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# Oligonucleotide Analogues as Modulators of the Expression and Function of non coding RNAs (ncRNAs) : Emerging Therapeutics Applications.

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# Oligonucleotide Analogues as Modulators of the Expression and Function of non coding RNAs (ncRNAs): Emerging Therapeutics Applications

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## Abstract

ncRNAs are emerging as key regulators of physiological and pathological processes and therefore have been identified as pharmacological targets and as markers for some diseases. Oligonucleotide analogues represent so far the most widely employed tool for the modulation of the expression of ncRNAs. In this perspective we briefly describe most of the known classes of ncRNAs and then we discuss the design and the applications of oligonucleotide analogues for their targeting. The effect of modifications of the chemical structure of the oligonucleotides on properties such as the binding affinity toward targets and off targets, stability to degradation and on their biological effect (when known) are discussed. Examples of molecules currently used in clinical trials are also reported.

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## Introduction

Since the discovery that small non-coding RNAs (ncRNAs) regulate gene expression, being involved in physiological and pathological processes, our knowledge on non-coding RNAs has grown enormously[1]. Non-coding RNAs represent the largest part of the transcribed genome, with lengths ranging from a few tens to several thousands of bases. The main effort of scientists is to understand their function, the role they have in diseases and the eventual networks they are involved in. Computational and experimental strategies led to the prediction and identification of ncRNAs, yielding a number of databases (noncode, miRbase, lncRNAdb, NONCODE, LncRNADisease ), which represent a valuable tool to approach the complex world of ncRNAs[2]. The fact that many ncRNAs regulate gene expression renders them ideal pharmacological targets. In principle we may either promote the expression of any gene which is deficient in a pathological situation as tumor suppressors and transcription factors or inhibit the expression of undesired genes by modulating the expression of the related ncRNA. In this context molecules such as antagomiRs, or mimics of microRNAs (miRNAs or miRs), which affect the function of miRNA or antagoNAT, which block the Natural Antisense Transcripts, have been explored. The relationships found between the alteration of expression of ncRNAs and diseases allowed the identification of ncRNAs as new biomarkers for specific diseases. Several studies have been reported so far on the modulation of ncRNAs' function by oligonucleotides, oligonucleotide analogues and small molecules; few molecules are currently being used in clinical trials.

In this perspective we will first describe a large part of the known classes of ncRNAs (grouped based on their length), with an emphasis on those molecules involved in diseases. We will then discuss the current strategies in designing ncRNAs-targeting therapeutics, as well as the associated challenges.

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## Short ncRNAs

We will describe, among the small ncRNAs, molecules composed of ≈ 18-200 bases (summarized in Table 1). Most of these molecules play key roles in the transcription of genes (small interfering RNAs, micro RNAs, small viral RNAs, transcription initiation RNAs), others are involved in post-transcriptional gene silencing (mirtrons, microRNA-offset RNAs).

Small interfering RNAs **(siRNAs)**, are small double stranded RNAs (dsRNAs), 20-24 nucleotides (nts) long that are processed from longer dsRNAs by the endonuclease Dicer. siRNAs can originate from transposons, heterochromatic sequences, intergenic regions, long-RNAs, and messenger RNAs (mRNAs). First discovered in plants[3], they were successively detected in flies and found to be ubiquitous in eucaryotes[4]. siRNAs are the main actors of RNA interference (RNAi), a natural cellular process which mediates the silencing of homologous transcripts through the full complementarity of siRNAs and target mRNAs, providing an innate defense mechanism against invading viruses and ensuring genome integrity via transcriptional silencing of undesired genomic loci (e.g. retrotransposons or repeat sequences)[5].

A widely studied class of small ncRNAs is represented by microRNAs**, miRNAs,** whose involvement in several human diseases has been intensively investigated. miRNAs are generated from longer precursors, pre-miRNAs, which are loaded and processed by the miR loading complex (miRLC) formed by Ago2, responsible for the loading and unwinding of the duplexes, and Dicer which cleaves the precursor into a smaller fragment[6]. Maturation of miRNAs may also occur through a different pathway not including Dicer: premiRs beginning with 5'U or 5'A are part of the miRNA precursor deposit complex (miDPC), in which Ago2 cleaves the precursors and binds the mature miRNAs[7].

The mature miRNAs are 19- to 21-nts in length, they mediate silencing of gene expression

through the imperfect binding to the 3' untranslated region (3' UTR) or the 5' UTR of protein-coding genes and cause translational repression[8]. In addition, they can function as positive regulators of gene expression by increasing the translation of a target mRNA through recruiting protein complexes, such as Argonaute 2 (AGO2) and fragile X mental retardation syndrome related protein 1 (FXR1), and indirectly up-regulating the translation of a target gene[9]. Deregulation of the expression of miRNAs has been found in a very large number of diseases, from cancer to cardiac failure, to metabolic disorders. Some cases will be discussed in the next paragraphs.

**Mirtrons** are short hairpin introns that use an alternative miRNA biogenesis pathway for their maturation: these short hairpin introns are in fact excised by the intron pathway before being cleaved by Dicer into a mature form of ~22 nts. They are indistinguishable from the typical miRNAs and some of them are evolutionarily conserved across similar species[10]. Mirtrons appear particularly susceptible to epigenetic regulation in some diseases like bladder cancer and in acute lymphoblastic leukemia[11].

MicroRNA-offset RNAs **(moRNAs)** originate prevalently from the 5' arm of miRNAs hairpin precursors; in their mature form they are 20 nts long . This suggests that the biogenesis of moRNAs and miRNAs might be linked, but not interdependent. The expression levels of moRNAs seem to be regulated in different developmental stages of *Ciona intestinalis*. Recently they have also been identified in humans and in herpes virus, but their biogenesis and possible functions remain to be determined[12]. Furthermore, some weakly expressed moRNAs have been found in solid tumors, together with other small RNAs[13] .

Small nucleolar RNAs (**snoRNAs)** of 60-300 bases are mainly found in the nucleolus; in mammals they are hosted in the introns of genes both coding for proteins and non-coding. They work as guide RNAs in the post-transcriptional modification of ribosomal and spliceosomal RNAs[14]; hybridization of the ribosomal RNA (rRNA) by snoRNAs is followed by the rRNA modifications (2'-O

ribose methylation or pseudouridylation) by specific proteins which associate to form the snoRNP complexes. SnoRNAs were hypothesized also to be precursors of miRNAs[15]. Several lines of evidence link mis-regulation of snoRNAs to cancer development[16]. Moreover, a loss in the expression of the snoRNA C/D box HBII was associated with the onset of the Prader Will syndrome[17]. Another family of ncRNAs is represented by the PIWI-interacting RNAs (**piRNA**s), oligomers of 26-31 nucleotides containing a 2'-OMe at their 3' end, originally named repeat associated small interfering RNAs (rasiRNAs)[18]. Unlike miRNAs, piRNAs do not require Dicer for their maturation and do not have a predicted hairpin structure. PiRNAs bind the PIWI proteins, a subfamily of the Argonaute family of proteins, while miRNAs interact with Ago. They play a key role in gametogenesis, heterochromatin modification and germline transposon silencing. Genome mapping of these small RNAs revealed that despite the extremely high diversity of piRNAs, they are localized in a relatively small number of genomic regions called piRNA clusters. PiRNA clusters were found to be bi-directionally transcribed as long primary transcripts. They are localized intergenic or in gene desert regions, and some piRNAs have recently been localized in the 3'UTR of protein coding genes. Several independent studies indicate that the piRNAs are involved in cancer development with oncogenic or tumor suppressor roles: the expression of piR-Hep1 was found up-regulated in hepatocellular carcinoma[19], while the level of piR-823 in gastric cancer tissues was significantly lower compared to normal tissues[20].

Small-viral RNAs (**svRNAs**), are composed by 22–27 nts, are derived from viruses and can control the viral switch between transcription and replication. In particular, svRNAs are detectable during replication of various influenza A viruses and their expression correlates with the accumulation of viral genomic RNA (vRNA)[21].

Transcription initiation RNAs (**tiRNAs**) are 18 nts tiny RNAs, localized into the nucleus and derived from sequences immediately downstream of the RNA polymerase II transcription start sites in

animals. They are generally associated with highly expressed genes and with G+C-rich promoters, bind to transcription-factors. Although the exact role of these molecules in the control of gene expression is poorly understood and their involvement in diseases at present is mostly unknown, their importance is generally recognized[22].

The Promoter-associated small RNAs (**PASRs**) of 26-50 nts are short transcripts localized in the promoter regions, identified in *H. sapiens* and belonging to the small ncRNAs. Transcription start site–associated RNAs (**TSSa**-RNAs), originally identified in *M. musculus*, are 20-90 nts long, flank active promoters[23] and have been hypothesized to regulate transcription; they are probably produced during the synthesis or degradation of longer upstream antisense RNAs (uaRNAs)[24]. Other small silencing RNAs that bind protein members of the Argonaute family are: QDE-2 interacting small RNAs (**qiRNAs**) about 20-21 nucleotides long, involved in the DNA damage response; telomere small RNAs ( **tel-sRNAs**) of approximately 24 nucleotides, engaged in telomere maintenance; Sno-derived RNAs (**sdRNAs**) which are predominantly 20–24 nts in length and modulate RNA silencing; splice junction associated RNAs (**spliRNAs**) 17- to 18-nucleotides long, involved in the post transcriptional gene silencing.

## Long ncRNAs

Long non coding RNAs (lncRNAs) represent the largest and most heterogeneous class of ncRNAs, arbitrarily defined as being longer than 200 nucleotides (Table 2). They constitute a significant fraction of mammalian transcriptome, whose biological role is now being discovered. The observation that more sophisticated organisms tend to have a higher number of non-coding RNAs renders investigations on lncRNAs even more challenging. Identification of such sequences, complicated by the fact that some lncRNAs contain short protein-like subsequences or long putative ORF, is carried out by RNA sequencing techniques and by bioinformatic tools. Recently a new tool to distinguish coding and non-coding RNA sequences has been reported and named

CNCI[25]; this tool classifies RNA sequences based only on their composition, allowing the annotation of lncRNAs for species lacking a whole or precise genome annotation. LncRNAs have been localized in inter and intragenic sequences, have been found both in the nucleus and in the cytoplasm, possess diverse functions and may act either in *cis* or in *trans*. Some long non coding RNAs have been demonstrated to interact with proteins (as transcription factors, PRC2), while others interact with oligonucleotides (mRNA or miRNA). Most of these lncRNAs are transcribed by RNA polymerase II, polyadenylated and expressed at low levels as compared to protein-coding genes. Predominantly lncRNAs have been found to be involved in the modulation of mRNA transcription, processing and in the modulation of the regulatory pathways at the post-transcriptional level[26]. In addition, lncRNAs have been shown to play a role in cell-cell signaling and in the organization of protein complexes.

Several lines of evidence indicate that there are essentially two modes of action through which lncRNAs exert their function: in *cis*- and *trans*. *Cis*-acting lncRNAs control the expression of genes located close (or sometimes far) to their transcription sites on the same chromosome, whereas trans-acting lncRNAs can either repress or activate gene expression at independent loci[27].

Recently, loss-of-function experiments have been used to investigate *cis*- versus *trans*-effects of long intergenic non coding RNAs (lincRNAs). These studies suggest that most of the lincRNAs identified and characterized so far act as trans-regulators[28], although some *cis*-regulators have been identified [27b, 28c, 29]. However, for both classes of lncRNAs, the targeting mechanism is still far from being understood completely.

Follows a brief description of a small number of lncRNAs.

X-inactive specific transcript RNA (**Xist**) is one of the first ncRNAs discovered[30], about 19 kb long in humans, with an intrinsic *cis*-regulatory capacity. It can function while remaining tethered to its

own locus by binding the Polycomb repressive complex 2 (PRC2), which is responsible for histone trimethylation at lys27, induces the formation of inactive heterochromatin on the X chromosome from which it is transcribed, with consequent initiation of inactivation of X-chromosome[31]. Xist is either positively or negatively regulated by other lncRNAs; alteration in the expression of Xist has been demonstrated to contribute to tumorigenesis. Loss of Xist after X chromosome inactivation results in the reactivation of X-linked genes, which promote tumorigenesis[32]. Other examples of lncRNAs regulating transcription after association with histone modifying complexes are represented by the Hox antisense intergenic RNA (**HOTAIR)**, the antisense noncoding RNA in the INK4 locus **(ANRIL)** and the KCNQ1 overlapping transcript 1 (**KCNQ1OT1)** [28b]. lncRNAs have also been found to show enhancer-like activity (**eRNA**)[33]. These lncRNAs, ≈2- kb in length, are mostly not polyadenylated. Their expression correlates positively with the accumulation of transcripts at the neighbour promoter, suggesting that they are involved in the activation of the synthesis of mRNA. eRNAs have been found in different species, with ≈2000 being identified in mouse and 3000 in humans, suggesting an extensive mechanism of gene regulation. Unlike the known enhancer molecules that are embedded in a DNA sequence, eRNAs are able to move around in the nucleus. Thus, they are also capable of modulating genes whose positions are far away on the DNA strand; recent studies suggest that the eRNAs are critical regulators of differentiation and development[34].

Regulation of the mRNA processing may occur through splicing modulation by the natural antisense transcripts (**NATs)** and the metastasis associated lung adenocarcinoma transcript 1 (**MALAT1)**. NATs are phylogenetically conserved transcripts, with lengths ranging from few tens to few thousand of base pairs, expressed at very low levels[35]. They modulate the expression of their cognate sense transcripts, stabilizing the protein coding sense transcript. Many functions have been hypothesized for NATs, ranging from epigenetic regulation of transcription, chromatin

modifications and monoallelic expression; these functions seem to be exploited through interactions of the NATs with DNA and chromatin. An alteration in the levels of expression of NATs has been demonstrated to be associated to different diseases: NATs have been identified in the Huntington disease repeat locus[36], in the brain derived neurotrophic factor[37] and in the apolipoprotein 1[38].

MALAT1, also known as nuclear-enriched abundant transcript 2 (NEAT2), is a very conserved ncRNA, retained in the nucleus, originally identified as a prognostic marker for metastasis and patient survival in lung adenocarcinoma[39]. Loss of function studies revealed that MALAT1 regulates the expression of genes associated to metastasis; in particular it suppresses inhibitors while activating promoter of metastasis[40].

Ultraconserved RNAs (**T- UCRs**) are lncRNAs characterized by a high degree of conservation within different species (ranging from rats, dogs to humans) [41]. They have been found in intra and inter-genic regions, are expressed in normal tissues and have also been associated to the pathogenesis of cancer[42]. These lncRNAs have been hypothesized to guide the RNA editing processes and to regulate post transcriptional events through the association to the 5' end or to the 3' UTR of a mRNA and the binding to miRNAs or to targets of miRNAs.

Some lncRNAs have also been found in the cytoplasm, where they bind to their partner by sequence complementarity. Competing endogenous RNAs (**ceRNAs)**, also known as miRNA sponges, are an example of cytoplasmatic RNAs acting as inhibitors of miRNAs. They have recently been demonstrated to participate to physiological processes as cell differentiation and pathological processes as tumorigenesis[43]. The understanding of the role of lncRNAs as endogenous microRNA sponges has been recently enriched by the discovery of a new class of lncRNAs, called circular RNAs **(circRNAs**). The majority of circRNAs arise from a 3'–5' ligation of

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both ends of a linear RNA strand. An extraordinary example is the circRNA CDR1 (also known as ciRS-7) a ~1,500-nucleotide-long circRNA, which is predominantly expressed in the human and mouse brain, that contains more than 70 binding sites for miR-7[44] .

Other examples of lncRNAs include the Promoter upstream transcripts (**PROMPTs**) with a length of 200-600 nts, [45] produced upstream of protein coding genes, present in the nucleus and quickly turned over by the RNA exosome. PROMPTs are capped at 5' and polyadenilated at 3' end (pA signals are linked to degradation)[46]. No relation has been found with cancer yet.

## Targeting ncRNAs by oligonucleotides and analogues

The number of ncRNAs with known functions is steadily increasing and in the last decade ncRNAs have come to represent an exciting avenue for the treatment of diseases[47]. By identifying and characterizing ncRNAs, it may be possible to manipulate the expression of a therapeutic gene for a range of indications. It is clear that the development of drugs able to modulate in a specific fashion the expression and function of ncRNAs represents an intriguing challenge, as it poses several issues related to the efficient delivery of the drugs to the targets, toxicity of the molecules, off target effects and last but not least cost of production. It is worthwhile highlighting that, thanks to the efforts carried out so far by the scientific community and pharmaceutical companies, some molecules are in clinical trials for the cure of cancer, HCV and heart failure. Most of the studies published so far refer to the gene silencing and to the interference in maturation or function of miRNAs (Figures 1 and 2), but in the last few years the interest toward regulators of the expression of lncRNAs has increased. Two approaches have been reported to modify production and function of ncRNAs: one is based on the use of small molecules, the other is based on the use of oligonucleotides and analogues.

Small molecules-based drugs usually interfere in the interactions of ncRNAs with proteins or DNA; they are derived from screenings of large libraries or are based on aminoglycosides showing structural and/or electrostatic complementarity to their RNA targets. Examples of small molecules as modulators of the functions of ncRNAs are so far restricted to inhibitors of the maturation of miRNAs. The second strategy, by far the most investigated, is based on the use of oligonucleotides (ONs), synthetic or expressed, or oligonucleotide analogues which bind for complementarity their targets. Standard oligonucleotides are not the perfect molecules for sequence-specific recognition of targets, as their binding affinity inversely correlates with their binding specificity. For this reason oligonucleotide analogues as Locked Nucleic Acids (LNAs) or Peptide Nucleic Acids (PNAs), showing a kinetically controlled selectivity against single-mismatch binding are currently being investigated[48]. The use of oligonucleotide analogues as LNAs, morpholinos, 2'-O-Methyl-RNAs (2'-OMe), phosphorothioates and combinations of them is actually preferred to the use of natural oligonucleotides, also due to their higher resistance to degradation *in vivo*. Follows a brief description of the properties of synthetic oligonucleotides analogues, which have been explored so far as tool to alter the functions of ncRNAs. Approaches for the modulation of the expression of ncRNAs are reported in Tables 3-7.

**Modified oligonucleotides**

The oligonucleotide analogues currently employed to regulate the expression of ncRNAs were originally developed as antisense or antigene agents, to overcome the stability and specificity drawbacks of standard oligonucleotides. Here, we discuss how the modifications affect the structure, the stability to degradation, the affinity towards the targets of these analogues. We will focus on analogues which find applications as modulators of the expression and function of ncRNAs. A very large variety of analogues has been explored, with modifications to the sugar-phosphate backbone or to the bases (Figure 3 and 4); those who have shown promising features

as drugs carry modification on the sugar-phosphate backbone and include molecules with a charged phosphate backbone as LNA, 2'-OMe, 2'-O-(2-methoxyethyl) RNAs (2'-MOE), 2'-deoxy-2' fluoro RNAs (2'-F) , and molecules with a neutral backbone as morpholinos and PNAs. Modified nucleotide bases have been explored in the context of siRNAs. Finally, a different strategy to protect oligonucleotides from degradation consists in the capping of the oligonucleotide 5' or 3' end (Figure 5); examples of such modifications are discussed in the paragraphs concerning the inhibition of miRNAs, the replacement of miRNAs and the gene silencing.

The first generation of antisense compounds based on oligonucleotide analogues includes the so-called phosphorothioate **(PS)** oligonucleotides, in which a sulfur atom replaces one oxygen atom in the phosphodiester bond. This substitution renders the P atom chiral, therefore oligonucleotide sequences containing *n* nucleotides and *n-1* PS linkages, have $2^{n-1}$ diastereoisomers. This fact might have implications on the activity of the molecules as in principle many similar species may have different biological targets.[49] The introduction of a PS linkage results in a greater resistance against endonucleases but causes a slight reduction of the binding affinity towards the RNA target. Stability studies carried out on PS-mRNA heteroduplexes showed that the melting temperature decreased by almost 0.5°C per nucleotide, with respect to that recorded for standard phosphodiester DNA/RNA duplexes[50]. Nevertheless PS analogues are still widely used as antisense agents and also in strategies aimed at interfering with the expression of ncRNAs; in fact the inclusion of a PS linkage improves the pharmacokinetic properties of the ON by promoting its binding to serum proteins and increasing its *in vivo* half-life. [51] Interestingly, some phosphorothioate oligonucleotides reached the market; this is the case of the vitravene (fomirvsen) commercialized by Isis Pharmaceuticals for the treatment of cytomegalovirus retinitis[52].

The second generation of analogues, obtained to overcome the relatively low affinity of phosphorothioate oligonucleotides toward the complementary RNAs, is characterized by modifications at the 2'-position of the ribose. Introduction of substituents in the 2' position of the sugar affects the conformation of the sugar and therefore the binding affinity of the modified strand toward its complementary. The conformations assumed by sugars are defined by the dihedral angles of the sugar ring as C3'-*endo*, C2'-*exo* (or North), assumed by sugars in A-form dsRNA, and C2'-*endo*, C3'-*exo* (South) typical of sugars in B-form ds DNA (Figure 6). Examples of 2' modified oligonucleotides are represented by 2'-**MOE** and 2'-**OMe** RNAs, showing an enhanced affinity toward complementary RNAs and also an enhanced stability to degradation as compared to 2'-deoxy and RNA oligonucleotides [53]. 2'- MOE-modified oligonucleotides have higher affinity and specificity to RNA than their 2'-OMe analogs[54]. The analysis of the crystal structure of a 2'-OMe RNA duplex suggests that this modified RNA arranges in a A-type RNA helix, with some deviations with respect to the canonical RNA structure consisting in a low base pair rise, elevated helical twist and inclination angles[55]. The ring of 2'-OMe sugars is found preferentially in the North conformation, and the 2'-OMe groups point toward the minor groove of the duplex; distinct hydration patterns, found in the major and minor groove, were supposed to play a structural role. The X-ray structure solved for a 2'- MOE RNA/RNA duplex revealed a conformational preorganization of the MOE substituents: all the modified riboses have a C3'-*endo* (North) conformation, and this is likely the reason of the high binding affinity and also of the extensive hydration of the substituents. The hydration pattern connecting the substituents and the oxygen atoms of the phosphate in the minor groove of 2'-MOE RNA has been hypothesized to contribute to the 2'-MOE resistance to nucleases[56]. Both 2'-OMe and 2'-MOE have been tested in different experimental contexts, both *in vitro* and *in vivo*; they have an elimination half-life of 25–30 days in all organs and species studied[57].

**2’-F** nucleotides are another example of 2’ modified RNAs. It was observed that the substitution in 2’ position of a hydroxyl group by a fluorine atom does not imply a substantial change in the conformation and in other structural features of the sugar. The increase in the binding affinity toward RNA complementary sequences has been explained with enhanced base-pairing and stacking interactions due to the electronegative fluorine atom[58]. The absence of the hydroxyl group in these molecules confers greater stability to chemical hydrolysis and high pH as well as an increased resistance against ribonucleases, leading to a higher survival in a biological environment, compared to the natural counterparts. An example of successful application of 2’-F analogues is represented by Macugen (pegaptanib), an RNA aptamer with 2'-F pyrimidine rings, approved by the U.S. Food and Drug Administration, that selectively antagonizes with VEGF and is currently clinically employed for the treatment of exudative (wet) age-related macular degeneration[59]. Morrissey and co-workers have recently shown that the combined use of 2'-F pyrimidine with 2'-OMe purine encapsulated in liposomes yields RNA duplexes with high stability in serum and improved activity *in vivo*[60]. Furthermore, phosphorothioate and phosphodiester analogues with 2'F-2’-deoxynucleosides were synthesized and used for antisense applications and ribozymes as they appear to adopt the most suitable form to interact with target proteins [61]. Between the other groups introduced at the 2’ position of ribose we also mention the **allyl** or the more hindered groups, as **benzyl** and **4-methylpyridine**[62].

2’-modified RNAs have been applied as inhibitors of the function and biogenesis of miRNAs.

**Locked nucleic acids** (LNAs) are a distinctive class of modified oligonucleotides containing a methylene bridge, which connects the 2’-*O* with 4’-*C* of the ribose, to form a rigid bicycle, locked into a C3’-*endo* (North) sugar conformation. The ability of LNA monomers to twist the sugar conformation from a South-type to North-type when introduced into an oligonucleotide sequence leads to the formation of thermodynamically more stable duplexes and results in a more effective

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biological activity. LNA oligonucleotides possess a binding affinity towards the target molecules higher than that of the natural counterparts and this is reflected in an increase in melting temperature of LNA hybrids of about 8 °C per included LNA monomer[63]. Structural investigations by NMR and X-ray crystallography performed on different LNA-RNA and LNA-DNA heteroduplexes demonstrated that the LNA-modified oligonucleotides adopt a perfectly A-type duplex form very similar to that adopted by the natural duplexes[64]. Furthermore, LNAs display excellent mismatch discrimination, resistance to enzymes and show a serum decay and a tissue distribution similar to that of phosphorothioate oligonucleotides[57]. LNA oligomers and mixed LNA/DNA oligomers are commonly employed to target natural oligonucleotides as antisense and siRNA agents [65, 66]. Several applications of LNA as antagomiRs are also reported.

Combination of 2'-OMe, 2'-MOE, 2'-F, LNA with the phosphorothioate backbone represents the latest frontier in the field of oligonucleotide analogues as drugs[67].

Between the analogues with a constrained backbone, we also report the 2', 4' constrained 2'-*O*-ethyl modified **cEt** [68]; the sugar adopts a C3'-*endo* pucker, thus cEt modified monomers confer an improved thermal stability to duplexes in a way which is comparable to LNAs. Also the stability to nucleases is higher than that found for LNAs and 2'-MOEs. Experiments in which cEt modified oligonucleotides were tested as antisense in animals demonstrate that they possess an enhanced potency and therapeutic profile[69]. cEt modified oligonucleotides have been employed for the knockdown of the lncRNA MALAT1.

Other examples of analogues are those with modifications only at the 4' position of the sugar ring: replacement of the oxygen by a sulfur atom as in 4'-thio-modified RNAs (**4'S-RNA)** results in an increase of the stability to nucleases[70]; combinations of 4'-thioribose and 2'-ribose modifications, as in **4'S-FANA**, provide molecules with an enhanced plasma stability and siRNA activity[71].

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Last but not least we will briefly describe oligonucleotide analogues lacking sugars on the backbone, as morpholinos and Peptide Nucleic Acids.

**Morpholino** oligonucleotides (MO) were synthesized by replacing the riboside backbone with a morpholinic moiety and the phosphodiester intersubunit linkage with a phosphoroamidate linker. The neutral backbone confers to this molecule a high binding affinity for complementary DNAs or RNAs and good enzymatic stability, also showing excellent solubility properties. Since morpholino oligonucleotides are not recognized even by signaling proteins, they have been applied very effectively for the inhibition of the RNA function by blocking its translation[72], redirection of splicing[73] and more recently as specific splice switching agents to alter the processing of pre-miRNAs [74]. The low toxicity and good uptake granted morpholino oligos an extensive use in the knockdown of target gene expression in developing zebrafish[74].

Other synthetic oligonucleotide analogues are **PNAs**, in which the naturally occurring sugar-phosphate backbone has been replaced by N-(2 - aminoethyl)-glycine units. A methylene carbonyl linker connects natural as well as unusual (in some cases) nucleotide bases to the secondary amine of the backbone [75] . Binding to complementary DNA, RNA and PNA oligomers occurs in a sequence specific fashion and also with a high affinity obeying to the Watson-Crick rules. Unlike DNA and RNA, PNA recognizes and binds complementary strands in both parallel and antiparallel orientations. However, the antiparallel orientation was found to be more stable. Although the chemical structure of PNAs is totally different from that of natural nucleic acids, the hybridization properties are not only preserved, but improved for PNAs . The uncharged scaffold of PNAs enhances thermal stability of PNA-RNA (PNA-DNA) duplexes and enables PNAs to form stable hybrids also in low-salt conditions [76]. For the inhibition of gene expression they appear to be more effective than other antisense molecules; because of their neutral backbone they are able to invade local RNA secondary structures, overcoming the issue related to the accessibility of RNA.

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Application of PNAs as drugs is hampered by their low cellular uptake which can be improved by the conjugation to carrier peptides or by further modification of the backbone, to introduce charges[77]. The use of PNAs as inhibitors of the biogenesis of miRNAs and as antagomiRs is described.

Modifications can also be introduced in the bases; in this context a large amount of work has been carried out on modified siRNAs[78] . Examples of modified bases include **2-thiouracil** and **pseudouracil**, characterized by a C3'-*endo* sugar pucker, **2,4 difluorotoluene** and **2,6 diamino** purine, in which the hydrogen bond pattern with the complementary base is modified and also bases with hindered substituents, as **propyl** and **cyclopentyl** on the bases which affect the ability of oligonucleotides to interact with proteins.

The application of oligonucleotides as drugs is still limited by the toxic effects deriving by their accumulation in the liver and kidney. We have to say that the toxicity of oligonucleotide analogues seems to be a function of the experimental system tested: experiments carried out in zebrafish demonstrated that LNA and 2'-OMe analogues are toxic to this organisms, unlike morpholinos[79]. In addition controversial data can be found in the literature: in experiments carried out in mice, some oligonucleotides bearing LNAs were reported to increase the serum levels of alanine-aminotransferase (ALT), which is a marker of hepatocellular injury, while other examples show no dose-limiting toxicity in preclinical and clinical studies[67b, 80]. A prediction of the toxicity of such molecules based on the nucleotide sequence, type and position of the modification is not possible yet, due to the lack of Structure-Activity-Relationship studies for the molecules tested in pre-clinical and clinical trials. An effort in this direction is represented by the work of Hagedom et al, reporting an analysis of the hepatotoxic potential of phosphorothioate LNAs carried out by an ensemble of biochemical and histopathological studies on mice, statistical modeling and sequence analysis[81]. The authors demonstrate that machine learning techniques may be employed to relate

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chemical features (modification and sequence) to the hepatotoxic potential of the modified oligonucleotides.

Another critical point for the therapeutic application of oligonucleotides is represented by their delivery. Efficient delivery of naked oligonucleotides has been demonstrated *in vivo* in few cases, for example for LNAs targeting miR-122 in non-human primates[82]. In most cases, the internalization of standard oligonucleotides is very poor and the efficiency of the oligo-based drug is limited by its rapid degradation. A large variety of delivery systems has been explored so far, ranging from peptides to liposomes, to nanoparticles[83]. The delivery systems have a dual function: drive the oligonucleotides to their target and protect them from degradation. Many delivery systems are optimized to increase the uptake of charged molecules; they are based on cationic lipids variously decorated with PEG, PEG-PLA, or PEG grafted with ligands which recognize specific receptors or antigens overexpressed on the surface of the cell[84]. In these cases the interactions between the oligonucleotides and the cargo are mainly electrostatic. Use of ligands conjugated to the termini of oligonucleotides as *N*-acetyl galactosamine and folate has been reported.

A different strategy is applied for the delivery of uncharged oligonucleotide analogues: in this case cargos are usually covalently linked to one end of the oligo. Peptides as polyarginine, Tat, Nuclear Localization Signals have been employed in different cases; we have to recognize that for molecules as PNAs there is not yet a general approach for delivery , as the efficiency of the uptake seems to be cell dependent[85].

**Strategies for the inhibition of maturation of miRNAs**

Different strategies to inhibit the maturation of miRNAs have been explored; one is based on molecules targeting precursors of miRNAs (pre-miRs and pri-miRs) such as small molecules and synthetic oligonucleotide analogues.

These studies are still in their infancy and in fact, as far as we know, despite the number of published papers, no clinical trial has been reported yet on molecules which inhibit the maturation of miRNAs [86]. A recent paper demonstrates that antagomiRs affect the maturation miRNAs too[87]. Small molecules have been identified by random screening of libraries or after testing aminoglycosides for the targeting of pre-miRs[88]; one striking example is represented by streptomycin which has been demonstrated to selectively inhibit miR-21 in living cells[89]. Inhibition of the maturation of miRNAs has been achieved also using apigenin, an Erk inhibitor, causing a decrease in the TRBP phosphorylation which is pivotal to the maturation of miRNAs[90]. Very recently an approach named Inforna was developed for the discovery of small molecules able to bind precursors of miRNAs and inhibit their maturation[91]. This approach relies on the complementation of bioinformatics and experimental data on the interactions of small molecules and annotated RNA motifs, leading to the identification of a) RNA targetable motifs chosen between the sites targeted by Drosha or Dicer, b) the lead small molecule and finally to a prevision of the fitness of the interaction. By Inforna, an inhibitor of miR-96 was found and validated by a luciferase model system; selectivity of the molecules was found comparable to that of an antagomiR.

Peptide based inhibitors of the maturation of miRNAs have also been tested[92].

A recently explored area is represented by the development of oligonucleotides and their analogues as inhibitors of biogenesis of miRNAs (Table 3). Such inhibitors target the apical region or the loop of pri-miRNAs which have been hypothesized to be devoted to recruit trans-acting factors as heterogeneous nuclear ribonucleoproteins (hnRNPs)[93]. These factors relax the stem loop and help the Drosha mediated cleavage. **LooptomiRs**, 2'-OMe oligonucleotides complementary to evolutionary conserved terminal loop of pri-miR18a, were found to abolish the

processing of miRNAs *in vitro* by Drosha[94]. RNA aptamers targeting the loop of pri-miR-18a have also been obtained and proved to successfully inhibit the maturation and function of miRNAs[95].

12-mer RNAs complementary to the apical region of the pre-miRNAs, designed to target pre-miR210 and pre-miR33, were demonstrated to bind pre-miRNAs and simultaneously sequester Dicer, affecting processing of miRNAs[96]; replacement of standard RNA with 2'-OMe units resulted in molecules forming with the pre-miRNAs complexes characterized by a higher melting temperature and still retaining the ability to inhibit the production of miRNAs.

Morpholino oligonucleotides designed to bind the Drosha and Dicer cleavage site on pri-miRNA 205 and pri-miRNA -375 were demonstrated able to efficiently block the processing of the pri-miRNA and the consequent maturation and function of miRNA in zebrafish embryos[97]. Interestingly the decrease in the expression of miR-375 observed using morpholinos targeting pri-miRNAs was higher as compared to that obtained using morpholinos targeting mature miRNA-375.

Peptide Nucleic Acids oligomers conjugated to carrier peptides were designed to target the miRNA-210 precursor with the aim to inhibit the maturation of miR-210 [85b]. It was found that such molecules down-regulate in a sequence specific fashion miR-210 and also pri-miR-210, leading to hypothesize that PNA based molecules strand invade the precursors of miR-210 and therefore inhibit their cleavage to mature miR-210. The observation that PNAs affect pri-miR-210 production suggests that these molecules reach the nucleus.

In a different approach chimeric molecules composed of DNA and LNA, **antagomiRzymes**, were found to target and cleave pre-miRNAs[98]. These molecules have a central region composed of 15 DNA bases, whose sequence corresponds to the catalytic core of the 10-23 DNAzyme, which is able to cleave at purine-pyrimidine junctions of RNA in the presence of divalent cations. The flanking regions of about 7-8 nucleotides, necessary to determine the binding specificity, are

complementary to the target pre-miRNA and contain two to four LNAs. Experiments were carried out with molecules targeting the stem of hsa-miR-372 and hsa-miR-373, in the region corresponding to the mature miRNA; in this way, both the precursor and the mature miRNA can be cleaved. AntagomiRzymes containing LNAs were demonstrated to be more effective as compared to those composed only by DNAs, although their activity was detected only at high concentrations of antagomiRzymes. It has been speculated that targeting of pre-miR loop might result in a more efficient inhibitor.

An alternative strategy to prevent the production of miRNAs relies on the use of proteins as transcription activator-like effector nucleases (TALEN). These proteins discovered in the plant pathogenic bacteria *Xantomonas*, contain a DNA binding domain, composed of 33-35 amino acids devoted to the recognition of base pairs, and a nuclease to induce DNA cleavage at pre-determined positions. Very recent papers report the **deletion of miRNA genes** by engineered endonucleases TALEN and clustered regularly interspaced short palindromic repeats (CRISP)/ CRISPR-associated systems in zebrafish[79, 99]. TALENs have also been tested to produce miRNA knockout mice[100]. Deletions of chromosomal segments by these strategies may result in the deletion of miRNA genes and clusters and can be useful to understand the role of ncRNAs.

**Strategies for the modulation of the function of miRNAs**

The function of miRNAs may be promoted or inhibited. Inhibition may be accomplished using synthetic oligonucleotides or vectors encoding inhibitors. One strategy is aimed at sequestering the mature miRNAs and preventing their binding to the 3' UTR regions of the target mRNAs and is achieved by synthetic oligonucleotides, or analogues, (the antagomiRs, tiny LNAs). In alternative, the function of miRNAs may be blocked using synthetic oligonucleotides complementary to the binding site of the miRNAs on the 3'UTR mRNAs, which hinder the binding of miRNAs to their

target RNAs with a consequent inhibition of the translational repression or degradation of the mRNA targets. This approach is defined as miRNA masking antisense technology[101]. Synthetic tough decoy have also been proposed as inhibitors of miRNAs[102]. A different strategy relies on miRNA sponges, transcripts expressed from strong promoters, containing repeated binding sites for heptameric seeds, which inhibit miRNAs sharing the same seed[103]. Endogenous miRNA sponges include the competing endogenous RNAs (ceRNAs)[43b], pseudogenes[43a] and also circular RNAs[104].

On the other hand it is possible to increase the amount of miRNAs available by mimics of miRNAs. Examples of inhibitors and mimics of miRNAs are summarized in Table 4.

**Inhibition of miRNAs.** The most studied molecules aimed at inhibiting the function of miRNAs are named **antagomiRs**; an antagomiR sequesters the mature miRNA, prevents the interaction of the miRNA with its mRNA target and consequently affects the mRNA regulation. The design of an efficient antagomiR requires several issues to be addressed[105]: the antagomiR is designed to be complementary to the mature miRNA; hybridization of the miRNA has to be effective when the miRNA is in a single strand form, or when it is in a duplex with the passenger strand and when it is part of the RISC complex. To address all these issues the antagomiR needs to have a binding affinity towards the miRNA target high enough to 1) avoid competition with the passenger strand, with other natural targets present into the cell, 2) prevent the action of the miRISC helicases which could open the miR/antagomiR duplex. AntagomiRs need to be highly specific, resistant to degradation by nucleases and non toxic. To satisfy all these requirements several oligonucleotide analogues have been tested; modifications regard mainly the oligonucleotide backbone, rather than the bases. AntagomiRs based on oligonucleotide analogues silence miRNAs by steric blocking, but the fate of the antagomiR/miR complexes has been suggested to be dependent on the composition of their backbones: high affinity LNA, 2'-F/2'-MOE, PNA-based antagomiRs sequester

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the miRNAs, which do not seem to be degraded; 2'-MOE antagomiRs instead, after binding the miRNAs, induce their degradation[106].

A considerable amount of work has been reported so far on the use of antagomiRs and importantly few of these are actually in preclinical development. AntagomiRs with different length and chemical composition have been explored; we will discuss few examples of antagomiRs, grouped based on their chemical modification.

The first generation of antagomiRs includes oligonucleotides containing 2'-OMe RNA units in their central part, as 2'-OMe confers resistance to endonucleases, phosphorothioates linkages at the 3' and 5' ends to protect the oligo from exonucleases and finally a 3' cholesterol modifier (Figure 5) to improve the cellular uptake. Such modified oligonucleotides were first tested by Krutzfeldt, who reported the efficient and selective inhibition of miR-122 in mice [107]. AntagomiRs based on 2'-OMe were found to sequester and inactivate miR-122 in Huh7 cells stably expressing the genotype 1b strain Hepatitis C Virus-N replicon NNeo/C-5B.

The antagomiR design reported by Krutzfeldt has found several applications : a 2'-OMe based antagomiR against miR-20a has been tested *in vivo* using the hypoxia-induced mouse model for pulmonary hypertension. It was found that inhibition of miR-20a yields the reinstatement of the bone morphogenetic protein receptor type II (BMPR2) signalling, whose disregulation is considered a hallmark of pulmonary hypertension, and prevents the vascular remodeling development[108]. An antagomiR for miR-17 based on 2'-OMe conjugated to cholesterol was tested *in vivo* in an experimental model of pulmonary hypertension: treatment with the antagomiR results in an improvement of the hemodynamic and structural changes caused by hypoxia in mice and MCT rats[109]. Recently a 2'-OMe-phosphorothioate-cholesterol conjugated oligonucleotide against miR-10b was tested in mice in a mammary carcinoma metastasis model: the antagomiR

preferentially targets metastatic cells, where miR-10b is expressed at a higher level; in primary tumor cells it inhibits metastasis and causes minimal toxic effects in normal animals[110]. The specificity of antimiR-10b was assessed observing that the levels of miR-9 and miR-21, which are overexpressed in breast cancer, and also the levels of miR10-a, differing for a single base from the mature miR10-b, were not altered. Another example of antagomiR based on 2'-OMe conjugated to cholesterol tested *in vivo* is represented by the antagomir for miR-24, which causes a reduction in the infarction size and improves heart function[111].

A 2'-Methoxyethyl (2'-MOE) modified oligonucleotide was tested for the inhibition of miR-21 signaling in gliomas: this antagomiR induces pro-apoptotic and cell cycle arrest genes, but does not change the viability of glioma cells, unlike other antagomiRs with different backbones[112].

2'-Fluoro-2'-methoxyethyl phosphorothioate mixmers have been proposed as antagomiRs: the 2' F is used to improve the binding affinity toward the complementary miRNA, while the MOE and PS modifications increase the stability to degradation. An oligomer containing 2'-F/2'-MOE-PS was reported as antagomiR for miR33a/b for the treatment of cardiovascular diseases; experiments carried out in mice knockout for the low density lipoprotein receptor with atherosclerotic plaques demonstrate that the antimiR-33 penetrates the lesions and reaches the plaque macrophages, promoting the expression of ATP-binding cassette transporter A1 (ABCA1), which regulates the high-density lipoprotein (HDL) synthesis and reverse cholesterol transport, and cholesterol removal [113]. A later study from the same group reports the effect of antimiR-33a/b on African green monkeys: systemic delivery of antimiR-33 enhances the expression of ABCA1, promoting an increase in the HDL levels in plasma[114]. Expression of genes target of miR-33, including those involved in fatty acid oxidation, was found increased, while the expression of genes related to fatty acids synthesis was found reduced; interestingly the plasma level of very-low-density-

lipoproteins (VLDL) associated triglycerides was reduced. These observations encourage the use of antimiR-33a/b for the treatment of dyslipidaemias.

An antagomiR composed of alternated 2'-F and 2'-MOE modified nucleotides with a full phospohorothioate backbone has been tested against miR-155, which was found up-regulated in Amyotrophic Lateral Sclerosis[115]. In the SOD1$^{G93A}$ rodent model the antagomiR slowed down the disease progression and extended survival.

The combination of 2'-MOE and 2'-F with a phosphorothioate backbone was also employed to produce an antagomiR designed to inhibit miR-27b, a marker of breast cancer metastasis; this was shown to bind with higher affinity and specificity than the corresponding 2'-OMe to RNA and to efficiently silence miR27-b in a mouse breast tumor model[116].

PNAs have been tested as antagomiRs in several cellular assays; they are usually conjugated to peptides or bear further backbone modifications to improve their uptake. PNAs conjugated to poly lysine targeting miR-122 were reported to inhibit miR-122 and up-regulate its targets in Huh7 cell lines and rat hepatocytes. In this cell system the effect of PNA antimiR-122 was found comparable to that of a LNA-2'-OMe antagomiR containing phosphorothioates linkages[85a]. Recently a PNA antagomiR was tested against miR-690, a miRNA over-expressed in functional Myeloid-derived suppressor cells (MDSCs) induced by delta-9-tetrahydrocannabinol. Inhibition of the miRNA-690 by the PNA antagomiR results in an increase of the expression of the transcription factor CCAAT-enhancer binding protein alpha (C/EBP$\alpha$), a regulator of cell differentiation. These studies highlight the role of miRNAs on the development and function of MSDC, suggesting the possibility to modulate the MDSC activity in cancer and inflammatory diseases[117].

Locked Nucleic Acid (LNA) based oligonucleotides have been successfully applied as antagomiRs. The LNA antagomiR against miR-195 is in pre-clinical trial for the remodeling of post myocardial infarction. miR-195 is a member of the miR-15 family, which contributes to loss of cardiac

regenerative capacity[118]; its inhibition is associated to an increased number of mitotic cardiomyocytes. Further examples of LNA based antagomiRs are represented by the antagomiR for miR-29 [119], employed to prevent aortic dilatation, antagomiRs for miR-208 and miR-499 for the cure of pathologic cardiac hypertrophy.

A LNA/DNA phosphorothioate mixmer, antagomiR for miR-122, also known as SPC3649 or miravirsen developed by Santaris Pharma, was tested on mice and monkeys and demonstrated able to efficiently silence miR-122 with a long lasting and reversible decrease in the accumulation of plasma cholesterol, without toxicity[82] . Miravirsen has also been tested on HCV chronically infected chimpanzee and found capable of suppressing the HCV viremia, with no evidence of viral resistance or side effects[120]. Very recent studies demonstrated that miravirsen inhibits miR-122 acting on precursors of miRNA[87]. Miravirsen invades the pre-miR122, binding to the 5p (antisense) strand, and inhibits the correct cleavage by Dicer to yield to the production of an extended miRNA; also miravirsen binds to the pri-miR, preventing its cleavage by Drosha in the nucleus. These data support the idea, proposed to explain the activity of LIN-28, that the activity of antagomiRs is related to their ability to bind to precursors, inhibiting their biogenesis.

The activity of miRNAs may also be temporally controlled by photocaged antisense oligonucleotides; 2'-OMe oligonucleotides were tested in a *C.elegans* model, and for this they are named **cantimirs.** These molecules are composed of an antagomiR strand connected through a bifunctional **coumarine** linker (Figure 5) to a "blocking" strand; after photolysis initiated by irradiation with light at 365 nm the linker is cleaved, the blocking strand is released and the antagomiR may exert its function[121].

**Tiny LNAs**, 8-mers targeting the seed region of miRNAs were introduced by Patrick in 2010[122] to inhibit miR-21; these studies suggested that this miR is not involved in cardiac hypertrophy or fibrosis, although contradicting results have been reported[123]. These oligomers have a fully

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phosphorothioate backbone and have been employed later in applications aimed at inhibiting miRNAs of the same family, sharing the same seed. Targeting of miR-122 in a mouse liver by phosphorothioate tiny LNAs resulted in silencing of miR-122, de-repression of the targets of miRNA, with effects *in vivo* comparable to those obtained using a 15-mer LNA antimiR-122[124]. The application of a tiny antiseed with a phosphodiester bond instead of phosphorothioate bond had no effect on miR-122 and its targets, highlighting the importance of phosphodiester modification on the delivery and activity of the tiny antiseed. A tiny LNA was successfully employed in a mouse model of Sonic Hedgegog medulloblastoma to inhibit the miR 17-92 cluster; treatment of mice with this antiseed results in a reduction of the tumor growth and prolonged survival of mice[125]. A seed targeting LNA has recently been employed in non-human primates for the inhibition of miR-33, a regulator of cholesterol/lipid homeostasis. In obese and insulin resistant non-human primates, inhibition of miR-33 by subcutaneously delivered tiny LNA yields de-repression of miR-33 targets and increase of the high density lipoprotein cholesterol[126].

An alternative approach to antagomiRs was proposed by Haraguchi in 2012, based on the use of synthetic 2'-OMe RNA duplexes, named synthetic tough decoys (**S-TuD**), containing binding sites (MBSs) for miRNA flanked at both ends by rigid stems[127]. The S-TuD design derives from the previously reported Tough Decoy RNAs, which have been reported to produce a long-term suppression of specific miRNA activity when expressed from lentivirus vectors in mammalian cells[102]. S-TuDs against miR-21, miR-200c and miR-106b transfected into HCT-116 cells silence the miRs with high efficiency. Experiments carried out with S TuD against miR-200c demonstrated that the inhibitory effect after a single transfection is long lasting and is retained even when the concentration is very low. The effects of S TuDs on silencing miRNAs sharing the same seed seem undetectable.

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Inhibition of the function of miRNAs by **miRNA masking** oligonucleotides, which interfere in the miRNAs/mRNAs interactions has been reported. Experiments carried out in zebrafish embryo demonstrate that 25-mer morpholino oligonucleotides complementary to the *sqr* or *lft* 3'UTR regions, which are targets of miR-430, inhibit the repression of the *sqt*-GFP reporter, which is normally induced by miR-430[101a].

**Replacement of miRNAs.** Sometimes restoring the function of miRNAs in cells might have therapeutic applications, for example when miRNAs silence unwanted or harmful genes, as when miRNAs act as tumor suppressors. The amount of miRNAs in cells may be restored using mimics of miRNAs or small molecules. As an example of small molecule we report the case of the antibacterial compound enoxacin, which is able to stimulate the expression of microRNAs tumor suppressor by enhancing microRNAs biogenesis machinery[128]; however this approach is not specific toward a single miRNA, unlike oligonucleotide-based approaches.

The function of miRNAs may be restored by **mimics of miRNA**, synthetic double stranded RNAs, which have one strand of the same sequence as endogeneous guide miRNA and the passenger strand either perfectly complementary or with mismatches. The guide is usually designed so that its 5' end is partially complementary to the sequence of the 3' UTR unique to the target gene. As siRNAs, mimics of miRNAs are processed by the RISC complex; while siRNAs are usually employed to silence a single mRNA inducing the degradation of its target, mimics target several transcripts, and potentially may regulate several genes.

Most of the studies are carried out with standard RNA duplexes, but there are also cases in which modified molecules are tested. For example hindered substituents on the bases, as **propyl** and **cyclopentyl**[78c] (Figure 4), which project in the minor groove, have been introduced in with the aim

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to reduce off target effects such as those derived from double-stranded RNA binding motifs and TLR-mediated immunostimulatory effects. Recent experiments carried out in peripheral blood mononuclear cell with mimics of miRNAs, aimed at restoring the activity of miR-122, demonstrated that cyclopentyl-guanines at positions 9 and 16 of the guide strand, in combination with cyclopentyl 2-aminopurine at positions 13 and 15 of the passenger strand, reduce the immunostimulatory activity of molecules, drastically decreasing the TNF-alpha levels[78c].

Interestingly some mimics of miRs are in clinical trials, as the mimics of miR-34 and let-7, both employed for the treatment of cancer. miR-34 antagonizes several oncogenic processes: it controls cell proliferation activating tumor suppressors pathways, inhibits cancer stem cells, which represent the seed of the tumor, metastasis and chemoresistance[129]. A liposomal formulation of a miR-34a mimic , named MRX34, is now in clinical trials at Mirna therapeutics Inc. for the treatment of patients with unresectable primary liver cancer or metastatic cancer with liver involvement (http://www.mirnarx.com/). The involvement of miR-34a in different cellular pathways led to hypothesize that miR-34a may act in synergy with conventional cytotoxic drugs: the combination of erlotinib, a tyrosin kinase inhibitor effective in a limited number of cases on non-small cell lung cancer, and a miR-34a mimic was tested in a panel of hepatocellular carcinoma cells (which are refractive to erlotinib)[130]. Interestingly, the drugs exert synergistic effect also on cells not sensitive to erlotinib.

Another example of mimic of miRNA, potential candidate as drug for lung cancer, is let-7, a tumor suppressor targeting a variety of oncogenes. Administration of let-7 mimic prior to implantation of the tumor xenograft in mice suppresses tumor formation while blocks tumor growth when injected into tumors developed prior to the treatment[131]. Experiments carried out in mice expressing an inducible mutant of the oncogene K-RAS, responsible for the formation of the non-

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small cell lung carcinoma (NSCLC), demonstrate that intravenous delivery of let-7 inhibits the formation of lung tumors[132].

In alternative, single stranded RNA mimics are employed as mimics of miRNAs; few studies on single stranded modified RNAs, **ss-siRNAs,** have been reported so far. ss-siRNAs are introduced as single strands and are designed for a seed-based recognition, as miRNAs. As siRNAs, ss-siRNAs are involved in the RNAi pathway, requiring Ago 2 for activity. Pioneering studies have been carried out by Chorn et al, who have tested 5' phosphorylated miR-124 mimics containing 2'-F and 2'-OMe[133]. The authors found that the single stranded mimics containing 2'-F nucleotides with a length of 19-22 nucleotides, were the most active in the inhibition of the target of the miR CD164 in HT116 cells. Substitution in position 2' of one F with one OMe caused a decrease in the activity of the mimic. Experiments carried out randomizing the 3' end bases of the mimic demonstrated that cassettes with a high percentage of pyrimidine perform better in the inhibition of the targets of the mimic as compared to cassettes with high purine content.

A very wide study has been proposed by Lima et al on the modifications which improve stability, pharmacokinetics and efficiency of ss-siRNAs[134]. Substitution of the 5'-phosphate by a stable 5'-E-vinylphosphate (VP) (Figure 5) increases the metabolic stability of the ss-siRNA. Structure-activity studies in which the chemical modifications of the RNA backbone and the Ago2 activity were evaluated, allowed to develop an active chimeric molecule composed of 2'-F phosphorothioates - 2'OMe phosphodiesters alternated nucleotides, a 5' VP followed by a 2'O-methoxyethyl nucleotide, and two adenosines 2'-Omethoxyethyl at the 3' end. Modified ss-siRNAs targeting Factor VII and ApoCIII mRNA showed a 50% reduction of the targets in the liver, but not of the off targets.

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ss-siRNAs were successfully tested *in vivo*, in the mouse central nervous system, to silence the mutant huntingtin and have proven to perform more efficiently than the ds-siRNA counterparts[135].

## RNAi for functional studies on ncRNAs

RNA interference **(RNAi)** is a naturally occurring pathway initiated by dsRNAs aimed at protecting the genome from the invasion of viruses and transposon. The RNAi machinery can be triggered by 21 nts duplexes directly introduced into cells (siRNAs) or engineered to be expressed in cells as short hairpins (shRNAs), which are then processed to siRNAs. siRNAs bind to the RISC complex which separates the siRNAs in two strands: the passenger strand, which is degraded and the guide strand which is carried from the RISC to its target mRNA, which will be cleaved. The introduction of synthetic siRNAs, as described by Zamore in 2000, is the methodology of choice for gene silencing in different experimental systems[4a]. siRNA duplexes have been modified in order to improve their silencing activity, which depends upon their stability to degradation, affinity and specificity of binding toward Ago2 and the target mRNA[136] (Table 5). Many companies started clinical trials using siRNAs for the cure of a number of diseases ranging from cancer to viral infections to hypercholesterolemia[137]. Although the exact composition of the drugs is not known, it appears that the main efforts in this field are devoted to the development of platforms for the targeted delivery of siRNAs, showing no accumulation in the target tissues, no sensitization during chronic dosing and durable knockdown of the target genes.

Interestingly siRNAs are also commonly employed in loss of function studies, aimed at understanding the biological role of ncRNAs. Few examples are described here.

siRNAs have been employed to unravel the function of the lncRNA AK139328, expressed at very high levels in normal mouse liver. AK139328 knockdown improved the ischemia/reperfusion injury in mouse livers, suggesting that this lncRNA may be a novel target to treat ischemia/reperfusion injuries[138].

The role of the lncRNA HOTAIR, deregulated in breast cancer progression, was discovered after HOTAIR depletion experiments by siRNAs in MCF7 cell[139]. These and other experiments suggested that HOTAIR promotes invasiveness in breast carcinoma cells.

In another example siRNAs were employed to target the lncRNA Fork head box C1 upstream transcript (FOXCUT), which was found overexpressed in oral squamous cell carcinoma: FOXCUT knockdown inhibited cell proliferation and migration and allowed to understand the positive correlation between the FOXC1 gene and the upstream transcript FOXCUT expression[140]. Finally we cite the example of siRNAs used to silence H19 RNA, an oncogenic lncRNA characterized in breast and colon cancer; H19 depletion by siRNAs resulted in suppression of U87 and U251 cell invasiveness and attenuation of migration[141].

## Modulation of the expression/function of long non coding RNAs

The knowledge we have at this time of the long non coding RNA world reveals that we are facing a very heterogeneous group of molecules, whose interactors have not yet been precisely defined, although some functions have been discovered, as described earlier. Interestingly, lncRNAs have already been recognized as unique targets for drugs as they offer the possibility of targeting a single location in the genome. The relationship emerging between the misregulation of the expression of ncRNAs and diseases also encourages studies aimed at discovering new tools to modulate their expression. The length of lncRNAs let us think of extensive secondary structures; therefore it is reasonable to suppose that efficient targeting of ncRNAs may be achieved by molecules with high affinity toward RNA, able to disrupt such structures. Experiments aimed at interfering with the expression of lncRNAs have been carried out in most cases with shRNAs and

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siRNAs, with the aim to understand their function[139]. Molecules employed for the targeting of lncRNAs are summarized in Table 6.

Single stranded oligonucleotide analogues have been used to target Natural Antisense Transcripts (NATs) to achieve the up-regulation of the corresponding protein. A recent paper by Modarresi reports that the inhibition of the NAT brain derived neurotrophic factor antisense transcript (BDNF-AS) by single stranded oligonucleotides (**antagoNATs**) results in the transient and reversible up-regulation of locus-specific gene expression[37]. The antagoNATs are 14-mers containing mixtures of 2'-OMe RNAs and LNAs; LNAs cap the oligonucleotide 3' and 5' ends and replace three more 2'-OMe bases within the sequence. BDNF-AS represses BDNF; inhibition of BDNF-AS causes the up-regulation of the BDNF mRNA transcript, alteration of the chromatin marks at the BDNF locus, increased protein levels, induction of neuronal outgrowth and differentiation *in vitro* and *in vivo*.

The antagoNAT strategy was applied also to inhibit the apolipoprotein 1-antisense, APOA1-AS[38]. APOA1-AS is a negative transcriptional regulator of the apolipoprotein 1 (APOA1) *in vitro* and *in vivo*, which modulates different histone methylation patterns. Phosphorothioates designed to cover all the APOA1-AS sequence were tested in humans and African green monkeys cells; active antagoNATs were found to cause the up-regulation of APOA1 mRNA. One 12-mer gapmer composed of two LNA nucleotides at the 5' end and three LNA nucleotides at the 3' end was injected intravenously into monkeys: the effect of this antagoNAT was an increase in the APOA1 mRNA expression, as judged by RT-PCR analyses of liver biopsies and an increase in the APOA1 circulating protein. These experiments demonstrate the effectiveness of antagoNATs in a primate model, suggesting the applicability of such strategy to induce gene up-regulation for a therapeutic purpose.

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Morpholino oligonucleotides were employed to target the recently identified HELLP lincRNA[142]. This long intergenic transcript is expressed in the placental extravillous trophoblast and is associated to HELLP syndrome, a disease associated to pregnancy inducing hemolysis, elevated liver enzymes and low platelets in the mother. Morpholinos targeting potential mutation site identified in HELLP families were demonstrated to reduce the invasion capacity of the trophoblasts.

LNAs have been used to knockdown eRNAs; it was found that LNAs caused the inhibition of induction of either the eRNA and the adjacent coding gene. It is therefore possible, by knocking down eRNAs, to modulate enhancers and related genes in a cell specific fashion[143].

Inhibition of the function of long non-coding RNAs by modified oligonucleotides may also be achieved with the aim to investigate pharmacological properties of modified oligonucleotides. The noncoding RNA gene metastasis associated lung adenocarcinoma transcript 1 (MALAT1) was employed as a target to study biodistribution, pharmacokinetic of modified oligonucleotides[144]. A 20-mer chimeric phosphorothioate oligonucleotide modified by 5-mer 2'-MOE at the 3' and 5' ends was tested in murine models while chimeric phosphorothioate oligonucleotides modified by ethyl bicyclic nucleic acid (cEt) in the three bases located at the 3' and 5' ends were tested in murine and monkey models. The concentrations of the oligonucleotide analogues were evaluated in different tissues and found to correlate with the MALAT1 knockdown; in general accumulation was higher in mice than in monkey organs and, accordingly, knockdown of MALAT1 was found more effective in mice than in monkeys. Liver was confirmed to be the primary target organ of the oligonucleotides systemically administered. 16-mer cEt was found more potent as compared to the 20-mer MOE in down-regulating MALAT1, although equally distributed across different species and organs.

With the aim to identify the functional region of the lncRNA Xist, Peptide Nucleic Acids oligomers have been employed in mapping experiments[145]. The technology named **Peptide Nucleic Acid-Interference Mapping** (P-IMP) based on the use of PNA oligomers conjugated to the carrier peptide transportan complementary to different regions of the first exon of Xist allowed the identification of a 19 nucleobases oligomer able to disrupt the binding of Xist to the X-chromosome, inhibiting the formation of Xi. These experiments demonstrate that Xist is organized in domains and that it exerts its function after binding to the X-chromosome; the Xist region responsible for the X-chromosome binding was also identified.

The function of long non coding RNAs was investigated by nuclease-induced segmental deletions of the corresponding genes. A recent paper reports the use of **Zinc Finger nuclease** and **TALEN** for the deletion of a lincRNA gene in zebrafish[146]. In studies on the function of MALAT1, Zinc Finger Nucleases were employed to silence its expression in human tumor cell; these studies revealed that the loss of the lncRNA is compatible with life and development[147].

## More applications of oligonucleotide analogues

Another aspect which deserves to be at least cited is the use of oligonucleotide analogues in studies devoted to the detection of ncRNAs and their targets. Peptide Nucleic Acids, for example, were employed to build molecular beacons for the detection of the long non coding Colon Cancer Associated Transcript 1 (CCAT1)[148]. The PNAs conjugated to poly-lysine and to the transdermal cell penetrating peptide contained as surrogate base the so called FIT probe, which acts as a light-up probe, allowing the imaging of CCAT1 in either cell lines and human biopsies. A little investigated but nevertheless interesting field is that of the imaging of nucleic acids using metal complexes; in fact, photophysical properties of metal complexes may be fine-tuned changing the metal ligands.

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3 Metal complexes of $^{64}Cu$ and $^{99}Tc$ conjugated to PNAs were applied to obtain scintigraphic imaging by PET and MRI of oncogene mRNA in tumors[149]. A paper from Mari et al proposed the use of luminescent rhenium complexes conjugated to PNA as tools for the detection of nucleic acids in cells; these complexes bearing bypiridine ligands were tested in HEK-293 cells and were demonstrated to possess good photoluminescence quantum yields[150]. The methodologies described may reasonably be applied to the imaging of ncRNAs in cells.

RNA oligomers modified by photo-activable moieties were recently described as tools to capture targets of miRNAs: a psoralen residue was incorporated into a biotinylated miRNA; upon irradiation the modified miRNA cross links to the target mRNA, which is isolated by biotin-streptavidin affinity column chromatography[151]. A similar application is described with RNA nucleotides bearing a diazirine containing nucleoside[152].

## Conclusions and perspectives

The recent findings on the functions of ncRNAs and on the networks they are involved in have substantially widened our prospects on gene regulation: ncRNAs are involved in RNA editing, work as scaffolds to help the assembly of multiple proteins, are gene repressors and recruit transcriptional regulators. Likely, many other biological activities will be discovered in the next future. The multiple functions discovered for ncRNAs highlighted new potential targets of pharmacological relevance; interestingly many of these functions are exploited through base pairing. The modulation of the expression of short and long ncRNAs by synthetic oligonucleotides appears therefore as a promising strategy to cure different diseases, from cancer to cardiovascular diseases. Although a detailed understanding of the mechanism of action of ncRNAs is not available yet, advanced studies in the field of short ncRNAs led to a number of clinical trials with either

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siRNAs, antagomiRs or miR mimics in a reduced time span. The design of unmodified oligonucleotides for siRNA, shRNA and antagomiR applications is aided by bioinformatics platforms, based on the combination of known or predicted thermodynamic parameters referred to the stability of siRNA and miRNAs' secondary structure, accessibility of the targets predictions and experimental data related to the activity of siRNAs/miRNAs. Unfortunately it is not as easy to design modified oligonucleotides and to predict how chemical modifications affect the stability and structure of the mimics and which modification will increase the potency of our molecules. Thermodynamic parameters may be obtained for 2'-OMe RNA/RNA and LNA-2'-OMe RNA/RNA duplexes using calculators developed by Kierzek et al, (http://rnachemlab.ibch.poznan.pl/) based on studies of the thermodynamic properties of synthetic modified duplexes. The next step will be that of performing structural studies of complexes formed by the oligonucleotides (or analogues) and ncRNAs, combining the structural and activity data available to obtain new bioinformatics tools useful to predict stable and bioactive molecules. The combination of structural data, biological activity data as derived from clinical trials and availability of thermodynamic predictions tools will contribute to the development of safe and potent oligonucleotides based drugs.

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**Biographies**

**Concetta Avitabile** received a B.S. degree in 2005 and a M.S degree in 2007 in Chemistry from the University of Naples "Federico II", Italy. She earned her Ph.D. in Chemical Sciences from the University of Naples "Federico II" in 2008, working with Alessandra Romanelli and Carlo Pedone on design, synthesis and characterization of Peptide Nucleic Acids and analogues. She is currently a postdoctoral scholar at the University of Naples "Federico II".

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**Abbreviations**

2’-OMe, 2’-O methyl RNA; 2’-MOE, 2’-O-(2-methoxyethyl) RNA; 2’-F, 2'-deoxy-2' fluoro RNA; 4’S-RNA, 4’-thio RNA; 4’S-FANA, 2’-deoxy-2’-fluoro - 4’ thioarabinonucleic acid; ABCA1, ATP-binding cassette transporter A1; Ago2, Argonaute 2; ALT, alanine-aminotransferase; ANRIL, antisense noncoding RNA in the INK4 locus; APOA1,apolipoprotein 1; AS, antisense; BDNF-AS, brain derived neurotrophic factor antisense transcript; BMPR2, bone morphogenetic protein receptor type II; CCAT1, Colon Cancer Associated Transcript 1; C/EBP$\alpha$, CCAAT-enhancer binding protein alpha; cEt, 2’, 4’ constrained 2’-*O*-ethyl modified RNA; ceRNA, competing endogenous RNA; circRNA, circular RNA; CRISPR, clustered regularly interspaced short palindromic repeats; dsRNAs, double stranded RNAs; eRNA, enhancer-like RNA; FIT, forced intercalation; FOXCUT, Fork head box C1 upstream transcript; FXR1, fragile X mental retardation syndrome related protein 1; HDL, high-density lipoprotein; hnRNPs, heterogeneous nuclear ribonucleoproteins; HOTAIR, Hox antisense intergenic RNA; KCNQ1OT1**,** KCNQ1 overlapping transcript 1; lincRNA, long intergenic non coding RNA; LNA, locked nucleic acid; lncRNA, long non coding RNA; MALAT1, metastasis associated lung adenocarcinoma transcript 1; MBSs, binding sites for miRNA; MCT, monocrotaline; MDSC, Myeloid-derived suppressor cells; miDPC, microRNA deposit complex; miRLC, microRNA loading complex; miRNA and miR, microRNA; MO, morpholino; moRNA, microRNA-offset RNA; NAT, natural antisense transcript; ncRNA, non-coding RNA; NEAT2, nuclear-enriched abundant transcript 2; NSCLC, non-small cell lung carcinoma; nts, nucleotides; ON, oligonucleotide; pA, polyadenilated; P-IMP, Peptide Nucleic Acid- Interference Mapping**;** PASR, Promoter-associated small RNA; piRNA, PIWI interacting RNA; PLA, polylactide; PNA, peptide nucleic acid; PROMPT, Promoter upstream transcripts; PS, phosphorothioate; qiRNA, QDE-2 interacting small RNA; PRC2, Polycomb repressive complex 2; pre-miRNA and pre-miR, precursor of microRNA; pri-miR, primary microRNA; rasiRNA, repeat associated small interfering RNA; RISC, RNA induced silencing complex; RNAi, RNA interference; shRNA, short hairpin; siRNA, small interfering RNA; snoRNA, small nucleolar RNA; snoRNP, small nucleolar ribonucleoprotein; spliRNA, splice junction associated RNA; ss, single strand; S-TuD, synthetic tough decoys; svRNA, small-viral RNAs; T- UCR, transcribed-ultraconserved RNA; TALEN, transcription activator-like effector nucleases; tel-sRNA, telomere small RNA; tiRNA, transcription initiation RNA; TLR, Toll like receptor; TSSa-RNA, Transcription start site–associated RNA; uaRNA, upstream antisense RNA; UTR, untranslated region; VLDL, very-low-density-lipoproteins; vRNA, viral RNA; VP, vinylphosphate; Xist, X-inactive specific transcript RNA.

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Table 1. Small RNA classes

| Name **(abbreviation)** | length, mechanism of action and function |
| --- | --- |
| **microRNAs** (miRNA) | ~19-25 nts, are produced by Dicer mediated cleavage of double- stranded portion of precursors of miRNAs. Associate with the miRISC complex and modulate, at the post-trascriptional level, protein expression by partial complementary base-pairing with target mRNAs; affect translation and stability of mRNAs . |
| **Mirtrons** | ~22 nts**,** belong to the splicing-derived miRNAs that comprise also 5'-tailed mirtrons, 3'-tailed mirtrons and sintroms. Function as miRNAs**.** |
| microRNA-offset RNAs (**moRNAs**) | ~ 20 nts**,** are produced by the hairpin precursor of miRNAs . So far, the knowledge on their possible functions is still fragmentary. |
| Piwi-interacting RNAs (**piRNAs**) | ~26-31 nts, are produced by a Dicer independent mechanism. They exclusively bind to the Piwi subfamily of the Argonaute proteins, are essential in the development of germ cells and regulate transposon activity. |
| Promoter-associated short RNAs (**PASRs**) | ~20-200 nts, are produced by a Dicer independent mechanism. Their localization at genomic level coincides with the 5' end of transcription start sites of both coding and non-coding genes. A role in the regulation of gene expression has been hypothesized. |
| QDE-2 interacting small RNAs (**qiRNAs**) | ~20-21 nts, in their mature form interact with QDE2, a member of the Argonaute protein family. Their role in the DNA damage response (DDR) remains to be clarified . |
| Small interfering RNA (**siRNAs**) | ~20-24 nts, produced by Dicer mediated cleavage of double-stranded duplexes. Form complexes with Argonaute proteins; by fully complementarity with their targets modulate gene expression, are involved in transposon control. siRNAs are involved in the immune response either in plants and in mammalian. |
| Small nucleolar RNAs (**snoRNAs**) | ~60-300 nts, are produced by a Dicer independent mechanism. Members of the larger family of snoRNAs, are localized to the nucleolus . Are involved in pseudouridylation and ribosomal RNA modifications. |
| Small-viral RNAs (**svRNAs**) | ~ 22–27 nts, derive from viruses, can control the viral switch between transcription and replication. In particular, svRNAs are detectable during replication of various influenza A viruses, their expression correlates with the accumulation of viral genomic RNA (vRNA). |
| Sno-derived RNAs (**sdRNAs**) | ~20-24 nts, are produced by Dicer cleavage of longer precursors, associate with Ago2. Possess regulatory functions, being involved in gene silencing. |
| Splice junction associated RNAs (**spliRNAs**) | ~17- 18 nts, hypothesized to be generated by a RNAP II dependent mechanism, are involved in the post-transcriptional gene silencing**.** |
| Telomere small RNAs (**tel-sRNAs**) | ~24 nts, are produced by a Dicer independent mechanism. Are involved in the establishment and maintenance of heterochromatin structures. |
| Trascription initiation RNAs (**tiRNAs**) | ~18 nts, are produced by a Dicer independent mechanism. Typically derived from sequences localized immediately downstream of transcription initiation sites**,** have been hypothesized to be involved in the transcription. |
| Transcription start site–associated RNA (**TSSa RNAs**) | ~ 20-90 nts, flank active promoters, probably regulate the maintenance of transcription. |

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Table 2. Long ncRNA classes

| Name **(abbreviation)** | length, mechanism of action and function |
|---|---|
| Antisense Non-coding RNA in the INK4 Locus **(ANRIL)** | Transcribed by RNA pol II, has alternatively spliced isoforms, the unspliced transcript is 34.8 kb termed p15AS. Plays an important role in cell cycle control, cell senescence, stem cell renewal and apoptosis and has been associated with a range of human diseases including cancer. |
| Competing endogenous RNAs (**ceRNAs**): | Natural decoys for microRNAs. The majority of validated ceRNAs are mRNAs, also some transcripts of lncRNAs have been described to have ceRNA activity. They play important roles in physiological and pathological processes, such as development and cancer. |
| Circular RNAs (**circRNAs**) | Endogenous RNAs, arising from a 3′–5′ ligation of both ends of a linear RNA strand, are characterized by multiply repeated miRNA binding sites, that can serve as sponge for miRNAs. Possess interesting properties as high cellular resistance to exonuclease digestion, and lack of a poly-A tail that would avoid miRNA-mediated destabilisation after the binding . Mammalian brain tissues appear to be enriched in ciRS-7 , a circRNA that contains multiple, tandem sequences for miR-7. |
| Enhancer RNAs **(eRNAs**): | ≈2- kb, involved in development and differentiation, are important regulators of their neighboring protein-coding genes. |
| Homeobox (HOX) transcript antisense RNA (**HOTAIR) :** | 2.3kb, is an intergenic ncRNA, spliced and polyadenylated; it has been found associated with the mammalian polycomb repressive complex 2 (PRC2). It is involved in primary and metastatic breast cancer. |
| KCNQ1 overlapping transcript 1 (**KCNQ1OT1**): | 91 kb, is an imprinting lncRNA, unspliced and polyadenylated transcript found in the nuclear compartment. It is transcribed in the antisense direction to Kcnq1 gene, it is important for bidirectional silencing of genes in the Kcnq1 domain and regulates the expression in *cis* of neighboring genes. |
| Metastasis-associated lung carcinoma transcript 1 (**MALAT1**) | About 7 kb, localized in nuclear speckles, is processed by RNaseP and RNaseZ to generate the small ncRNA mascRNA, which is then exported to the cytoplasm. The larger MALAT1 RNA seems to have a role in regulating alternative splicing machinery. Studies have demonstrated implication of MALAT1in multiple cancers. |
| Natural antisense transcripts (**NATs**) | Have partial or full sequence complementarity to other transcripts. Although the function of the majority of NATs is still unknown, experimental evidences suggest that they can be subdivided in *cis*- and *trans* NATs . They are involved in a broad range of gene regulatory events. |
| Promoter upstream transcripts (**PROMPTs**) | 200-600 nts, are produced immediately upstream of TSS . Are transcribed in both sense and antisense direction respect to the TSS of gene. Their function is largely unknown. |
| Ultraconserved RNAs (**T- UCRs)** | 200-3000 nts, are transcription products of UCRs, localized in genomic regions, absolutely conserved (100% identity with no insertions or deletions) between orthologous regions of the human, rat, and mouse genomes. Their function are still undefined but evidence supports their modulation on gene expression that includes an antisense inhibitory role for other ncRNAs or protein coding genes, or a role as enhancer of gene expression, or as a sponge for microRNAs. |
| X-inactive specific transcript RNA (**XIST**) | 17 kb in mouse and 19 kb in human, is a polyadenylated long ncRNA that plays a critical role in X-chromosome inactivation. |

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Table 3. Examples of oligonucleotide analogues inhibitors of the maturation of miRNAs

| Name | Function | Modification/architecture | Mechanism of action |
|---|---|---|---|
| LooptomiR | Inhibition of the biogenesis of miRNAs | 2'-OMe/ss | Bind to pri-mi loop, abolishing Drosha activity |
| Antipre-miR | Inhibition of the biogenesis of miRNAs | PNA/ss | Bind to pre-miR, preventing its processing |
| Antipri-miR | Inhibition of the biogenesis of miRNAs | Morpholino/ss | Bind the pri-miR, preventing its processing |
| AntagomiRzyme | Inhibition of the biogenesis of miRNAs | DNA,LNA/ss | Bind and cleave the pre-miR |

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Table 4. Examples of oligonucleotide analogues modulators of the function of miRNAs

| Name | Function | Modification/architecture | Mechanism of action |
|---|---|---|---|
| AntagomiRs | Inhibit the function of miRNAs | LNA, 2'-F, 2'-OMe, 2'-MOE, PNA, PS, cholesterol /ss | Sequester the mature miRNAs and prevent the interaction of miRNAs and mRNAs |
| Tiny LNAs | Inhibit the function of miRs sharing the same seed | LNA-PS/ss | Bind the seed region of miRNAs and prevent interactions of miRNAs and mRNAs |
| CantimiRs | Controlled Inhibition of the function of miRNA | 2'-OMe (stems)-coumarin (loop)/asymmetric stem loop | See AntagomiRs |
| S-TUDs | Inhibit the function of miRNAs | 2'-OMe/ds | Bind to miRNAs |
| miR-masks | Inhibit the function of miRNAs | Morpholino/ss | Bind the 3'UTR mRNA regions which are occupied by miRs and prevent interactions of miRNAs and mRNAs |
| miRNA mimics | Enhance the activity of miRNAs - regulate several transcripts | LNA, 2'-F, 2'-OMe,PS /ds | Are incorporated into the RISC and do not induce degradation of mRNAs |

Table 5. Examples of oligonucleotide analogues for gene silencing

| Name | Function | Modification/architecture | Mechanism of action |
|---|---|---|---|
| siRNAs | Silence potentially a single gene | LNA, 2'-F, 2'-OMe, 2'-MOE, PNA, PS, allyl, cholesterol/ds | Are incorporated into the RISC and induce mRNA degradation |
| ss-siRNAs | Silence potentially a single gene | 2'-F, 2'-OMe , PS, 5' phosphate, 5'-E-vinylphosphate/ss | Are incorporated into the RISC and bind the target mRNA by their seed |

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Table 6. Examples of oligonucleotide analogues as modulators of the function of lncRNA

| Name | Function | Modification/architecture | Mechanism of action |
|---|---|---|---|
| antagoNAT | Up-regulation of locus-specific gene expression | 2'-OMe, LNA, PS/ss | Bind Natural antisense transcripts |
| Other Antisenses to lncRNAs | Inhibition of lnc (linc, MALAT1) | Morpholino, 2'-MOE, PS, cEt/ss | Bind selected lncRNAs, inhibiting their function |

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Table 7. Examples of non oligonucleotidic molecules as modulators of the function of ncRNAs

| Name | Function | Mechanism of action |
|---|---|---|
| Small molecules (e.g. aminoglycosides) | Inhibition of maturation of miRNAs | Bind to Drosha or Dicer sites on pre-miRNAs |
| peptoids | Inhibition of maturation of miRNAs | Bind to loops of pre-miRs |
| TALEN | Inhibition of production of miRNAs | Deletion of ncRNA genes |
| CRISPR-associated (Cas) | Inhibition of production of miRNAs | Deletion of miRNA genes |
| Zinc Finger Nuclease | Inhibition of production of miRNAs | Deletion of lncRNA genes |

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**Legends to Figures**

**Figure 1.** Selected examples of clinical applications of antagomiRs of: **A**: miR 92-a, which targets Janus kinase 1 (Jak 1) and integrin $\alpha$5 (Itg$\alpha$5), results in proangiogenic effects[153]; **B**: miR-21, which binds to Spry1, a negative regulator of the MAP kinase signaling in cardiac fibroblasts, reverting interstitial fibrosis and alleviating cardiac disfunction[123a]; **C**: miR-33a, which targets the adenosine triphosphate-binding cassette transporter A1 (ABCA1), acting on cholesterol transport[114]; **D**: miR-122, causes a reduction in the HCV RNA replication[82].

**Figure 2**. Selected examples of application of siRNAs for the knockdown of: **A**: B-cell CLL/lymphoma 2 (BCL-2), an antiapoptotic protein overexpressed in Chronic lymphocytic leukemia (CLL)[154]; **B**: protein kinase N3 (PKN3), resulting in the inhibition of tumor progression and metastasis formation in patients with advanced solid tumors[155]; **C**: MALAT and HOTAIR which results in the inhibition of invasion and migration of lung metastases[40139]; **D**: polo-like kinase 1 (PLK-1) which inhibits liver metastases progression[156].

**Figure 3.** Representation of the chemical structure of oligonucleotide analogues with modification on the backbone. B: nucleobase.

**Figure 4.** Representation of the chemical structure of the modified bases. R= ribose.

**Figure 5.** Representation of the chemical structure of the “end capping” tools.

**Figure 6.** Representation of two conformations assumed by the sugar rings in oligonucleotides.

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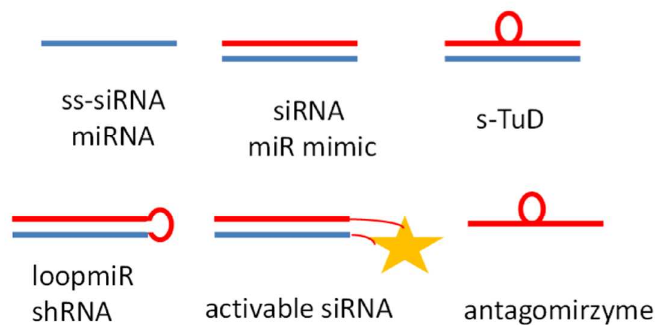
## Table of Contents graphic



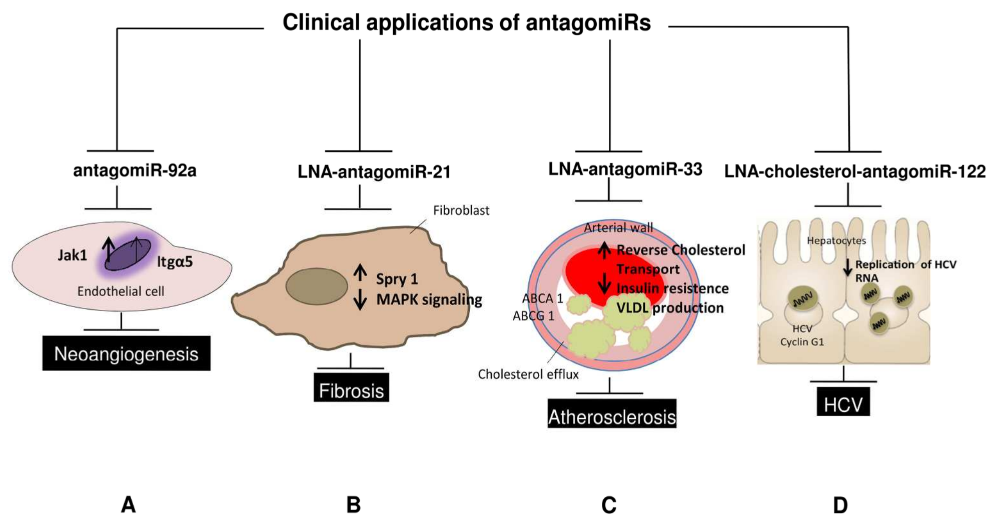



Figure 1. Selected examples of clinical applications of antagomiRs of: A: miR 92-a, which targets Janus kinase 1 (Jak 1) and integrin α5 (Itgα5), results in proangiogenic effects153; B: miR-21, which binds to Spry1, a negative regulator of the MAP kinase signaling in cardiac fibroblasts, reverting interstitial fibrosis and alleviating cardiac disfunction123a; C: miR-33a, which targets the adenosine triphosphate-binding cassette transporter A1 (ABCA1), acting on cholesterol transport114; D: miR-122, causes a reduction on the HCV RNA replication82.
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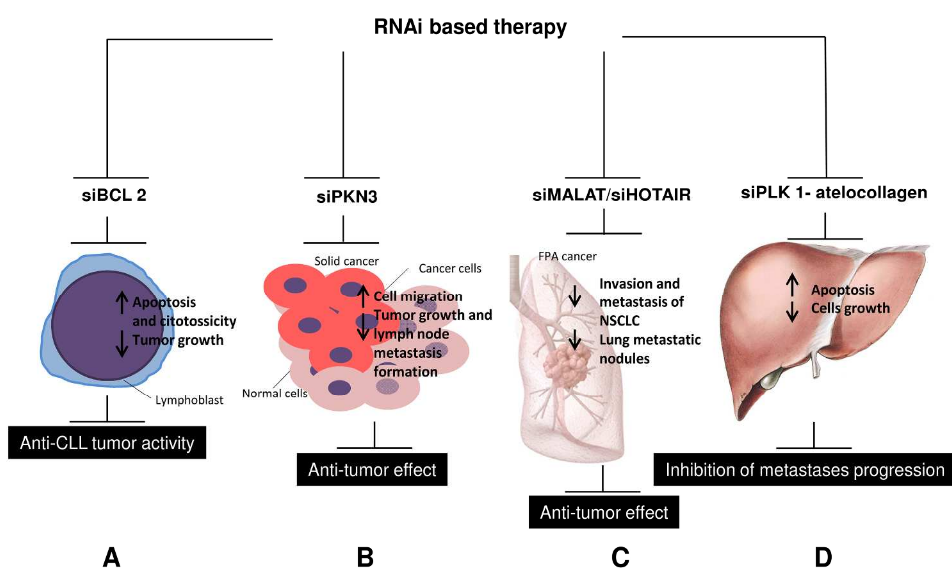


Figure 2. Selected examples of application of siRNAs for the knockdown of: A: B-cell CLL/lymphoma 2 (BCL-2), an antiapoptotic protein overexpressed in Chronic lymphocytic leukemia (CLL)154; B: protein kinase N3 (PKN3), resulting in the inhibition of tumor progression and metastasis formation in patients with advanced solid tumors155; C: MALAT and HOTAIR which results in the inhibition of invasion and migration of lung metastases40139; D: the polo-like kinase 1 (PLK-1) inhibits liver metastases progression156.
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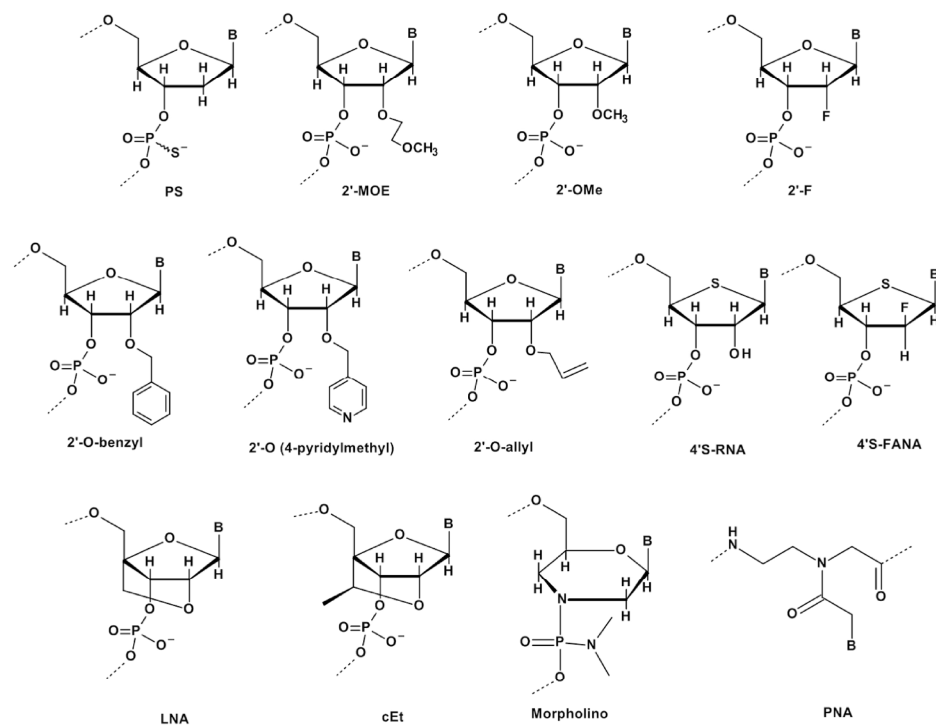


Figure 3. Representation of the chemical structure of oligonucleotide analogues with modification on the backbone. B: nucleobase.
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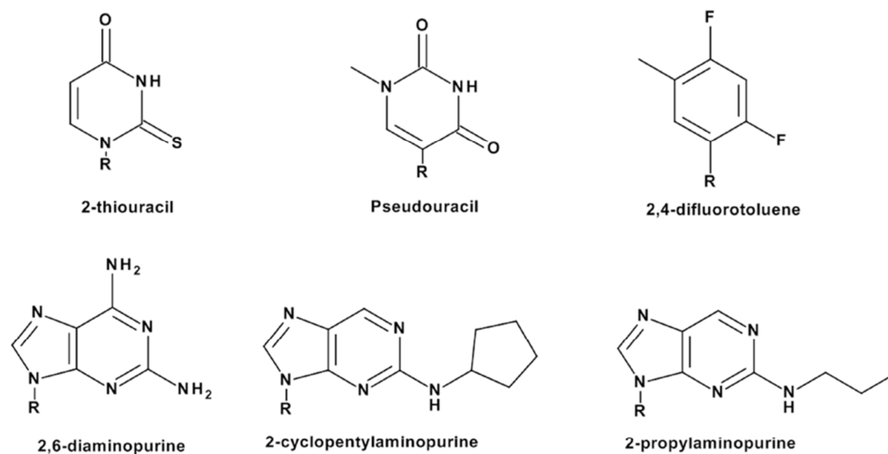


Figure 4. Representation of the chemical structure of the modified bases. R= ribose.
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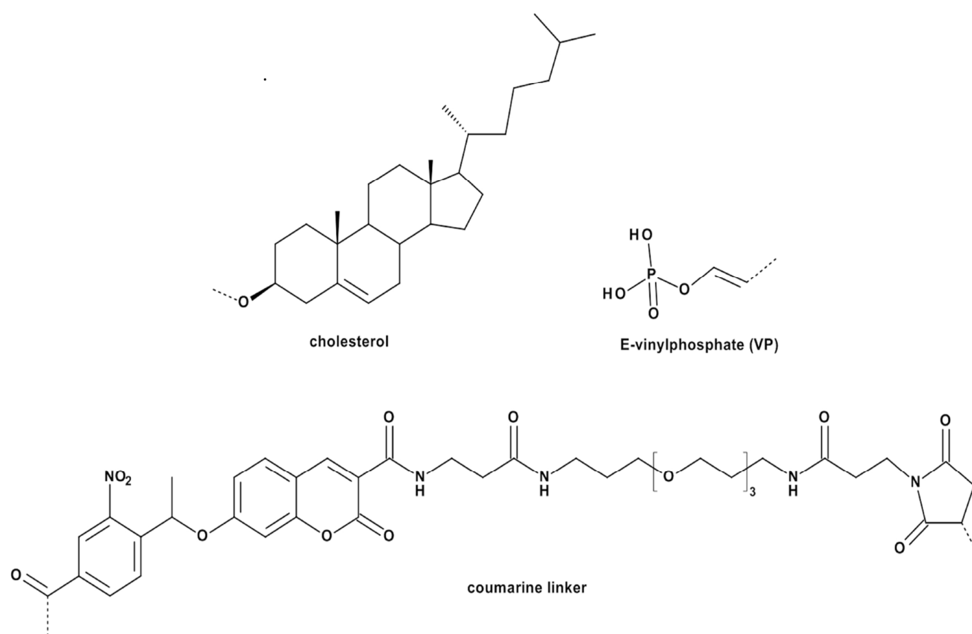


Figure 5. Representation of the chemical structure of the "end capping" tools.
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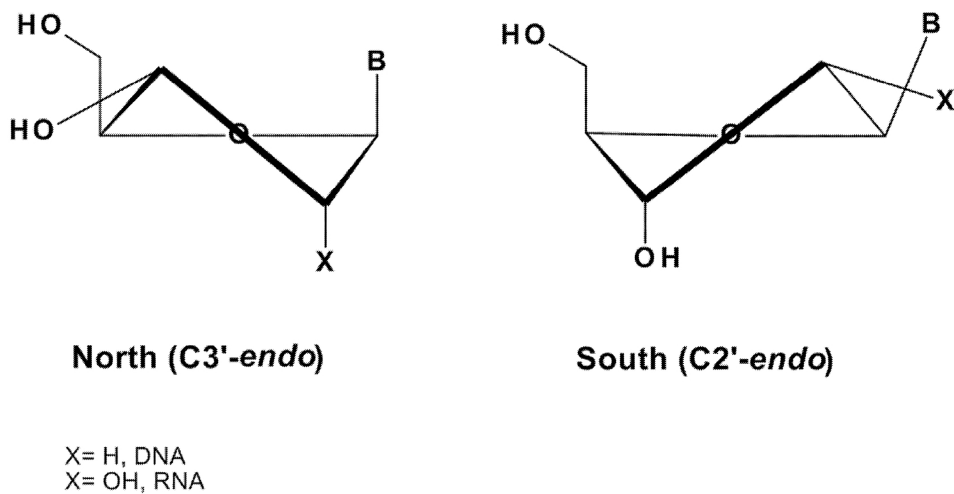


Figure 6. Representation of two conformations assumed by the sugar rings in oligonucleotides.
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