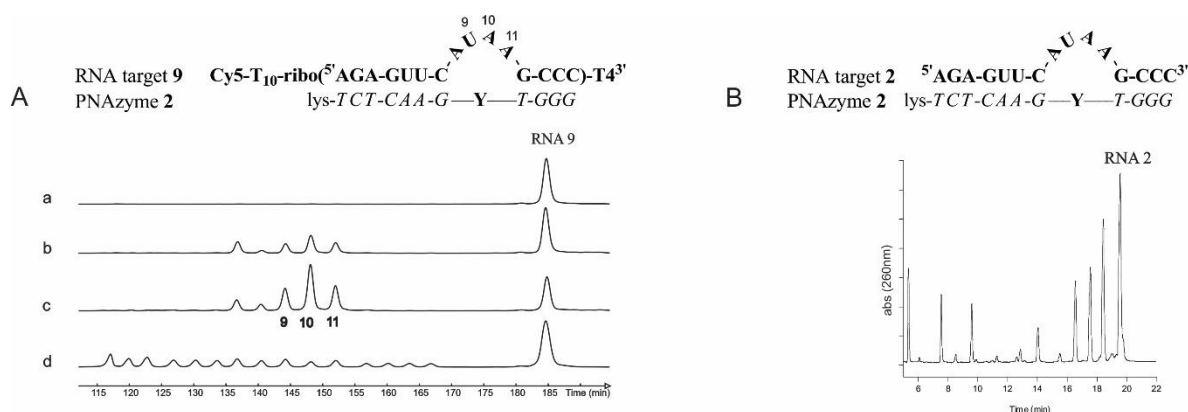


Figure 41: Comparison of two techniques examining the cleavage of the target RNAs 3 and 7 induced by PNAzyme 2. A: 4  $\mu$ M of RNA target 3, 4  $\mu$ M PNAzyme 2, 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 37°C, (a) 0min, (b) 24h, (c) 40h (75 % cleaved RNA target 3), (d) Hydrolysis ladder of substrate 3; B: 4  $\mu$ M of RNA target 7, 4  $\mu$ M PNAzyme 2, 10 mM HEPES buffer, pH 7.4, 100 mM NaCl, 37°C, 24 h.

PNAzyme 2 was first incubated with Cy5 labelled RNA target 9 in equimolar amounts at pH 7.4 (Figure 41A). The cleavage of the RNA occurs mainly in the bulge region, specifically in positions U9, A10, A11. This result was confirmed by cleavage of the corresponding RNA target 2 used when evaluating cleavage with the IE-HPLC based analysis (Figure 41B). It is clear that PNAzyme 2 causes bond breakage only in the bulged out region of the RNA substrates 9 and 2 but without much selectivity for a single position. The cleavage rates obtained are also comparable for these two substrates (ca 22 and 17 h respectively). Two kinetic studies with non-complementary RNA sequences 11 and 12, were performed in order to exclude natural nuclease cleavage. In both cases there was no RNA degradation.

Since PNAzyme 2 has the advantage of being metal-free, we considered to analyze it in more detail at different pH and to vary the RNA bulge, as done for PNAzyme 1 (Figure 42).[86]

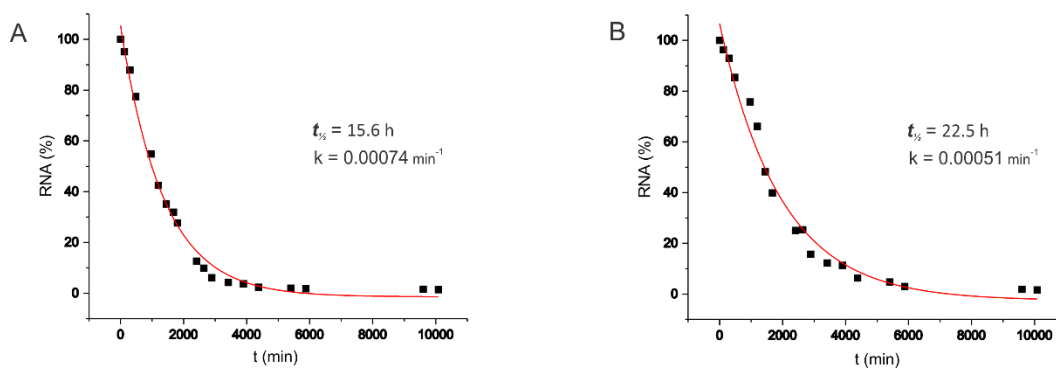


Figure 42: Cleavage kinetics of RNA 9 in the presence of PNAzyme 2 (from sequencer analysis). Conditions: 4  $\mu$ M of RNA 9, 4  $\mu$ M PNAzyme 2, 50 mM Tris-HCl, 100 mM NaCl, 37°C; A: pH 8; B: pH 7.4

As already shown by the previous described system with tris(aminobenzimidazole) cleaver attached at the end of the PNA, the rate of cleavage is higher at pH 8. Changing the bulge sequence from AUAA to AAAA (RNA substrate 1) we observed a rate improvement (Figure 43A) which is opposite to what we observed for the Cu(II)-phenanthroline based PNAszymes. The same relation between the different bulges was confirmed by electrophoresis/sequencer based analysis of cleavage of RNA sequences 10 (Figure 43).

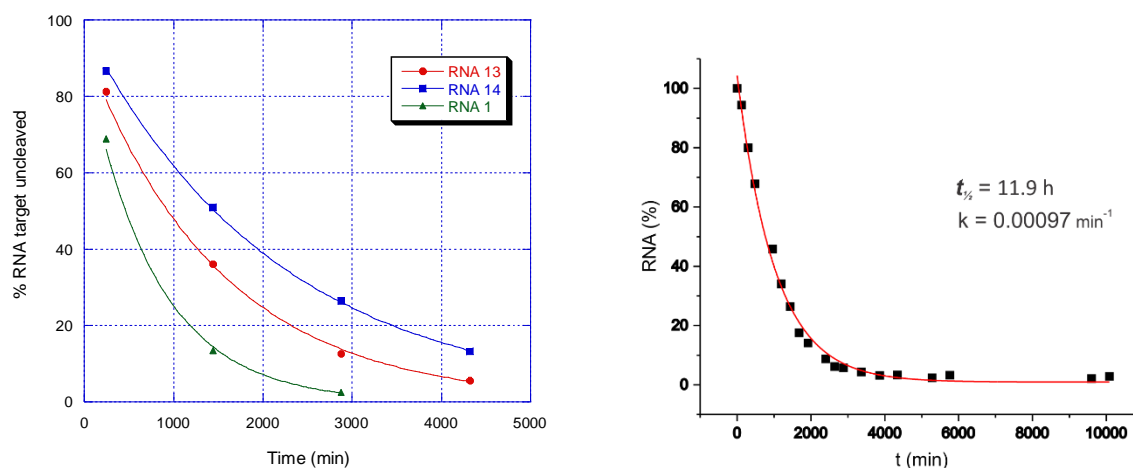


Figure 43: A) Extent (%) of uncleaved RNA substrate **1**, **13**, **14** as a function of time (as analyzed by IE-HPLC). Conditions: 4  $\mu\text{M}$  of RNA targets **1**, **13** and **14**, 4  $\mu\text{M}$  PNAzyme **2**, 10 mM HEPES buffer, pH 7, 100 mM NaCl, 37°C. Half-lives for substrates **1**, **13** and **14** are about 9, 17 and 25 h respectively. B) Cleavage kinetics of RNA **10** in the presence of PNAzyme **2** (from sequencer analysis). Conditions: 4  $\mu\text{M}$  of **4**, 4  $\mu\text{M}$  PNAzyme **2**, 50 mM Tris-HCl, pH 8, 100 mM NaCl, 37°C. Half-life for substrates **10** is about 12 h.

RNA substrate **13**, which has two uridines in the bulge, was cleaved at a rate similar to RNA substrate **2** and when the wobble GT pair next the bulge was changed for an AT Watson Crick base pair (RNA substrate **14**) the rate dropped further (Figure 43). Use of an imidazole buffer gave similar results as use of a HEPES buffer in cleavage of RNA substrate **2** by PNAzyme **2**, suggesting that imidazole in solution does not co-act in the catalysis. The addition of  $\text{Cu}^{2+}$  ions retards substantially the reaction probably because of an interference with the tris(aminobenzimidazole) action.

### 5.2.3 Turnover experiments with PNAzyme 2

In additional experiments, PNAzyme **2** was incubated with five times excess of RNA substrate **9**. The result shows that after the first week most of the substrate was degraded (Figure 44).

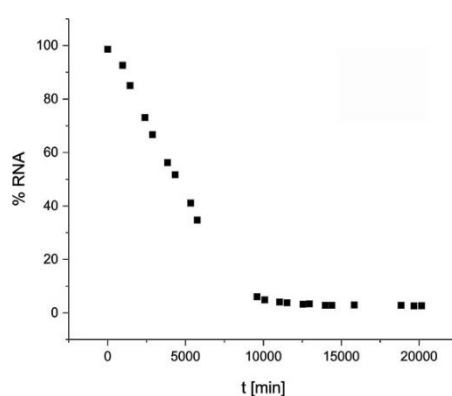


Figure 44: Turnover kinetics of substrate RNA **9** in the presence of conjugate **2**. Conditions: 4  $\mu\text{M}$  substrate RNA **9**, 0.8  $\mu\text{M}$  conjugate **2**, 50 mM Tris-HCl, pH 8, 100 mM NaCl, 37°C.

The newly designed tris(2-aminobenzimidazole) based PNAzyme **2** is thus demonstrated to be an artificial nuclease that turns over the RNA substrate and is the first tris(2-aminobenzimidazole) system where this is shown. This non-metal ion artificial nuclease preferably cleaves in the bulge region formed upon hybridization to the RNA substrate and

displays sensitivity to both bulge sequence and to the bulge closing base pair. Tris(2-aminobenzimidazole) PNA cleaver showed cleavage action towards a microRNA substrate (miRNA 20a), which may be a starting point for future in vitro and in vivo experiments.

## 6 Clamping of RNA with PNA

### 6.1 Triplex structure: Hoogsteen hydrogen bonds and parallel/antiparallel orientation.

Nucleic acid triplex structures are complexes formed by a double stranded nucleic acid and a third single strand, called 'triplex forming oligonucleotide', TFO. The TFO binds usually in the major groove of the duplex forming Hoogsteen base pairs. The binding can be with parallel orientation (parallel triplex) or in antiparallel orientation with reverse Hoogsteen base pairing (antiparallel triplex) (Figure 45).[118, 119]

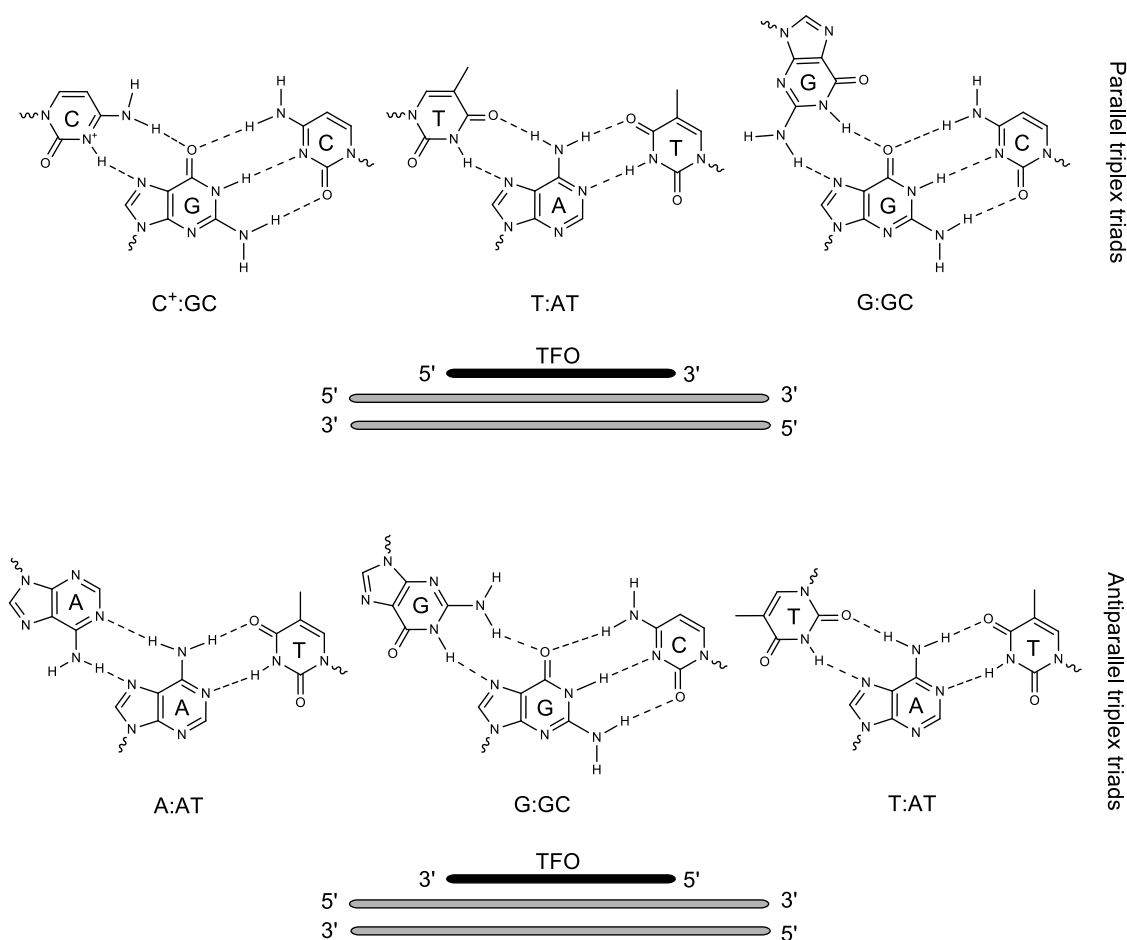


Figure 45: Schematic presentation of parallel and antiparallel triplex complexes.

The Hoogsteen base pair geometry, was observed for the first time in crystal structures with monomeric adenine and thymine base derivatives [120, 121]; the Hoogsteen binding is characterized by hydrogen bonds between the side of the purine base that faces the major groove of the duplex and the Watson Crick base pairing face of the pyrimidine in the third strand. There are essentially six different triads that can form a triplex. In the case of C<sup>+</sup>:GC triad, the formation of a second Hydrogen bond between the G of the duplex and the C of the TFO requires protonation of the cytosine N3 to form two hydrogen bonds. This makes the triad dependent on pH [122] and its stability is quite weak at physiological pH. One of the ways to

circumvent the problem is to substitute the natural C with a synthetic pseudoisocytosine ( $\Psi$ iC), which can form two hydrogen bonds also at neutral pH (Figure 46).[123]

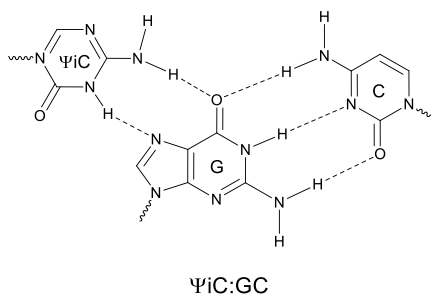


Figure 46: Structure of the  $\Psi$ iC:GC triad.

Oligonucleotides can bind in a sequence specific way to both double stranded DNA and RNA to form triplex structures. Two of the problems for these complexes is the stability and the dependence on pH. Several modified nucleobases has been synthesized to increase the stability of the dsDNA:DNA or dsDNA/RNA triplexes at neutral pH. Examples are the 5-methylcytidine,[124, 125] 2'-O-methylpseudoisocytidine,[126] 8-aminopurines[127], at neutral pH.

## 6.2 DNA:PNA or RNA:PNA triplexes.

There are many reported examples of PNA/DNA triplexes like the  $\text{PNA}_2$ :DNA. This type of triplex is formed when a purine containing DNA strand forms Watson-Crick base pairs with a homopyrimidine PNA strand in an antiparallel orientation and deploys a second set of base-pairing hydrogen bonds, according to a Hoogsteen base pairing scheme, to the second PNA strand of identical sequence running parallel to the DNA.

Double stranded RNA helixes have a deeper and narrower major groove which could hinder the binding with a third strand. However, Rozners et al. has shown that PNA can form stable triplexes with dsRNA at physiological pH [128], and even at physiological pH when pseudo isocytosine or 2-aminopyridine bases are included.[129] Homopyrimidine PNAs, due to their ability to form thermally stable duplexes and triplexes with DNA and RNA, are suitable to form  $\text{PNA}_2$ :RNA triplexes with the complementary RNA target. [82, 130]

The  $\text{PNA}_2$ :RNA triplex synthesis project started with an idea to obtain a preorganized structure where the extremities of the PNA can be used for further conjugations. This will allow modification of the physical properties of the obtained triplex. One of the aims of the designed triplex is to increase the rate of cleavage of our artificial ribonuclease PNAs (PNAzyme) providing a new arm that can reach the active site, without interfering with the catalytic activity of the  $\text{Cu}^{2+}$ -neocuproine site.

There are essentially two ways to form the triplex to obtain the effect we aim to: one with the RNA target in the center of the triplex structure and the other one with one strand of PNA in

the center of the triplex. We started the study from structure a in Figure 47 and by testing the best length for the triplex forming unit for the clamping PNA.

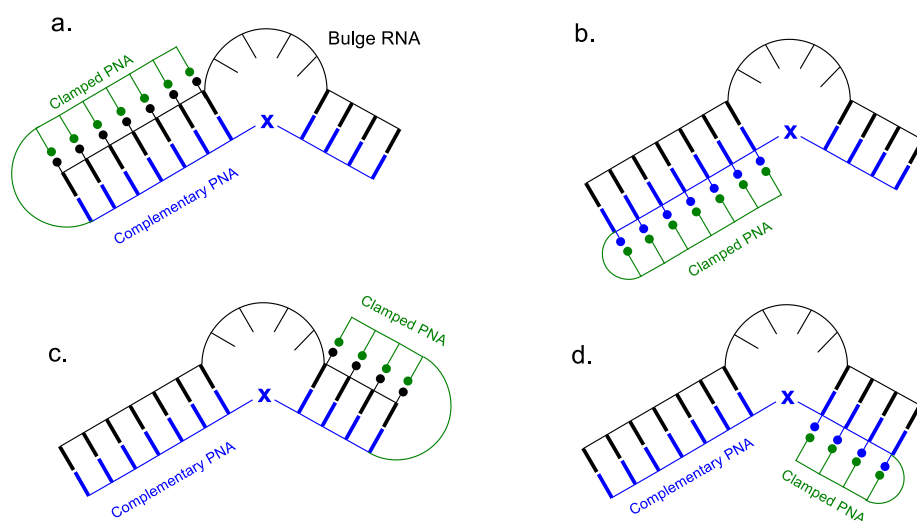


Figure 47: Schematic representation of the PNA<sub>2</sub>:RNA triplexes (bold lines are Watson Crick bonds and dots are Hoogsteen bonds).

The first triplexes designed has only TAT triads. We initially avoided the use of G in the RNA sequence to avoid pH dependence or introduction of a non-natural base in the PNA strands that should form the Hoogsteen pairing of the triplex.

### 6.3 The challenge of targeting microRNA

Since its discovery in 2000,[131] micro-RNA (miRNA) which is a non-coding, short regulatory RNA molecule, has gained a lot of interest because of its post-transcriptional regulation control of gene expression. More than 2500 miRNAs has been described for humans, covering around 3% of all human genome and controlling at least 40% of the protein coding genes, and the numbers are increasing.

Mature microRNA is generated through a primary miRNA, called pri-miRNA, characterized by a complex secondary structure of loops, (Figure 48). These loops are the target of a RNase endoribonuclease, DROSHA, and DGCR-8, which is a second enzyme involved in the cleavage process.[132] The product of the enzymatic cleavage are 70 nt hairpin RNA miRNA precursors (pre-miRNAs). The pre-miRNAs are then exported to the cytoplasm with the aid of an exportin and processed by Dicer into the mature short miRNA duplexes. One strand of the mature miRNA will be loaded on the RISC complex.[133]

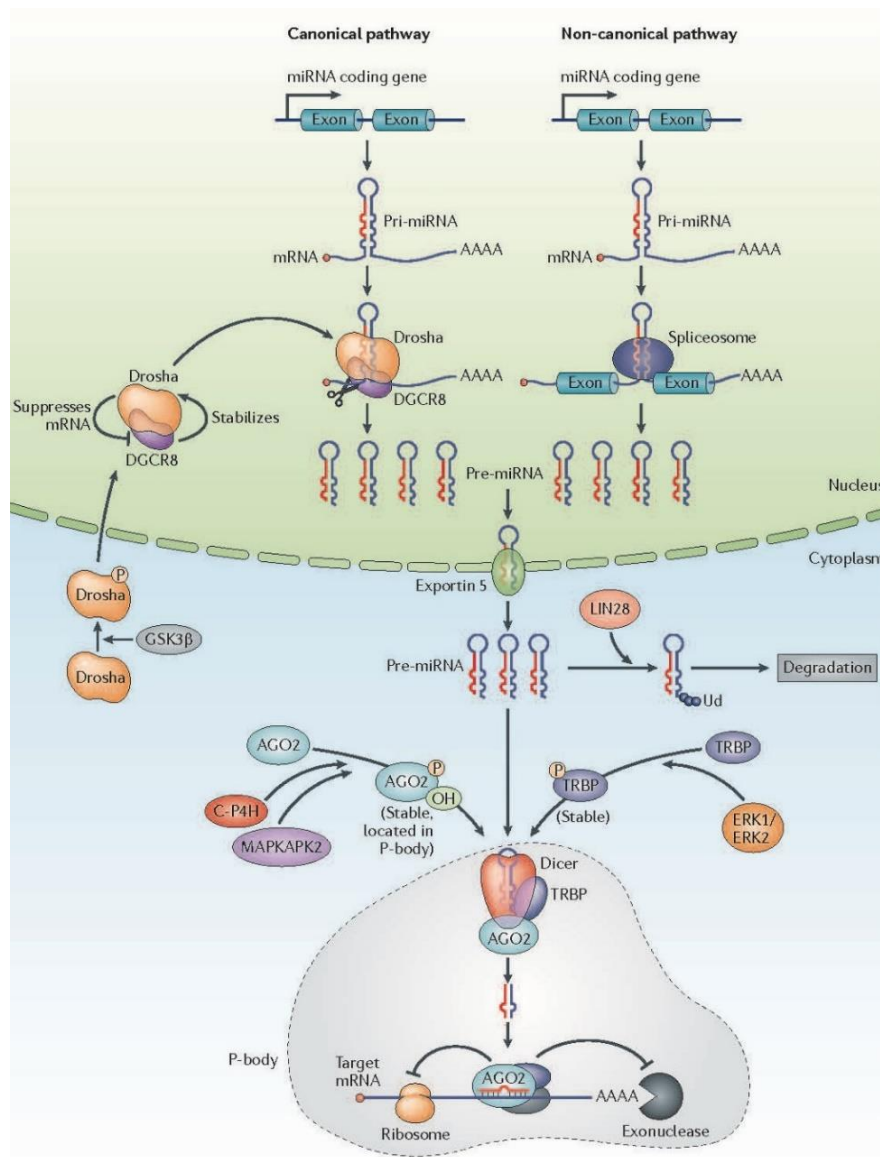


Figure 48: Biogenesis of the mature miRNA. MicroRNAs are also produced through non-canonical pathways, such as spliceosome-dependent mechanisms, as shown here. Picture adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Drug Discover] [134], copyright (2014).

MicroRNAs play a crucial role in human diseases, and are therefore, interesting targets for disease therapy. Until today, many diseases were not curable because of non-efficient protein regulation by drugs. The possibility to regulate them through miRNAs initiates a new way of designing the therapy: miRNA inhibitors can induce selective upregulation of one protein population and miRNAs and mimics can induce gene silencing, thus resulting in downregulation of the target protein, or in the other way around depending on the role of the microRNA.[135]

There are mainly three approaches that so far have been used for therapeutic targeting of miRNA (Figure 49) [134]:

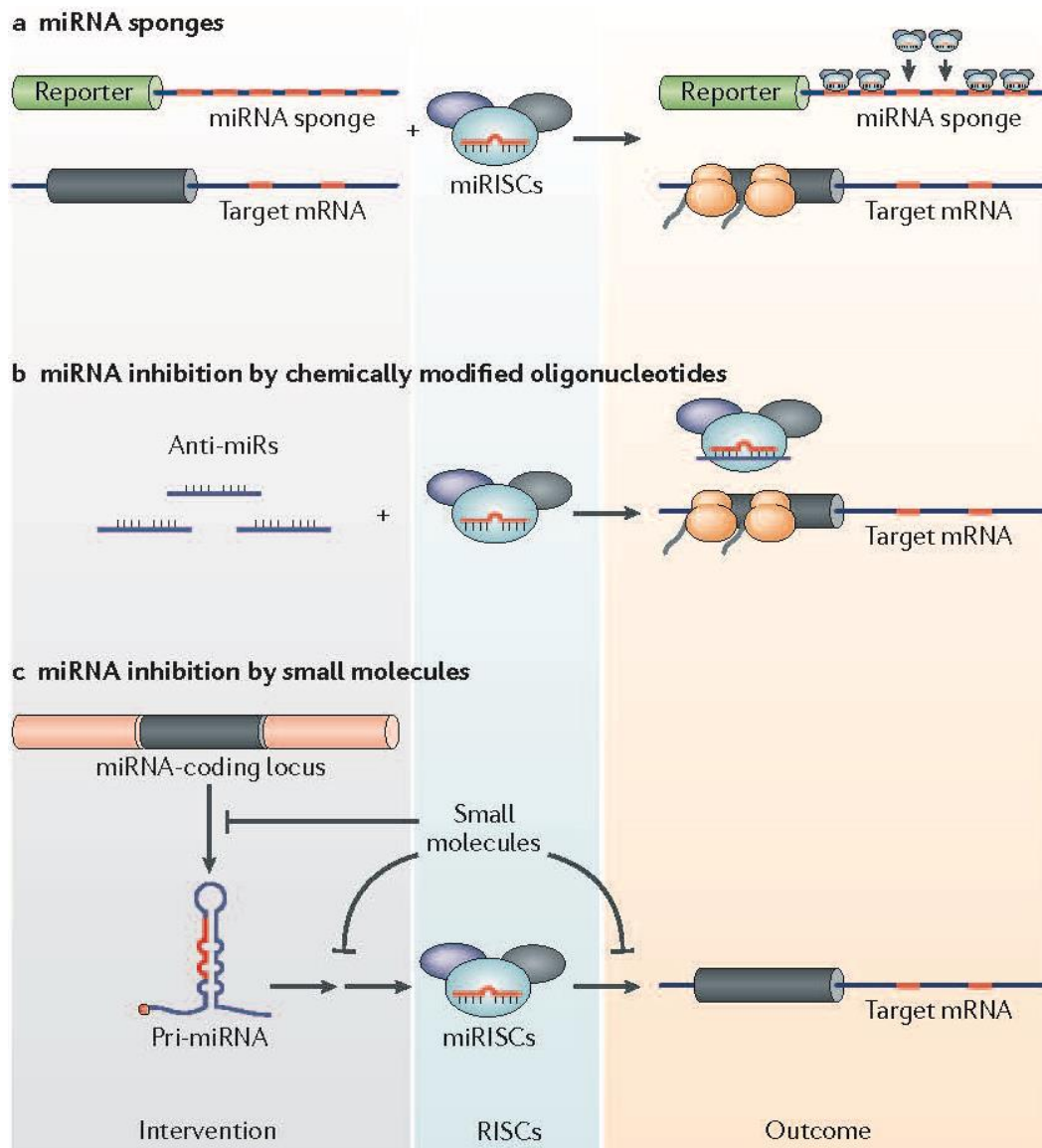


Figure 49: miRNA inhibition strategies. a) MicroRNA sponges; b) Antisense oligonucleotides (ASO); c) Small-molecules inhibitors. Picture adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Drug Discover] [134], copyright (2014).

- a) MicroRNA sponges, which are microRNA inhibitors expressed from strong promoters, containing multiple binding sites to a microRNA of interest. Overexpression of the miRNA sponges, reduces the endogenous microRNA, starting the expression of the mRNA.[136, 137] MicroRNA sponges contain four to ten binding sites which are separated by a few nucleotides. Their efficacy depends mostly on the concentration of sponge RNAs relative to the concentration of the miRNA, which is a limitation for their use: when miRNA concentration is high, the treatment requires an unachievable dose of sponge RNA. Another limitation is the success evaluation of the treatment since there is no miRNA deletion, but an inhibition of mature miRNA.
- b) Antisense oligonucleotides (ASO) work as competitive inhibitors of microRNAs, annealing to the mature microRNA strand after the RISC complex has removed the second strand. The first example of microRNA ASO is from 2004, where a 2'-OMe RNA was



successfully tested as a RISC blocker.[138] In 2008 the first clinical trial targeting a microRNA to treat hepatitis C started. The targeting agent used was an LNA based oligonucleotide complementary to miR-122 and, two years after, the therapy was judged efficient and the phase II was started.[139]

- c) Small-molecule inhibitors, which act as transcriptional regulators. In 2008 Gumireddy et al. reported a cellular screen for miRNA-pathway inhibitors and the first small-molecule modifiers of miRNA function using miR-21 as target, which is known as a antiapoptotic factor in cancer cells.[140] Over the years many small-molecules inhibitors have been designed but their use is usually limited because of the high EC<sub>50</sub>.

## 6.4 Clamping of RNA with PNA enables targeting of microRNA (paper IV)

MicroRNA is, at most stages of maturation, generally in a duplex or in hairpin form. Oligonucleotides with the ability to perturb the double stranded RNA or form a stable triplex structure with the targeted miRNA should be efficient in the process of up or down regulation. PNAs are good candidates as ASO agent because of their stability to nucleases and their ability to perturb a double stranded DNA. In 1995, Dias et al. showed that short PNAs, containing contiguous pyrimidine residues, can disrupt a targeted 27-mer RNA hairpin structure upon Hoogsteen binding.[141] The translation process of an mRNA was blocked with a BisPNA clamp.[142] Few reports can be found on use of PNA for targeting microRNA although generally by duplex formation [143, 144], and often focused on promoting uptake of the PNA.[145-147]

There are not many examples of complexes of two strands of PNA with RNA reported and there is no data on thermal melting/stability, only a single biological readout, on BisPNA clamps binding to RNA. Thus, we decided to investigate the stability of complexes between BisPNA clamps and RNA, and then to try to target and strand invade a hairpin microRNA.

### 6.4.1 Initial studies on (PNA)<sub>2</sub>:RNA

We started the study by synthesizing several BisPNAs and then measuring the melting temperature of the BisPNA:RNA complexes where the single strand RNA target is an alteration of our most common PNAzyme target (M-BCR/ABL mRNA). The RNA has six adenosines at the 5' end, which is the polypurine strand of the designed triplex. The first BisPNA (BisPNA **1**, Figure 50a) synthesized has a polyT strand with a linker in the middle (two AEEPs, Linker **1**). Comparing the thermal melting of the PNA:RNA target **1** complex (which is the reference duplex) and BisPNA **1**:RNA target **15** complex, we observed an increase of 8 °C, showing that the thymines added after the linker did improve the affinity of BisPNA **1** to the RNA target.

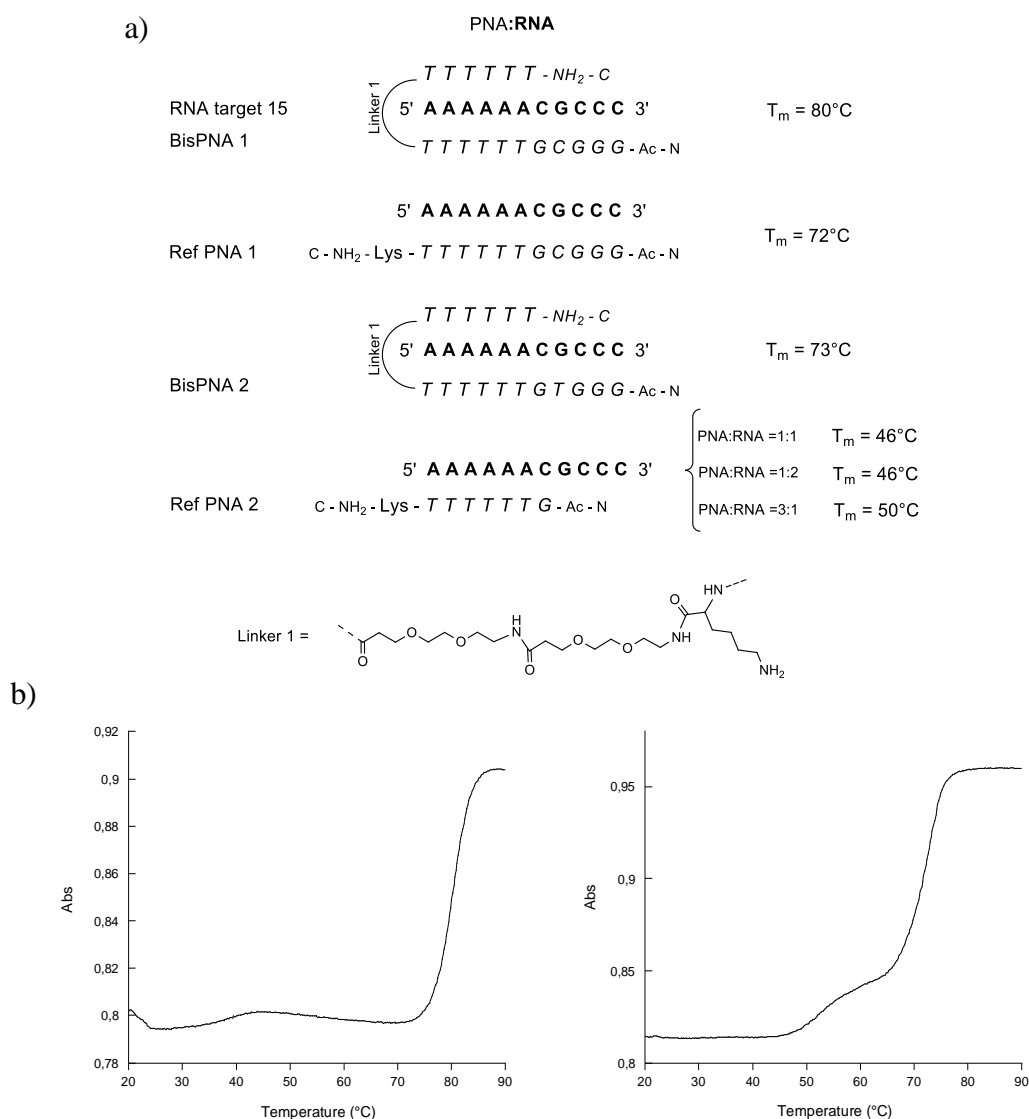


Figure 50: a) Schematic representation of BisPNA:RNA complexes and corresponding melting temperatures; b) thermal denaturation analysis of BisPNA 1:RNA target **15** (left panel) and BisPNA 2:RNA target **15** (right panel).

The melting curve of the BisPNA 1:RNA target **15** complex was almost outside of the recordable region (Figure 50b left panel). Therefore we synthesized a construct where the GC base pair is swapped for a GT wobble pair (BisPNA 2) in order to lower the melting of the complex. The thermal denaturation analysis of the complex with BisPNA 2 shows a clear bi-phasic transition (Figure 50b, right panel) which further indicates that a triplex is formed in the complex. The triplex formation is supported also by the results obtained from the interaction of RNA target **15** with a shorter PNA with only six T and one G (Ref PNA 2, Figure 50a): when the T<sub>m</sub> was measured at a PNA:RNA ratio of 3:1 we got a 4 °C increase, compared to the T<sub>m</sub> obtained at 1:1 ratio.

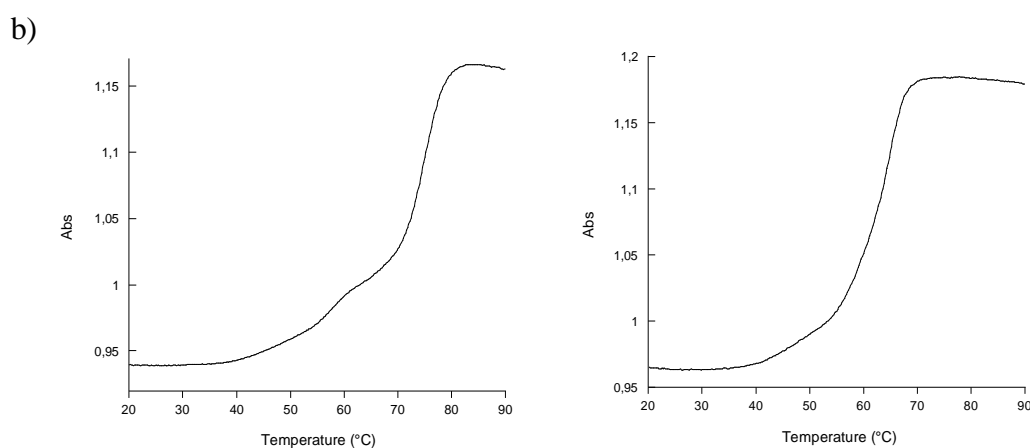
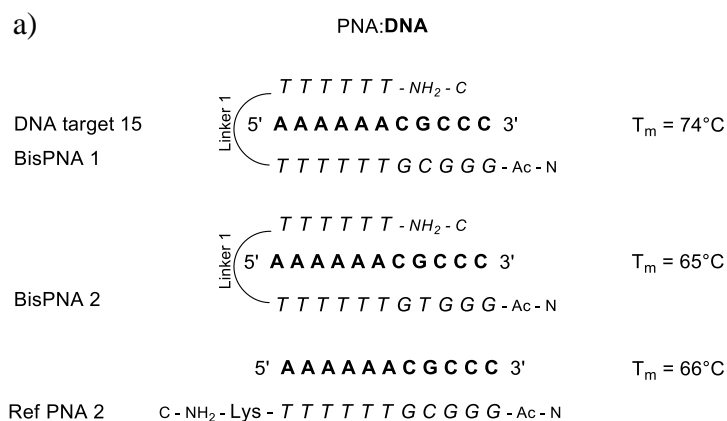


Figure 51: a) Schematic representation of BisPNA:DNA complexes and corresponding melting temperatures; b) thermal denaturation analysis of BisPNA 1:DNA target 15 (left panel) and BisPNA 2:DNA target 15 (right panel).

Comparing the melting data obtained for RNA complexes with those obtained for PNA:DNA complexes (Figure 51b) it is immediately clear that the BisPNA is more easily dissociated when using DNA as a target. Whereas the differences in  $T_m$  values between the clamped complexes and the reference duplexes are similar, the difference between the RNA complex and the DNA complex is 6 °C. The stabilization obtained adding the clamped strand is more or less the same for RNA and DNA complexes but with the RNA the BisPNA complex is more stable one, as we would interpret it, mainly due to the more stable PNA:RNA duplex part.

The initial idea was to synthesize a clamp PNA which could form a stable triplex with a feasible target RNA. Since it would be quite rare to find a natural RNA target which is composed of a 6nt polyA, we proceeded testing the same type of complex with a polypurine sequence composed of both guanosines and adenosines.

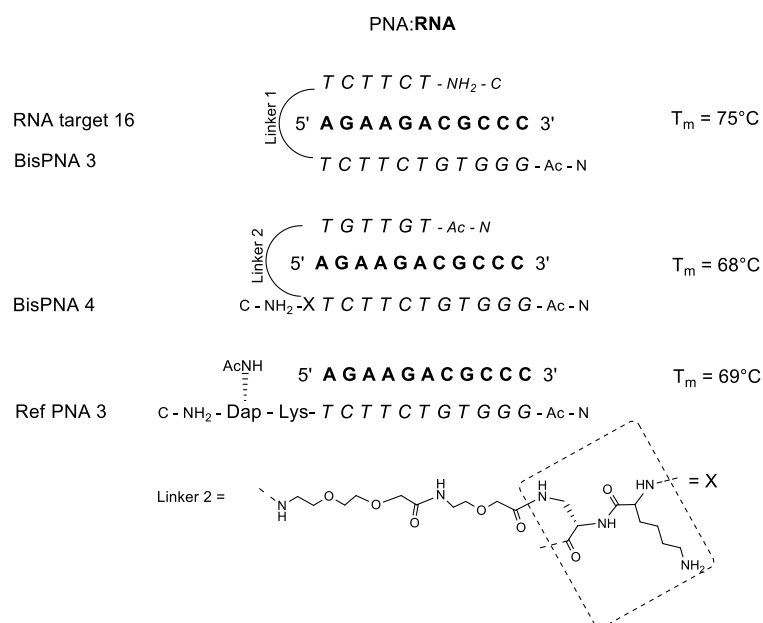


Figure 52: Schematic representation of the parallel and antiparallel BisPNA:RNA complexes and the corresponding melting temperatures.

The complex BisPNA **3**:RNA target **16** also gave a higher  $T_m$  than the duplex with Ref PNA **3** (Figure 52) showing a stabilization with the PNA clamp, as was already observed for complex with the polyA RNA target. As described in the chapter 6.1, at physiological pH and without exchange of the C to a pseudoisocytosine, there will be only one Hoogsteen H-bond in the CGC triad, so we expected some loss in stability, but not more than that the clamp is substantially more stable than the corresponding duplex. We also synthesized BisPNA **4**, designed to form an antiparallel triplex, by switching direction of the PNA during synthesis and using a diaminopropionic acid branching point to link the two C-terminals of the duplex. The BisPNA **4**:RNA target **16** complex shows no stabilization if compared to the duplex, which is quite unexpected since the G:GC triad has one hydrogen bond more than the triad C:GC at physiological pH.

We don't have a clear proof of (PNA)<sub>2</sub>:RNA triplex formation in the above complexes but from the CD analysis there are some structural differences present in the spectra. Results are still difficult to interpret with no reported CD studies on PNA:RNA:PNA triplexes. The stabilization by adding the clamping nucleotides as well as the increased melting with excess of Ref PNA **2** also does suggest triplex formation.

#### 6.4.2 Targeting miR-376b

From the results of the thermal denaturation analysis, we concluded that it should be worthwhile to target a double stranded hairpin microRNA. A truncated version of miR-376b was chosen. MiR-376b is a human microRNA which is known to control autophagy,[148] the degradation pathway which is used by the cells to degrade long-lived proteins and organelles like mitochondria and generate necessary nutrients and building blocks (e.g., amino acids and fatty acids).[149] Autophagy is also a key process for intracellular defense in response to

infection [150, 151], not least in connection of bacteria and viruses causing global health problems such as tuberculosis [152, 153] and HIV infection.[154]

Abnormalities in the autophagy process have been reported to be associated to cancer and neurodegenerative diseases and it is targeted for drug development.[155] Hsa-miR-376b was for example shown to be expressed in anomalous way in a number of human cancer types, including lung cancer, renal cell carcinomas, and ischemia. Korkmaz et al. showed that autophagy suppression is usually promoted during tumor formation and it is therefore an interesting target for a duplex invasion experiment.[148]

The shortened version of miR-376b we are targeting, is a hairpin with a 9 nt long polypurine stretch and it contains the entire region of the mature miRNA after processing by Dicer (Figure 53). Two AntimiR PNA clamps, differing from each other in the triplex forming unit, were synthesized: AntimiR **1**, is designed to form a parallel triplex with TAT and CGC triads, whereas AntimiR **2**, an antiparallel one with TAT and CGG triads.

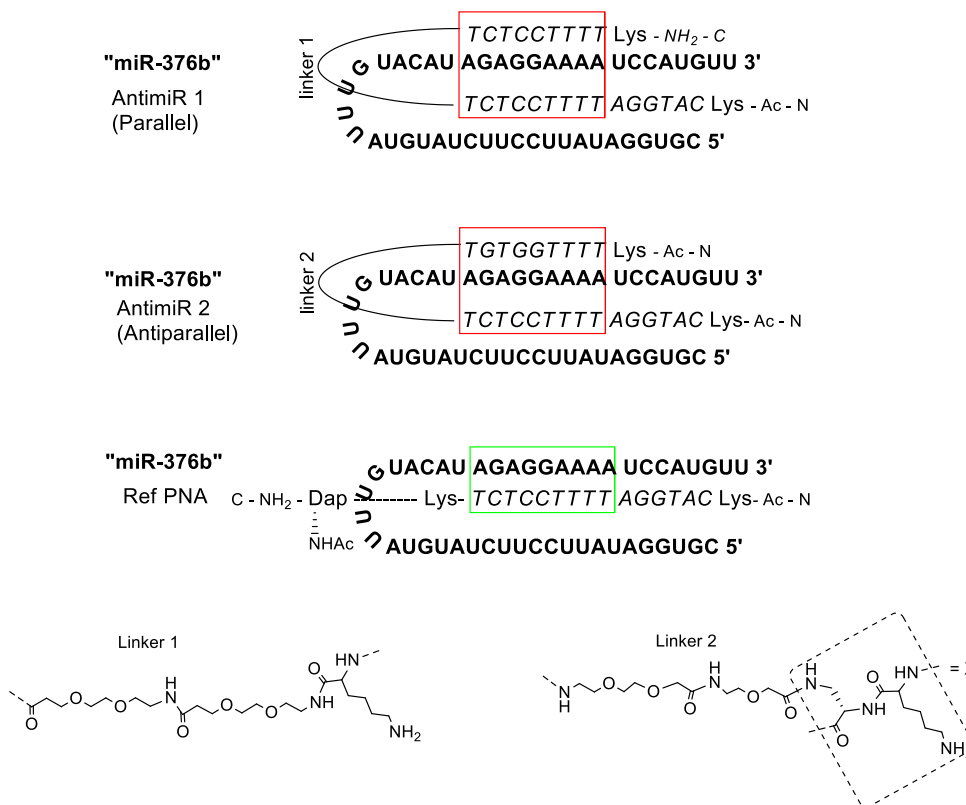


Figure 53: Schematic representation of the parallel and antiparallel AntimiR:miR-376b complexes.

The binding experiments with  $^{32}\text{P}$  labelled target RNA followed by analysis using an electrophoretic mobility gel shift assay (EMSA) shows that after only 1 h incubation AntimiR **1** interacts with the ‘miR-376b’ target. The initial microRNA band (Figure 54, Panel a, arrow 1 and 2) is much less intense and new bands appear and these increase with time (Figure 54, Panel a and panel b, arrow 3 and 4). Incubation with AntimiR **2** also result in new bands (Figure 54, Panel c, arrow 3 and 5) already at ratio 1:10, but less intense than with AntimiR **1** and it requires a longer incubation time to clearly visualize them.

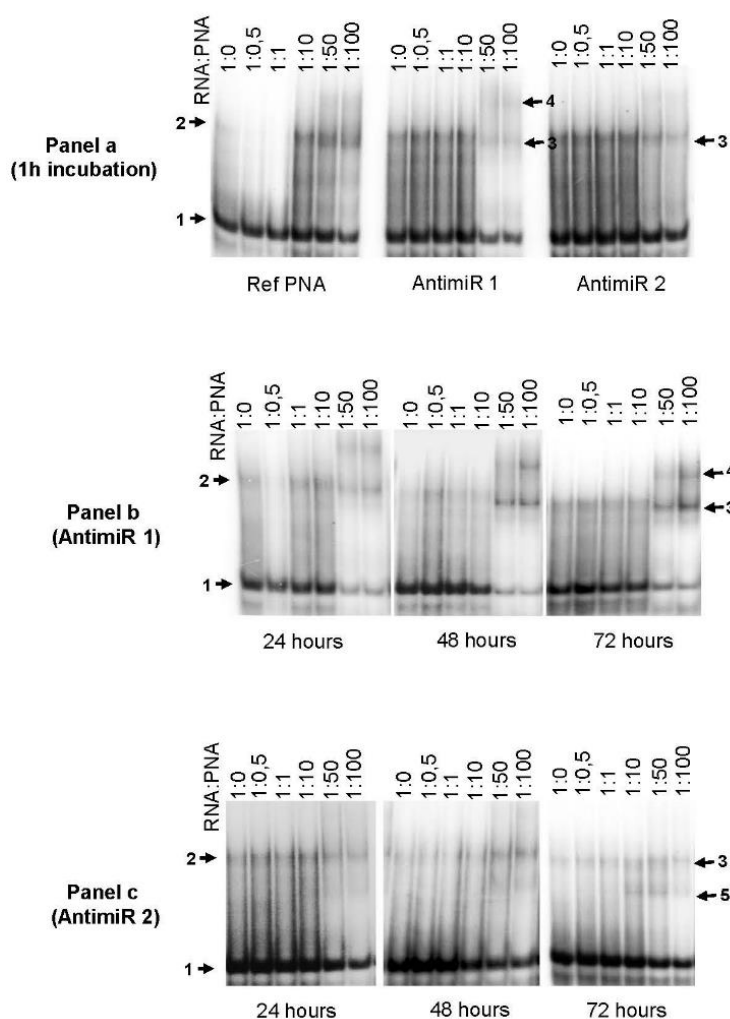


Figure 54: Complex formation between PNA and “miR-376b”. Electrophoretic mobility shift assay displaying migration of  $^{32}\text{P}$ -labelled “miR-376b” on PAGE after incubation during 1, 24, 48 or 72 hours in the presence of increasing concentrations of AntimiR 1, AntimiR 2 or Ref PNA.

### 6.4.3 Conclusions and future perspective

We have shown in this study that synthesized PNA clamps designed to form triplexes with RNA single strands and to invade double/hairpin strands. The complexes of RNA with a clamping PNA are considerably more stable than the PNA:DNA counterparts. The higher stability of the complex with RNA makes the BisPNA a valuable tool for microRNA targeting.

Since miRNAs control the levels of hundreds of proteins, careful studies are needed and caution should be exerted in their usage as a treatment modality. Further *in vivo* studies should reveal possible beneficial effects of autophagy modulation by miR-376b, antagomirs and their derivatives, vs. side effects of these treatments. In the nearest future we plan to investigate to what extent we can affect autophagy by our PNA AntimiRs and similar constructs in *in vitro* cell assays.

Currently we are testing the stability of the triplex at pH 5.5. At this pH, the cytosines of the Hoogsten PNA strand will be protonated, enhancing the stability of the triplex towards the duplex formed with the Ref PNA. In order to get a further increase in stability keeping the

physiological pH, we would like to exchange the C in the PNA strand involved in the Hoogsteen binding with the ΨiC, described in chapter 6.1, or the G base, an α-guanidine modification of PNA, whose backbone derived from arginine instead of glycine and which has already been shown effective in stabilization of triplex PNA: RNA<sub>2</sub> and in enhancement of cellular uptake.[156]

## 6.5 PNazymes designed to form a triplex with RNA (*paper V, second part*)

### 6.5.1 Evaluating the length of the triplex forming unit of the clamping PNA

The addition of an oligoether improved the catalytic rate for a PNzyme but the positions where to attach one or several oligoethers without interfering in a negative way, remains a problem to be solved [paper II].

In paper IV we showed the formation of a BisPNA:RNA triplex where a BisPNA formed by two linked strands clamps the RNA target. Connecting together the two concepts, PNzyme and clamping BisPNA, in one structure, we could be able to reach back with oligoether units (or other entities) to the centrally positioned RNA bulge leaving the nearest connections to the neocuproine site unchanged. As explained in the previous chapter, there is not much information about PNA/RNA triplexes in the literature, therefore the length of the triplex forming arm of the clamping PNA had to be tested.

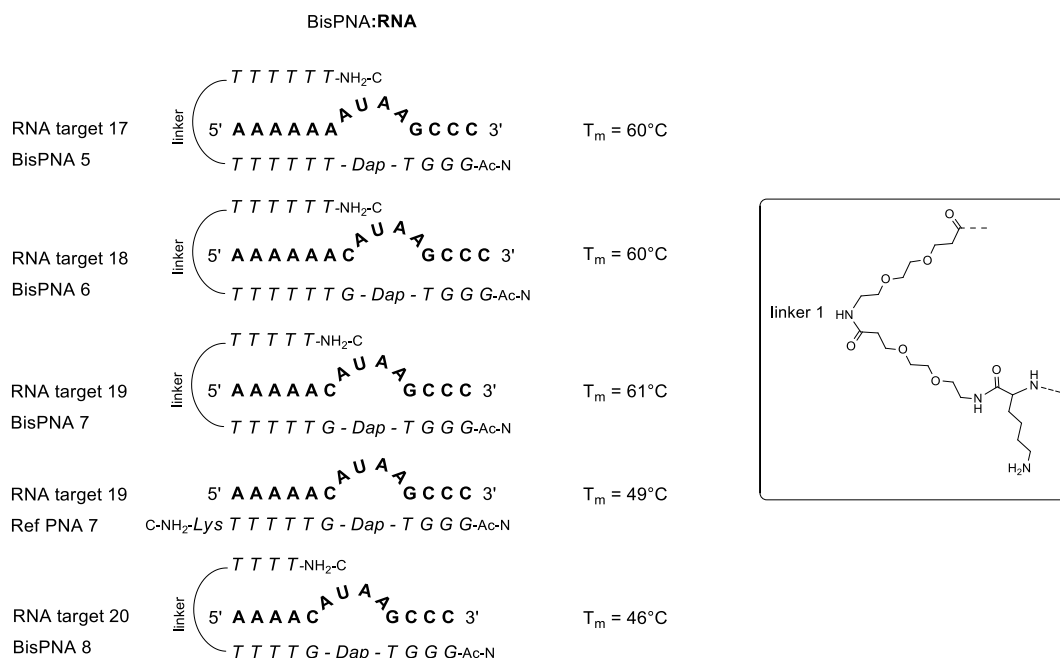


Figure 55: Non-conjugated Bis-PNAs for determination of complex stability.

BisPNA's **5-8** (Figure 55) were synthesized, and the respective complexes with RNA were subjected to thermal denaturation analysis. Complexes with 5 or 6 TAT triads (BisPNA **5-7**) formed stable complexes with the RNA targets **17-20**, displaying thermal meltings of 60-61 °C. On the other hand, the construct with only four potential TAT motifs (BisPNA **8**) was

considerably less stable, having a  $T_m$  of 46 °C. The duplex between RNA target **19** and Ref PNA **7** was 12 °C lower than for the corresponding BisPNA complex suggesting that a triplex is likely to have formed.

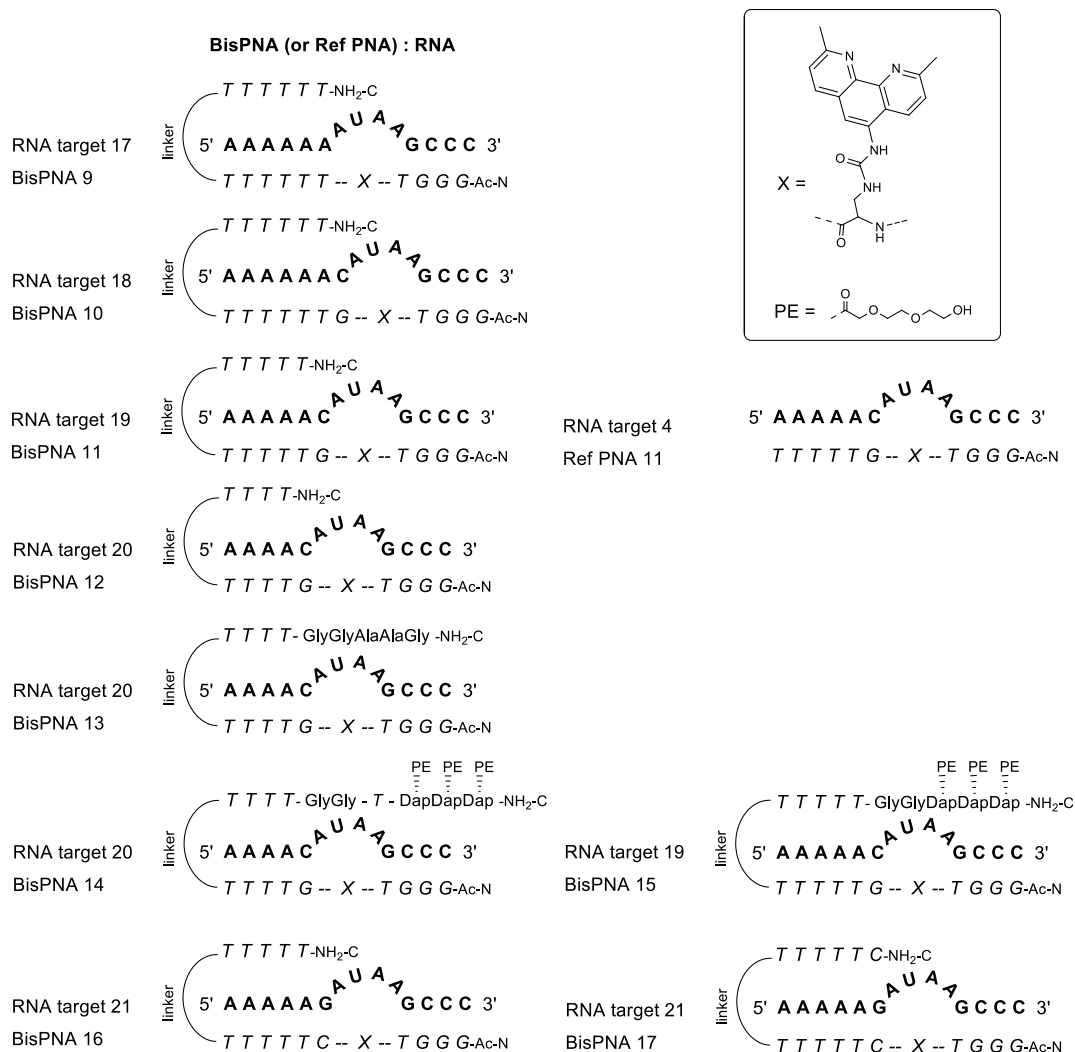


Figure 56: BisPNA neocuproine conjugates designed to clamp the RNA target by triplex formation at the 5'-stem part of the RNA.

Since BisPNA with 6 TAT triads showed an increase in stability, BisPNA **9** and BisPNA **10**, conjugated to the cleaving unit (neocuproine), were synthesized (

Figure 56). The kinetics of RNA cleavage for BisPNA **9** and **10** were difficult to analyse, but it was clear from turnover experiments that BisPNA **10** acted as an artificial enzyme (Figure 57). The HPLC profile of the 1:1 reaction with RNA was indicative of RNA precipitation or strong binding with the BisPNA. This, together with a lower rate than we would have expected, indicates that the rate limiting step with this PNzyme is the release of the substrate.



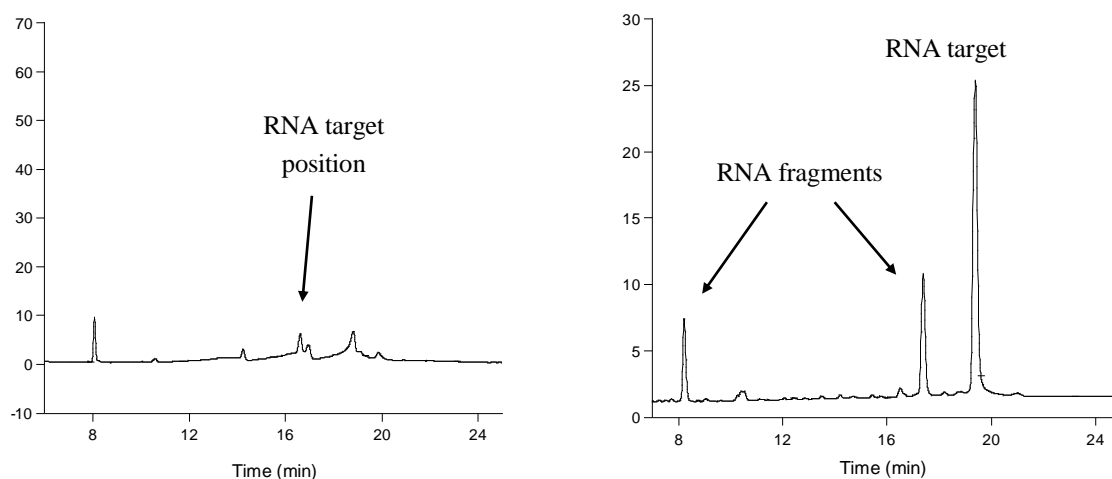


Figure 57: IE-HPLC chromatograms of the cleavage of the RNA target 18 by BisPNA 10, at pH 7,4 and 37 °C. Left panel, incubation with RNA and BisPNA in equimolar amounts, right panel, turnover experiment with 5 times excess of RNA.

Table 3: Full length RNA remaining at different times after incubation of RNA targets with BisPNA or Ref PNAs 9-17 (4  $\mu$ M of each, pH 7.4, 37°C).

<i>PNAzyme</i>	<i>15 min</i>	<i>30 min</i>	<i>60 min (or *45 min)</i>
<b>BisPNA 9</b>	Complexing made analysis difficult		
<b>BisPNA 10</b>	Complexing made analysis difficult		
<b>BisPNA 11</b>	81.7	66.9	44.4
<b>Ref PNA 11</b>	95.1	91.7	86.4
<b>BisPNA 12</b>	77.7	61.7	38.2
<b>BisPNA 13</b>	79.7	60.3	36.4
<b>BisPNA 14</b>	78.3	65.4	39.6
<b>BisPNA 15</b>	79.4	63.9	39.3
<b>BisPNA 16</b>	72.4	51.4	27.1
<b>BisPNA 17</b>	59.9	43.0	33.6 *

On the other hand, constructs with 5 and 4 TAT motifs, BisPNA **11** and **12**, cleaved the RNA with a half-life in the range between 30 and 60 minutes, which is comparable to the previously mentioned non-clamping PNAzymes. Comparing BisPNA **11** with its reference Ref PNA, which doesn't have a triplex forming unit, the BisPNA **11** give a substantially higher rate of cleavage (

Table 3). The reason for the different cleavage rates cannot be attributed to the instability of the duplex, since the thermal melting of Ref PNA **11** with RNA target **19** is well above the temperature of incubation. It appears that the presence of the PNA Hoogsteen strand is

somehow perturbing the structure of the complex with the RNA target, enhancing the cleavage activity.

### *6.5.2 Addition of oligoethers to the BisPNAs and interchange of a GC base pair.*

The next step was to add entities such as oligoethers and short peptides at the end of the triplex forming part of the PNA, to see if this would influence the cleavage.

BisPNA **13** is an elongation of BisPNA **12** with a GlyGlyAlaAlaGly peptide at the C-terminal of the BisPNA, whereas BisPNA **14** and **15** are extended with a peptide carrying with 3 oligoether units. As compared to the unconjugated BisPNA **11** and **12**, conjugation of these different entities to the PNA did not hamper the RNA cleaving activity of these PNAs, but they also did not improve the rate of cleavage. The idea was that triplex formation would guide the direction of the oligoethers or peptides towards the RNA bulge but, it is likely that due to the flexibility of the conjugates they were mostly directed into the surrounding water rather than interacting with the bulge, where we hoped that they would replace water molecules in the vicinity of the cleavage site.

We then decided to change the direction of the bulge closing GC base pair to a CG pair, which would enable a CGC triplex (although the triplex part would only have one hydrogen bond at physiological pH). Consequently BisPNA **16** and BisPNA **17** were synthesized, BisPNA **17** having an extra cytidine in the Hoogsteen strand. Comparing RNA cleavage rates with the corresponding BisPNA **16** and BisPNA **11** RNA complexes, where the only difference is that a base pair is swapped from a GC to a CG, we could see an enhancement in the PNAzyme efficacy. Furthermore, the construct with the additional cytidine at the C-terminal of the triplex forming unit, BisPNA **17**, gave an even higher rate of RNA cleavage. The rate of cleavage at a 1:1 ratio of the PNAzyme BisPNA **17** to RNA is in fact the highest rate of RNA cleavage reported so far in any artificial ribonuclease construct, with a half-life of between 15 and 30 minutes.

### *6.5.3 Conclusions and future perspective*

We have designed new BisPNAs which are able to clamp the RNA target by forming a triplex. A positive effect on the cleavage was observed when the clamping part is not too long. Changing the direction of a closing base pair, from a GC to a CG pair, enhances the rate of cleavage with a clamping PNAzyme, without compromising the selectivity, leading to the so far most efficient artificial nuclease reported. Future developments in the project involve evaluation of the effect that the exchange from GC to CG has on RNA target **22** cleavage using PNAzyme **3** as cleaver (Figure 58). Comparing the rate of RNA target **22** cleavage with the cleavage obtained for RNA target **21** would give us an idea if the enhancement obtained using BisPNA **17** as cleaver was due to the combined effect of triplex formation and base-pair exchange or if it is mainly due to the latter.

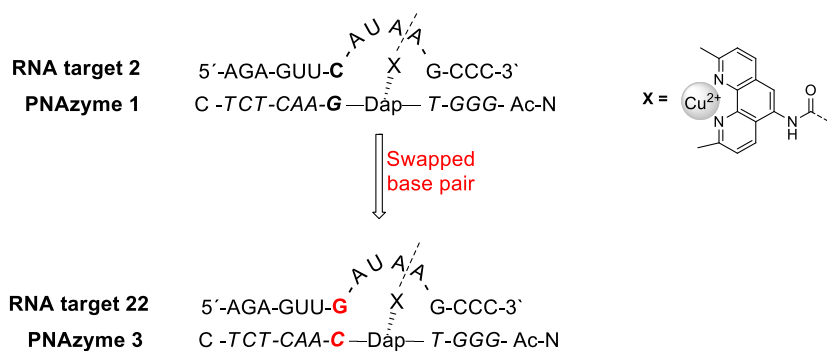


Figure 58: Structure of complex PNAzyme 3:RNA 22.

The clamping property can be used also to test the efficacy of BisPNA PNAzyme which are composed of different cleaver units. I started testing the activity of BisPNA **18** with two neocuproine attached (Figure 59) but, as already observed in previous experiments with dual neocuproine conjugation, the activity was completely lost.

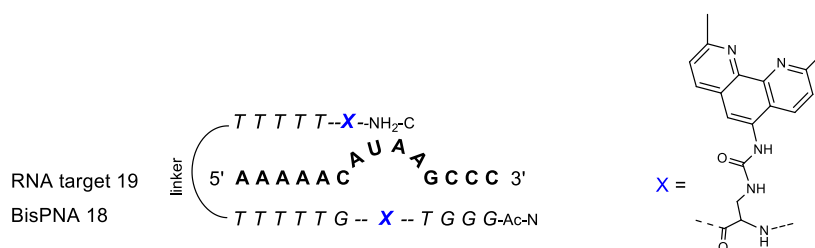


Figure 59: BisPNA 18 complex with RNA target 19.

There is work ongoing with alternative cleavers, based on other phenanthroline derivatives and we have already achieved even higher rates of cleavage with these systems and will try to target Malarias parasites using these PNAzymes.



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