

REVIEW



Anti-miRNA oligonucleotides: A comprehensive guide for design

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ABSTRACT

MicroRNAs (miRNAs) are small, non-coding RNA molecules that regulate gene expression post-transcriptionally. As a consequence of their function towards mRNA, miRNAs are widely associated with the pathogenesis of several human diseases, making miRNAs a target for new therapeutic strategies based on the control of their expression. Indeed, numerous works were published in the past decades showing the potential use of antisense oligonucleotides to target aberrant miRNAs (AMOs) involved in several human pathologies. New classes of chemical-modified-AMOs, including locked nucleic acid oligonucleotides, have recently proved their worth in silencing miRNAs. A correct design of a specific AMOs can help to improve their performance and potency towards the target miRNA by increasing for instance nuclease resistance and target affinity. This review outlines the technologies involved to suppress aberrant miRNAs. From the design strategies used in AMOs to its application in novel miRNA-based therapeutics and detection methodologies.

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Introduction

MiRNAs are small, single-stranded and noncoding RNAs, with approximately 21 nucleotides (nt) that regulate gene expression at a posttranscriptional level. They complementarily bind to the 3' untranslated region (3'UTR) of their target messenger RNAs (mRNAs) causing their degradation, translational repression, and/or deadenylation (Fig. 1) [1–3].

In most eukaryotic cells, miRNA processing unleashes a silencing response specific to a target RNA sequence, so-called RNA interference (RNAi). The RNAi pathway is an important cellular process to control post-transcriptional gene expression, triggered endogenously by the conversion of double-stranded RNA sequences into miRNAs [4]. MiRNA transcription starts in the nucleus, typically from intronic or intergenic regions of DNA, with the formation of a primary structure called pri-miRNA as a result of the action of RNA polymerase II or RNA polymerase III [5,6] (Fig. 1). The pri-miRNA has a long stem and loop structure that is shortened to a pre-miRNA with ~60 nt by ribonuclease-III Drosha together with the DGCR8 protein (Microprocessor complex subunit DGCR8: DiGeorge syndrome critical region 8) [7,8]. The pre-miRNA is then exported to the cytoplasm via the Exportin-5/Ran complex, [9] where it is further processed by another ribonuclease-III enzyme named Dicer (Double-strand-specific ribonuclease) [10–12] into an imperfect miRNA duplex with unpaired nucleotides (Fig. 1). In the cytoplasm, the pre-miRNA is recognized and cleaved by Dicer with the assistance of TRBP

(Transactivation-responsive RNA-binding proteins), forming a smaller double-stranded miRNA (ds-miRNA) with 21–27nt. After the cleavage, the ds-miRNA dissociates from and re-associates with Dicer at a different position with the help of TRBP. Although, there are some studies reporting that TRBP is dispensable for miRNA biogenesis, recent evidences reinforce that the TRBP increases the RNA-binding affinity of Dicer and enhances cleavage accuracy, especially in a crowded environment [13]. The protein argonaute-2 (Ago2) [14] is further associated to this complex, forming a loading complex (RLC), [15] which assists in the assembling of an RNA-induced silencing complex (RISC). The ds-miRNA is then transferred to the Ago2 in a process mediated by Hsp90 (heat shock protein 90-KDa), which maintains the Ago2 in an open conformation in order to receive the miRNA, forming a pre-RISC complex. Inside the Ago2, the two miRNA strands are separated and the passenger strand is removed. The selection of the guide strand from a ds-miRNA is not arbitrary. The strand whose 5' end is less stable serves as guide, where the other is discarded (the “asymmetry rule”). It is believed that TRBP senses the asymmetry of the ds-miRNA, and positions the miRNA in an orientation that allows a correct strand selection and loading to Ago2 proteins.

Finally, the RISC complex is then conducted by the mature miRNA to the complementary mRNA (Fig. 1) [16]. In the case in which the RISC binding is perfectly complementary to the target mRNA, the mRNA will be cleaved by Ago2. This

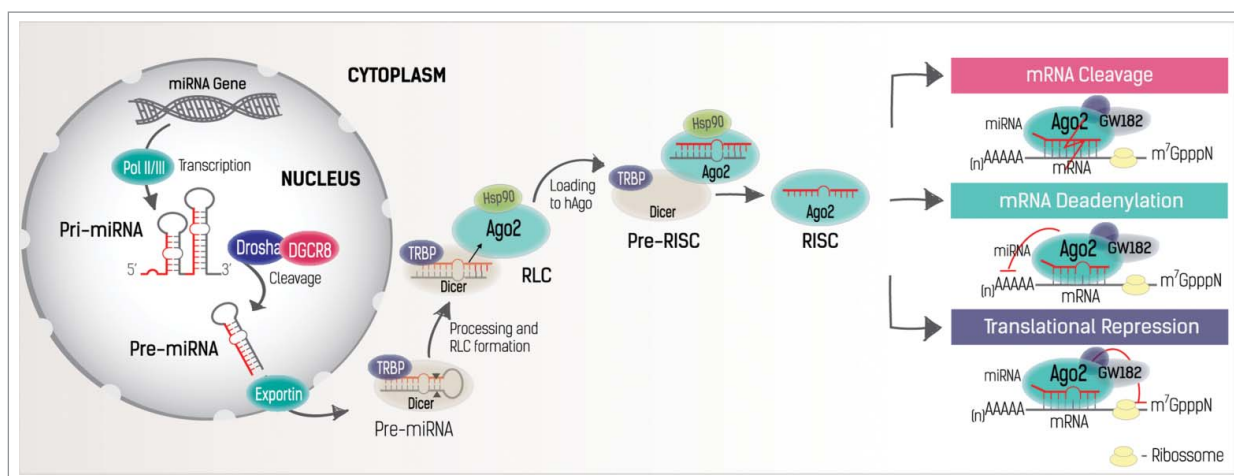


Figure 1. MiRNA biogenesis. Formation of a primary miRNA structure (pri-miRNA) by RNA polymerase II and III (Pol II/III), followed by Drossha's cleavage, resulting in a pre-miRNA. The pre-miRNA is then exported to the cytoplasm by Exportin-5/Ran complex (Exportin), where it is transformed in a loop-free homoduplex miRNA by the action of Dicer-TRBP. After association of the RLC complex comprised essentially by Dicer-TRBP-Ago2, the ds-miRNA is loaded into Ago2 (mediated by Hsp90), where is further processed to a mature single-stranded miRNA (guide strand). The RISC complex is then guided by the miRNA to the target complementary mRNA, leading to mRNA cleavage, translational repression or deadenylation. Upon RISC binding, other important proteins may associate to the Ago2, like GW182, to assist on the silence of the mRNA. DGCR8: Microprocessor complex subunit DGCR8 (DiGeorge syndrome critical region 8); Dicer: Double-strand-specific ribonuclease; TRBP: Trans-activation-responsive RNA-binding protein; Ago2: protein argonaute-2; Hsp90: Heat shock protein 90-KDa; RLC: RISC-loading complex; RISC: RNA-induced silencing complex; (n)AAAAA: poly-A tail of RNA; m⁷GpppN: 7-methyl guanosine cap structure of RNA.

process is frequently found in plants, where miRNAs share high homology to the 3'UTR of mRNAs. Conversely, in mammals, the RISC normally binds partially to the complementary mRNA. The seed region at positions 2–7 from the 5'-end of the miRNA sequence, is often fully paired and essential for the interaction. Due to this partial complementarity, translation repression and/or mRNA deadenylation pathways (Fig. 1) [17–19] are preferred rather than mRNA cleavage. After silencing, the miRNA remains intact and able to target other mRNA molecules with the same complementarity degree.

Translation repression and/or mRNA deadenylation processes are intimately connected and although the precise translation repression mechanism is still unclear, the deadenylation and consequent mRNA decay mediated by miRNA is better understood. The RISC complex of miRNAs can trigger mRNA deadenylation that leads to decapping and ultimately to mRNA decay. The deadenylation process starts with the association of Ago2 proteins present in the RISC complex to a GW182 protein which is responsible for the recruitment of cytoplasmic deadenylase complexes: PAN2–PAN3 (PAB-dependent poly (A)-specific ribonuclease subunit PAN2 and PAN3) and CCR4–NOT (transcription complex subunit) [20]. Deadened mRNAs by the deadenylase complexes are then decapped by DCP2 (m⁷GpppN-mRNA hydrolase) [21]. Without the protection of the cap structures mRNAs are ultimately degraded by the cytoplasmic 5'-to-3' exoribonuclease XRN1.

To understand how miRNAs may repress translation and consequently lead to mRNA decay, it is desirable to go a step back and have a short overview of the normal mRNA translation mechanism. Translation is a multi-step process beginning with the recruitment of ribosomal subunits to the mRNA, leading to the initiation, elongation, and termination of the translation. A cap-dependent translation, which is the most frequent process for translation, requires an mRNA with a 5'-cap and a poly-A tail. The 5'-cap is recognized by the cap-binding protein, eIF4E (Eukaryotic translation initiation factor 4E), part of

the eIF4F initiation complex. The eIF4F complex is responsible for recruiting the 40s ribosomal subunit to the mRNA, which is then checked for the initiation codon to start translation. The assembly of the 60s subunit is then followed by the elongation phase. The cytoplasmic poly-A-binding protein (PABPC) also play an important role in the translation. PABPC proteins recognize the poly-A tail and interact with eIF4F complex, forming a circular structure essential to protect the mRNA from degradation and thus leading to an effective translation [22].

Several studies are trying to elucidate how miRNAs interact with the mRNA translation machinery and their role in the mRNA deadenylation and decay [23–26]. A common ground of understanding is that miRNAs can inhibit cap-dependent translation at the initiation phase. More likely, miRNAs seem to interfere with the 5'-cap eIF4F complex. First studies indicate that deadenylation is the cause of the translation depression. Deadened mRNAs fail to assume the circular conformation due to the action of GW182 proteins on PABPC [27,28] and therefore contribute to the repression and decay of mRNA. However, in 2006 it was demonstrated that although the two mechanisms are intimately connected, deadenylation and translation repression can function independently [29]. MiRNAs can repress the translation before the deadenylation step occurs, albeit the extension of the repression on deadened mRNAs seem to be higher in comparison to the one observed in mRNAs containing the poly-A tail [26].

Clearly, post-transcriptional mechanisms of miRNA play an important role in gene control, and in this way can influence different biological processes. For instance, they are able to trigger inflammatory responses, to control cell proliferation, apoptosis, DNA repair, and DNA methylation processes. It comes with no surprise that many miRNAs are associated with several human diseases and have already been identified in different pathologies including neurological disorders, cardiovascular diseases, inflammatory processes, and cancer. In the latter case, more than half of the miRNA genes are located in specific

genomic regions frequently associated with cancer development and progression [30]. MiRNAs are able to control the expression levels of oncogenes and tumor suppressor genes, modulating apoptosis, cell migration, and angiogenesis (Fig. 2).

In cancer, miRNAs can act both as oncomirs (Fig. 2A) and as tumor suppressors (Fig. 2B) [31,32] depending on which genes they control. Downregulation of tumor suppressing genes or miRNAs that regulate oncogenes, promote tumor development, either by the overexpression of miRNAs with oncogenic activity (oncomirs) (Fig. 2A) or by the low expression of miRNAs with tumor suppressor activity (Fig. 2B) [33–37].

Furthermore, the same miRNA can be identified in different types of cancer, and within the same type of cancer it can have both roles, depending on its expression levels and target gene (s). For instance, miR-9 was found deregulated in leukemia, cervical, brain, breast, gastric, and colorectal cancers (Table 1) [38–46]. In the case of gastric cancer (GC), there is evidence that miR-9 is under-expressed, functioning as a tumor suppressor by targeting the oncogenes *RAB34*, *NFKB1*, *CCND1*, and *ETS1*; miR-9 was also reported to function as an oncomir by targeting the *CDX2* gene (Table 1) [42,43,47,48]. In 2009, Hongchun Luo et al. [47] showed that miR-9 expression was directly linked to the *RAB34* gene expression, encoding the Ras-related protein Rab-34. Rab-34 is a small GTPase protein

member of the RAS oncogene family, which regulates aggregation, junction, and fusion of vesicles in exocytosis and endocytosis. The authors observed that the stimulation of the expression of miR-9 in gastric cell lines led to a decrease of Rab-34 expression. The same was observed in the work of Hai-Ying Wan et al, [48] regarding the *NFKB1* gene, which is responsible for the regulation of immune responses, embryo and cell-binding development, cell-cycle progression inflammation, and oncogenesis. The authors observed that the re-expression of miR-9 in gastric cell lines led to a decrease in the expression of the NF-kB1 factor, which in its turn downregulates VEGF, resulting in the inhibition of the invasion and metastasis of tumorigenic cells.

The *CCND1* gene encodes Cyclin D1, an oncoprotein from the family of G1 cyclins that inhibits members of the retinoblastoma (RB) protein family and regulates the cell cycle during the G1/S transition. Overexpression of Cyclin D1 is often associated with carcinogenesis at early stages and is a prognostic indicator of poor survival in cancers like breast cancer [49]. Ets-1 is a transcription factor that directly controls the expression of a wide variety of genes that regulate differentiation, proliferation, and invasion [50]. Restoring the expression levels of miR-9 in GC cells, attenuated the proliferation, migration, and invasion of cancer cells, by knocking down the expression of Cyclin D1 or Ets-1⁴².

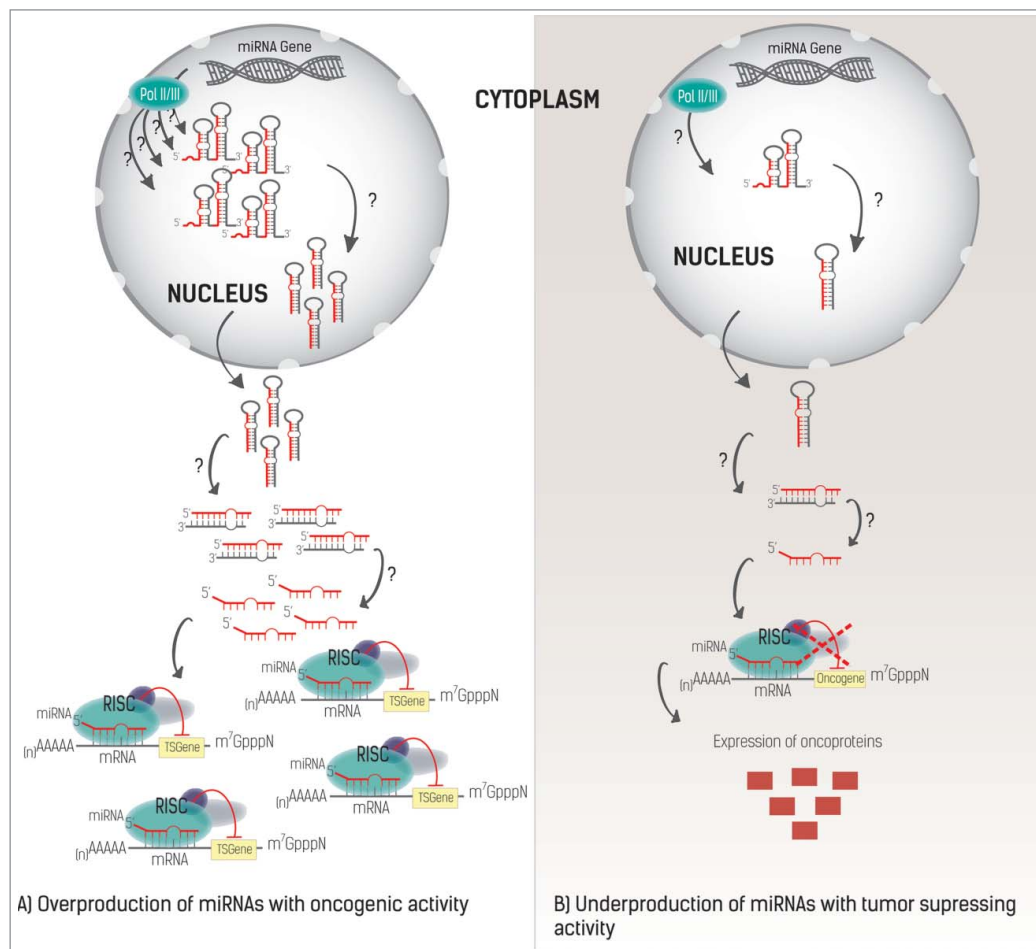


Figure 2. The different roles of miRNA in gene expression. A) The overexpression of miRNAs that target tumor suppressing genes, thus being responsible for the development of cancer (oncomir). B) Underexpression of miRNA with a tumor-suppressing profile leads to an increase of oncoproteins (red squares) promoting tumor formation. The question marks represent any perturbation on the miRNA level, which can occur at any stage of the miRNA biogenesis, leading either to its overproduction or degradation.

Table 1. Role of miR-9 and its validated targets in different cancer types.

miR-9 role	Type of cancer	miR-9 targets	References	
Onco-miRNA	Colorectal Cancer	<i>CTNNA1</i>	[128]	
	Breast Cancer	<i>CDH1</i>	[129-131]	
	Gastric Cancer	<i>CDX2</i>	[43]	
	Liver Cancer	<i>TLN1, CDH1, KLF17</i>	[132-134]	
	Bladder Cancer	<i>LASS2</i>	[135]	
	Leukemia	<i>HES1, MLL</i>	[38,136]	
	Osteosarcoma	<i>GCI</i>	[137]	
	Glioblastoma Multiforme (GBM)	<i>MAPKAP/MAPK14/MAPKAP3</i>	[138]	
	Squamous cell carcinoma: Esophageal Cancer and Skin Cancer, respectively	<i>CDH1, CTNNA1</i>	[45,139]	
	Tumor Suppressor	Colorectal cancer	<i>TM4SF1, UHRF1</i>	[40,140]
		Breast Cancer	<i>NOTCH1</i>	[41]
		Gastric Cancer	<i>NFKB1, RAB34, CCND1 and ETS1</i>	[47,48,139]
		Liver Cancer	<i>WWTR1, IL6, AP3B1, TC10, ONECUT2, IGF2BP1, MYO1D, ANXA2</i>	[141,142]
Cervical Cancer		<i>CKAP2, HSPC159, IL6, and TC10</i>	[39]	
Lymphoblastic leukemia		<i>FGFR1 and CDK6</i>	[143]	
Ovarian Cancer		<i>NFKB1</i>	[144]	
Oral squamous cell carcinoma		<i>CXCR4</i>	[145]	
Melanomas		<i>NFKB1</i>	[146]	
Medulloblastoma		<i>HES1</i>	[147]	
Neuroblastoma		<i>MMP14</i>	[46]	

Regarding the oncogenic activity of miR-9, Rotkrua et al. [43] suggested that the downregulation of *CDX2* by miR-9 might contribute to cell proliferation in GC. The *CDX2* gene encodes a homeobox protein involved in the differentiation and maintenance of the intestinal epithelial lining [51]. It is still unclear the role of *CDX2* in GC, whether it functions as a tumor suppressor or an oncogene. At an initial stage, *CDX2* gene expression is associated with the development of intestinal metaplasia of the stomach, a premalignant lesion that increases the risk of the patient to develop GC [52,53]. During cancer progression, the expression of *CDX2* progressively decreases, suggesting it might function as a tumor suppressor gene and a prognostic marker [49].

The double role of miRNAs is an interesting example that highlights the importance of carrying out a comprehensive characterization of the role of miRNAs in cancer and their post-transcriptional mechanisms in mRNA regulation. This would allow to develop new strategies in cancer treatment and cutting-edge therapies specifically designed to control oncomirs or to promote the expression of miRNA with tumor suppressing activity.

2. Controlling miRNAs activity

It is quite straightforward that, in order to control miRNA activity, it could be desirable to restore miRNA expression with tumor suppressing activity (gain-of-function) or to block miRNAs with oncogenic activity inhibiting its function (loss-of-function) [54].

Gain-of-function strategies mainly rely on either the incorporation of double-stranded chemically synthesized miRNA mimics or on viral vectors to mediate miRNA overexpression. Chemically synthesized miRNA mimics are generally not the exact natural miRNA duplex. The sense strand is altered to be perfectly complementary to the mature miRNA (to increase stability), and should be chemically modified to inactivate this now non-natural sense-strand. MiRNA mimics can be designed to target a single mRNA [55] or to incorporate multiple miRNA units for targeting different mRNAs and silence

multiple target genes [54,56,57]. Viral vector-mediated miRNA overexpression consists on the use of an integrating vector system, containing short hairpin RNAs (shRNAs) driven by Pol III promoters [57]. Upon delivery, these systems are able to sturdily express the miRNA of interest.

The loss-of-function modulating system of miRNAs is mainly achieved by applying miRNA sponges; miRNA-Masking Antisense Oligonucleotides; or by using antisense oligonucleotides targeting miRNAs (AMOs) [54].

MiRNA-sponge technology consists of the expression of mRNA molecules with multiple binding sites for the target miRNA that will function as a decoy or a “sponge” to trap the desired miRNAs. That way the endogenous target mRNA will be preserved and able to function normally [58]. This approach is intensively used to inhibit functional classes of miRNAs *in vitro*, but so far its use *in vivo* has been limited [54]. MiRNA sponges are specific to a miRNA target and not to a gene of interest. Using a miRNA sponge to knockdown a miRNA or a miRNA seed family could affect all target genes of its targeted miRNAs, leading to undesirable effects.

MiRNA-Masking Antisense Oligonucleotides technology (miR-Mask, also known as BlockmiR, target protectors or as target site blockers) relies on an inverted approach: instead of blocking the target miRNA, these molecules shield the mRNA which function is desirable to preserve. In this way, the miR-Mask are able to block the access of the miRNA to its target mRNA, impeding its action to the target gene. Moreover, miR-mask technology targets the miRNA in a gene-specific manner, i.e. the oligonucleotides are designed to protect the mRNA, and consequently, the expression of the protein of interest, leaving intact the miRNA responsible for the repression, which might have other important roles in the system [59]. MiR-Mask is an alternative and valuable supplement to AMOs technology [54].

Also, this approach can be further combined with miRNA sponges (sponge-miR-mask technology) to block the access of multiple miRNA members to their binding sites on mRNA, leading to the re-expression of proteins. A sponge miR-mask is designed to bind by partial complementarity to the 3'UTR of

all target mRNAs of a miRNA seed family site of 8 nt. However, sponge-miR-Mask technology has poor gene-specificity, since these molecules might block the expression of all genes associated with the same seed binding site of the miRNA family [54].

The most popular approach to correct aberrant miRNA expression is based on the synthesis of antisense oligonucleotides with a complementary sequence [60–62]. Antisense oligonucleotides (ASOs) are being used to suppress dysfunctional mRNAs for more than four decades, [63] and are currently being used to target miRNAs. In antisense oligonucleotides targeting mRNAs, ASOs mostly stimulate an RNase-dependent degradation of the target RNA. They are designed to harbor continuous DNA monomers along the sequence in order to promote RNase H activity. When applying antisense technology to miRNAs (AMOs), one may consider that the mechanisms through which AMOs act may follow the same pathway of ASOs – the binding of antisense oligonucleotides to miRNA should promote degradation via RNase, in the same way ASOs cleave mRNA. However, it is now believed that the anti-miRNAs cause miRNA silencing mostly by steric blocking the target miRNA. These findings were supported by the work of Davis et al. [64] where they tested a designed ASO sequence known to trigger the RNase H-dependent degradation to target the miR-21. The antisense oligonucleotide, despite being effective promoting mRNA degradation, failed to inhibit miR-21 activity, suggesting that the miRNA-ASO duplex may not be accessible to the RNase H.

Targeting aberrant miRNAs with AMOs is a promising approach for post-transcriptional gene control in future cancer therapies. Specific and strong AMOs' recognition and binding to the miRNA target is accomplished through Watson-Crick base pairing, which may be optimized by modifying AMOs at the chemical level.

The design of Anti-miRNAs

Anti-miR oligonucleotides-based mechanism relies on the complementary base pairing of the oligonucleotide sequence to its target miRNA. AMOs are typically designed to be a perfect match to the miRNA target, [65] especially to the seed region of the miRNA, which has been shown to influence anti-miRNA specificity and activity.

In therapeutics involving antisense oligonucleotides, AMOs must display some desirable characteristics in order to be applied successfully in a biological system. It is important to design and select AMOs with enhanced potency to silence the target miRNA and with increased binding affinity to the target. The potency of the AMOs is intrinsically associated with its capacity to efficiently reduce the expression of the target miRNA. Whereas, the binding affinity is related to the stability of the duplex upon AMO binding to its target, and thus related to the melting temperature (T_m) of the oligonucleotide. A higher T_m value represents an improved binding affinity and a stronger duplex, because more energy is required to destabilize the connection between both molecules. Overall, it is also desirable that AMOs exhibit improved extracellular and intracellular stability, and thus be resistant to exo- and endo-nucleases. They should also exhibit improved cellular uptake rates to be

efficiently delivered to cells and tissues, and have no toxicity or other side effects, such as the knockdown of non-target molecules important to maintain biological function or the stimulation of undesired auto-immune responses [66].

Unmodified DNA oligonucleotides present several disadvantages that preclude their applicability as antisense technologies. As such, several sets of modifications were introduced to improve structural properties of the AMOs.

3.1. Chemical modifications – AMOs evolution

The first study reporting the use of antisense oligonucleotides applied to miRNAs used unmodified DNA sequences complementary to several miRNAs injected to *Drosophila* embryos [67]. Antisense oligonucleotides composed only of DNA bases are rapidly degraded in a biological system by endo- and exonucleases and fail to connect with the majority of the target miRNA in mammalian cells. The necessity to overcome DNA-AMOs limitations emerged rapidly and a vast array of chemical modifications have been studied applied to antisense technologies.

Chemical modifications are mainly introduced in the sugar ring, especially at the C2' position, and/or in the backbone of the oligonucleotide structure. Backbone substitutions include either internucleotide linkage modifications or the substitution of the entire backbone for structures analogous to the DNA. Internucleotide modifications are often combined with sugar modifications to improve potency and resistance to the oligonucleotide sequence. These modifications will be further discussed in the following sections.

3.1.1. Sugar modifications

Chemically-modified sugars are a remarkable way to enhance binding affinity and nuclease stability. The C2' position defines the conformation of the sugar ring and modifications at this position that are able to shift the conformation of the sugar moiety from a C2'-endo (southern conformation, typical of DNA duplexes) to a C3'-endo sugar pucker (northern conformation, typical of RNA duplexes), improves the binding affinity of AMOs for RNA complements [68]. Furthermore, in this conformation the 2'-modification is closer to the 3'-phosphate group, conferring higher nuclease resistance to the oligonucleotide [69].

The most popular alterations applied to miRNAs studies involve modification at the 2' carbon of the ribose. It is the case of 2'-OMe, 2'-MOE and 2'-F alterations, that are all reported to confer AMOs increased binding affinity (ordered by increased potency: 2'-OMe \cong 2'-MOE < 2'-F) [64] and nuclease resistance compared to DNA (Fig. 3). The use of locked nucleic acids (LNA) is very popular in AMO designs, due to their improved performance on the stabilization of the duplex by its locked configuration [64,65]. All these substitutions can be combined to improve AMOs potency. For instance, MOE/LNA, 2'-OMe/LNA or 2'-F/MOE are successful examples of oligonucleotides mixmers with improved binding affinity when compared to oligonucleotides harboring only one type of substitution [64,65,70].

The first generation of modified-AMOs appeared in 2004 and contained a methylated hydroxyl group at the C2 carbon

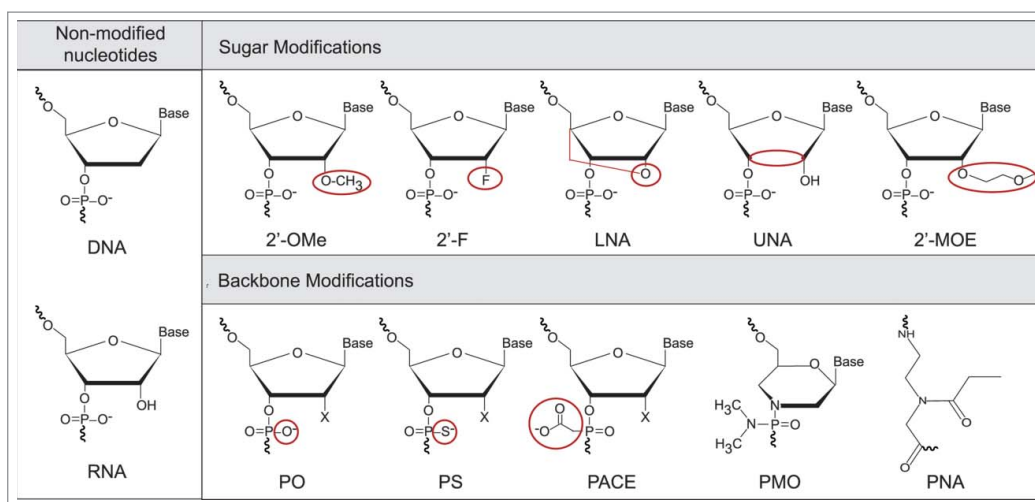


Figure 3. Chemical modifications applied in the AMOs design. Unmodified nucleotides – DNA: deoxyribonucleic acid, RNA: ribonucleic acid; Sugar modifications – 2'-OMe: 2'-O-methyl, 2'-F: 2'-fluoro-RNA, LNA: Locked Nucleic Acid, UNA: Unlocked Nucleic Acid, and 2'-MOE: 2'-O-methoxyethyl; Backbone modifications – PO: phosphodiester, PS: phosphorothioate, PACE: phosphonoacetate, PMO: Phosphorodiamidate Morpholino Oligomers, and PNA: Peptide Nucleic Acid. In red is highlighted the site and the type of modification.

of the sugar ring (2'-OMe RNAs, Fig. 3) [71,72]. Hutvagner and his co-workers [71] showed that 2'-OMe oligonucleotide are nontoxic and can efficiently suppress *Caenorhabditis elegans-let-7* miRNA *in vivo*. In the same year, Meister et al. [72] also proved that it is possible to control miR-21 activity by blocking the RISC complex. 2'-OMe oligonucleotides offer several advantages comparing to DNA-counterparts. They increase thermal stability to the target by approximately +0.8°C/insert [73]. Also, 2'-OMe is a natural modification that occurs in mammalian RNA conferring minimal or no toxicity when used *in vivo* [71]. While 2'-OMe oligonucleotides are more resistant to nuclease activity when compared to DNA counterparts, they are still susceptible to exonucleases in the serum. Successful inhibition of miRNAs *in vivo* using 2'-OMe-AMOs was only achieved by applying the AMO directly into the cells [71]. In order to overcome this problem, additional non-oligonucleotide-modifiers can be placed near both ends of the oligonucleotide sequence to protect it from the attack of nucleases. Lennox et al. [70] described a novel nontoxic compound, N, N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine (“ZEN”) placed at or near 5'- and 3'-ends of a phosphodiester (PO) 2'-OMe oligonucleotide complementary to a targeted miRNA. The 2'-OMe-ZEN, with the modifier placed in both ends of the oligonucleotide sequence (dual-ZEN AMO), completely blocks endo- and exo-nuclease attack, and shows increased potency, binding affinity, good specificity and low-toxicity *in vitro* when compared to the unprotected 2'-OMe-PO design. In the same work, the ZEN modifiers were also compared to other non-nucleotide blocking groups (C3 spacer) placed in the same manner in an AMO sequence. Both ZEN and C3 AMO variants showed improved potency when the modification was performed at the 5'-end of the sequence rather than at the 3'-end, and in the case of the C3-AMOs, the binding affinity of the two AMO variants was the same. The 3'-end modification was expected to give a more stable AMO, and therefore increased potency. The opposite outcome led to the speculation that the potency of an AMO depends not only of its binding affinity and nuclease resistance, but also of the

role that the 5'-end plays on the ability of the AMO to invade the miRISC complex. Furthermore, the dual-ZEN AMO showed higher potency than the dual-C3 AMO, which offered limited improvement in potency regarding the unmodified 2'-OMe [70].

In a similar line of work, Vermeulen et al. [74] demonstrated that flanking a single-stranded 2'-OMe-AMO sequence with double-stranded duplexes capable of forming hairpin structures highly improves the inhibitory effect of the AMO by several fold, possibly by helping the AMO invasion to miRNA-RISC. More recently, it has been reported that cross-linked duplexes flanking the single-stranded AMO inhibited miRNA functions more efficiently than AMOs with normal single or double strands. The interstrand cross-link of DNA or RNA not only contributed to improve 2'-OMe resistance and potency properties, but also to stabilize the duplex avoiding the risk of dissociation into single strands [75].

The 2'-MOE (2'-O-methoxyethyl) oligonucleotides harbor a methoxyethyl modification at the RNA 2'-OH position (Fig. 3). This configuration increases nuclease resistance and target binding affinity by approximately +1°C/insert⁷³ when compared with unmodified DNA oligonucleotides. Moreover, 2'-MOE oligonucleotides are effective in silencing miRNAs and display improved anti-miRNA activity when compared to 2'-OMe oligonucleotides [64,76]. Esau et al. [76] demonstrated that miR-122 function can be modulated in mice using a 2'-MOE-AMO. The inhibition of the miR-122, a miRNA overexpressed in the liver, resulted in the reduction of plasma cholesterol levels, increase of hepatic fatty acid oxidation, and a decrease of hepatic fatty acid and cholesterol synthesis rates.

The miR-122 is associated with Hepatitis C virus (HCV) infection in the liver. Currently, a company called Regulus Therapeutics is validating a 2'-MOE-AMO-122 (RG-101) conjugated with an N-Acetylgalactosamine sugar (GalNAc) for the treatment of HCV infection in humans [77,78]. The GalNAc carbohydrate structure shows high binding affinity to the asialoglycoprotein receptor (ASGPR) on hepatocytes, improving cellular uptake [79] and enhancing the potency of RG-101 by

up to 10–20 times compared with unconjugated oligonucleotide. In the beginning of 2016, RG-101 entered the phase II of a clinical study, but despite the promising results in phase I, the drug is currently on a clinical hold by the American Food and Drug Administration (FDA). The same company has other promising AMOs to be applied in miRNA therapeutics. Another GalNAc-conjugated oligonucleotide, the RG-125 (AZD4076) targeting miR-103/107, is currently in phase I of clinical development. RG-125 is expected to contribute to the treatment of non-alcoholic steatohepatitis in patients with type 2 diabetes/pre-diabetes.

The 2'-Deoxy-2'-fluoro-nucleoside (2'-F) is also an RNA mimic with a fluorine 2'-hydroxyl group at the C2 carbon of the ribose (Fig. 3). Depending on the sequence, oligonucleotides with 2'-F-RNA residues show increased thermal stability of around $+1.6^{\circ}\text{C}/\text{insert}^{73}$, which is higher than when compared with 2'-OMe-RNA and DNA. 2'-F-AMOs with PO linkages do not have significant resistance to exonucleases, while PS linkages are highly resistant, conferring some protection to 2'-F-RNA AMOs. For *in vivo* applications, 2'-F-RNA can be further conjugated with other modifications, like 2'-MOE substitutions, to confer more stability [80]. In this study, a 2'-F-AMO was able to efficiently inhibit miR-122 activity, showing a mild dose-dependent immunostimulatory effect in mice. The 2'-MOE modification presents a lower binding affinity than the 2'-F, but the introduction of additional 2'-MOE modifications into the 2'-F/MOE AMO was able to minimize this immune response without affecting anti-miR-122 activity [80]. Nevertheless, the 2'-F-RNA substitution is a potent, and well-tolerated chemical modification that can be used to efficiently silence abnormal miRNAs. In addition, 2'-F-RNA residues are an effective alternative to LNA-modified oligonucleotides, since they provide a similar thermal stability and nuclease resistance, but at a lower cost [81].

LNA is a 2'-O, 4'-C-methylene- β -D-ribofuranosyl nucleotide where the ribose is locked in a 3' endo/N-type sugar conformation with a methylene bridge between the oxygen at 2' position and the 4'-C (Fig. 3). LNA monomers are also compatible to other RNA and DNA monomers and with phosphorothioate (PS) and PO linkages, respecting the natural base pairing binding. Due to their chemical structure, LNA-modified oligonucleotides have high resistance to nucleases and the highest binding affinity demonstrated so far of $+2$ to $+5^{\circ}\text{C}/\text{insert}$ when pairing with DNA sequences and $+4$ to $+10^{\circ}\text{C}/\text{residue}$ when pairing with a RNA strand [82]. The high binding affinity of LNA-AMOs makes it possible to design shorter sequences without compromising efficiency, which are useful to target the seed region of entire miRNAs families with similar biological functions. Obad et al. [83] were the first to describe tiny LNA-sequences of 8 nucleotides (so called "tiny LNAs") targeting the seed region of both individual miRNAs and co-expressed miRNA families. Also, systemic administrations of tiny LNAs showed unassisted uptake and long-term knock-down of miR-21 in a breast cancer mouse model, emphasizing the potential use of LNA-AMOs *in vivo*. More recently, Zhang et al. [84] successfully applied tiny-LNAs to silence miR-155 in low-grade B-cell lymphoma cells both *in vitro* and *in vivo*. Safe *in vivo* applications of LNA sequences are highly dependent of

sequence context, length and an overall optimized design. Shorter sequences had shown lower toxicity *in vivo* and *in vitro*, whereas long sequences had demonstrated severe cytotoxicity *in vitro* [70]. Also, shorter sequences are more likely to improve mismatch discrimination [85].

In terms of market, there are two drugs using LNA-AMO technology currently in clinical trials. The first drug called Miravirsen from Santaris Pharma, was developed to treat HCV infection by targeting miR-122 [86] and entered phase 2 of clinical trials. It is presently in an extension study to provide additional long-term safety and efficacy data. Another company (miRagen Therapeutics) entered phase 1 of clinical studies for MRG106-11-101 LNA-antimiR[®], which was designed to control the activity of miR-155. In hematological malignancy, miR-155 has key roles in the differentiation, function, and proliferation of blood and lymph cells. *In vitro* tests using the developed AMO successfully inhibited miR-155 activity in lymphoma cells, restoring normal function and reducing aberrant cell proliferation.

Unlocked Nucleic Acid (UNA, Fig. 3) is another analogue of RNA, in which the C2'-C3'-bond has been cleaved, resulting in an unlocked configuration of the furanose ring. The UNA inserts additively decrease duplex stability, and depending of the configuration, the T_m can decrease -5°C to $-10^{\circ}\text{C}/\text{modification}$ [87]. However, despite the decrease of T_m , the UNA unlocked configuration makes this molecule very flexible. UNA monomers can be placed strategically within the AMO sequence to enhance mismatch discrimination, making this substitution very interesting for detection purposes (further reviewed in the "miRNA detection using AMOs" section).

3.1.2. Backbone modifications

Phosphodiester bonds (PO) are the natural internucleotide linkage present in DNA and RNA (Fig. 3). Oligonucleotides designed to harbor the natural PO bonds are in this way more tolerated *in vivo*, displaying less toxicity. Yet, they are more prone to be cleaved by endo- and exonucleases in mammalian cells. Chemical modifications of these bonds have been extensively studied, and several chemical strategies have been very successful on improving nuclease resistance [88–91].

The first described internucleotide modification in antisense oligonucleotides was a phosphorothioate (PS) linkage, where the non-bridging phosphate oxygen is substituted by a sulfur in the phosphodiester bond (Fig. 3) [88]. PS bonds are more resistant to the activity of exo- and endonucleases and they can be placed throughout the oligonucleotide sequence (to improve endonuclease resistance) or at strategic points within the sequence, leaving some unmodified PO linkages. Generally, the PS linkages should be added near both the 5' and 3' ends [65] to improve the AMO resistance to exonucleases.

The major drawback associated to PS linkages is their tendency to bind to off-target proteins. PS binding to nuclear proteins may be potentially toxic to normal cells and compromise several cell functions [92]. However, the affinity of PS oligonucleotides to serum albumin, reduces plasma clearance and increases the AMOs lifetime in serum from hours to days [93].

Moreover, PS-DNAs present a slightly reduced binding affinity towards complementary RNA molecules in comparison to their corresponding phosphodiester oligodeoxynucleotide. The substitution to a PS-AMO decreases the melting

temperature of the heteroduplex by approximately -0.5°C per insert. Davis et al. [64] compared the effect of the PS backbone substitution on anti-miRNA activity. Uniform 2'-MOE and 2'-OMe sequences, with PS or PO backbones, were tested to evaluate their potency to silence miR-21 over time. 2'-MOE-PO-AMO displayed an increase in activity compared to 2'-MOE-PS-AMO, being more active in many later time points. The 2'-OMe showed little or no improvement in both designs (PS or PO), due possibly to the poor resistance of 2'-OMe-PO-AMO in cells. The decrease in terms of thermal stability offered by PS oligonucleotides is compensated by a greater hybridization specificity, higher nuclease resistance, and an improved cellular uptake both *in vitro* and *in vivo* as compared with unmodified DNA oligonucleotides. The potency of PS-oligonucleotides can be further enhanced by combining other substitutions, especially sugar modifications.

Other internucleotide linkage modifications used to silence miRNAs include phosphonoacetate (PACE) and thio-PACE. PACE synthesis replaces the nonbridging oxygen for an acetate group in the internucleotide phosphate linkage (Fig. 3). Thio-PACE is an analog of PACE comprising a PS substitution instead of a PO bond. Both modifications are reported to be completely resistant to nuclease activity and are able to enter the cells without any assistance as neutral esters [94]. In spite of these modifications they show a slight decrease of binding affinity to complementary RNA targets when compared to PO linkages, in the order of $-1.3^{\circ}\text{C}/\text{insert}$ for PACE duplexes and $-1.8^{\circ}\text{C}/\text{insert}$ for thio-PACE (under high salt concentrations) [94]. Threlfall et al. [95] tested the potency of 2'-OMe oligonucleotides with PACE and thio-PACE backbones to inhibit miR-122. In most cases, both types of AMOs formed stable duplexes with their target miRNA, showing higher melting temperatures than simple DNA oligonucleotides. Furthermore, single-stranded 2'-OMe thio-PACE oligonucleotides were notably more efficient in the cellular uptake and significantly improved the potency of a 2'-OMe-based anti-miRNA targeting miRNA-122 in the absence of a transfection agent [95].

Phosphorodiamidate Morpholino oligomers (PMOs) are uncharged substitutes of the PO bonds (Fig. 3), where the morpholine ring is linked through phosphorodiamidate groups instead of phosphates [90]. Given their neutral charge, PMO oligonucleotides are more resistant to nuclease activity and exhibit a similar or a slightly improved binding affinity for target miRNAs. Morpholino oligonucleotides have proven particularly successful as anti-miRNAs in Zebrafish systems, [96,97] exhibiting specific and non-toxic inhibition of both pre-miRNA and mature miRNA activity by blocking Drosha or Dicer nucleolytic processing sites [97]. The uptake of this configuration into mammalian cells is challenging, and as such further conjugation with cell-penetrating peptides (CPPs) is required. This allows both the *in vitro* and *in vivo* uptake, enlarging the potential use of PMOs in anti-miRNA studies. It is worth noticing that the uncharged nature of these molecules facilitates their conjugation with charged cationic molecules, such as the CPPs designed to promote the delivery.

Peptide Nucleic Acid (PNA) is an uncharged synthetic DNA analog composed of N-(2-aminoethyl) glycine monomers linked by peptide bonds [91] (Fig. 3). PNAs follow the natural base-pairing rules and exhibit a significant nuclease resistance,

and increased binding affinity towards DNA or RNA targets. PNA is particularly useful in anti-miRNA applications. Fabani and Gait [98] reported that PNA molecules used to silence miR-122 showed improved activity when compared with a 2'-OMe oligonucleotide. Like the PMOs, the cellular uptake of PNA-AMOs is challenging. PNA shows a more hydrophilic profile than lipophilic, which compromises the diffusion of unmodified-PNA molecules through cell membranes [99]. The PNA oligonucleotides designed by Fabani and Gait [98] had an amino-terminal cysteine to facilitate conjugation with a R₆-penetratin peptide (CPP). They also tested an unconjugated AMO with four lysine residues to improve solubility. The four lysine residues were sufficient to confer charge, so that the PNA would be able to be delivered into cells using electroporation or CPPs.

4. Anti-miR oligonucleotides: Design Strategies

Apparently, the design of complementary anti-miRNAs sequences targeting miRNAs is quite straightforward. Yet, in reality, the whole process might go into a tortuous and long road before reaching a functional and non-toxic anti-miR. While highly dependent on the strategy defined for the study, some guidelines should be followed to assist in the design of AMOs. The development of a chemically-improved anti-miR oligonucleotide passes through several stages, from the search of target miRNA sequences to the design of the AMO (including the selection of the most adequate chemical structure) and its validation (Fig. 4).

First, the miRNA sequence of interest needs to be obtained from databases like TargetScan v7.1 [100] or miRBase [101,102]. The search should be directed to the mature sequence of the miRNA and the guide strand. The passenger strand is not an active sequence of miRNA and is generally not considered in the design of AMOs. Nonetheless, the passenger strand sequence can be used to synthesize a negative control oligonucleotide.

Next, by base-pairing, it is possible to design an anti-miR oligonucleotide sequence that is fully complementary to the 5' end of the mature miRNA, especially matching the seed region. The designed sequence can have the same length, be shorter or even longer than the target [64,71,103]. Some examples of shorter and longer sequences were already discussed above, such as in the work of Obad et al. using tiny LNAs, [83] and in the work of Vermeulen et al. using longer 2'-OMe-AMOs with flanking nucleotides next to the complementary antisense miRNA region [74]. After selecting the desired AMO sequence, it is also advisable to perform an alignment for the human genome using for instance a Basic Local Alignment Search Tool (BLAST) [104] to check for possible off-targets of the oligonucleotide sequence.

Due to the poor stability *in vivo* and insufficient binding affinity of DNA sequences, chemical modifications should be employed into the design of AMOs. From all the evidence stated above, it is possible to sum-up some specific characteristics of the different chemical-modified AMOs (Table 2), especially in terms of binding affinity, nuclease resistance, cellular uptake and cytotoxicity. It is also worth mentioning that unmodified AMOs may lead to an

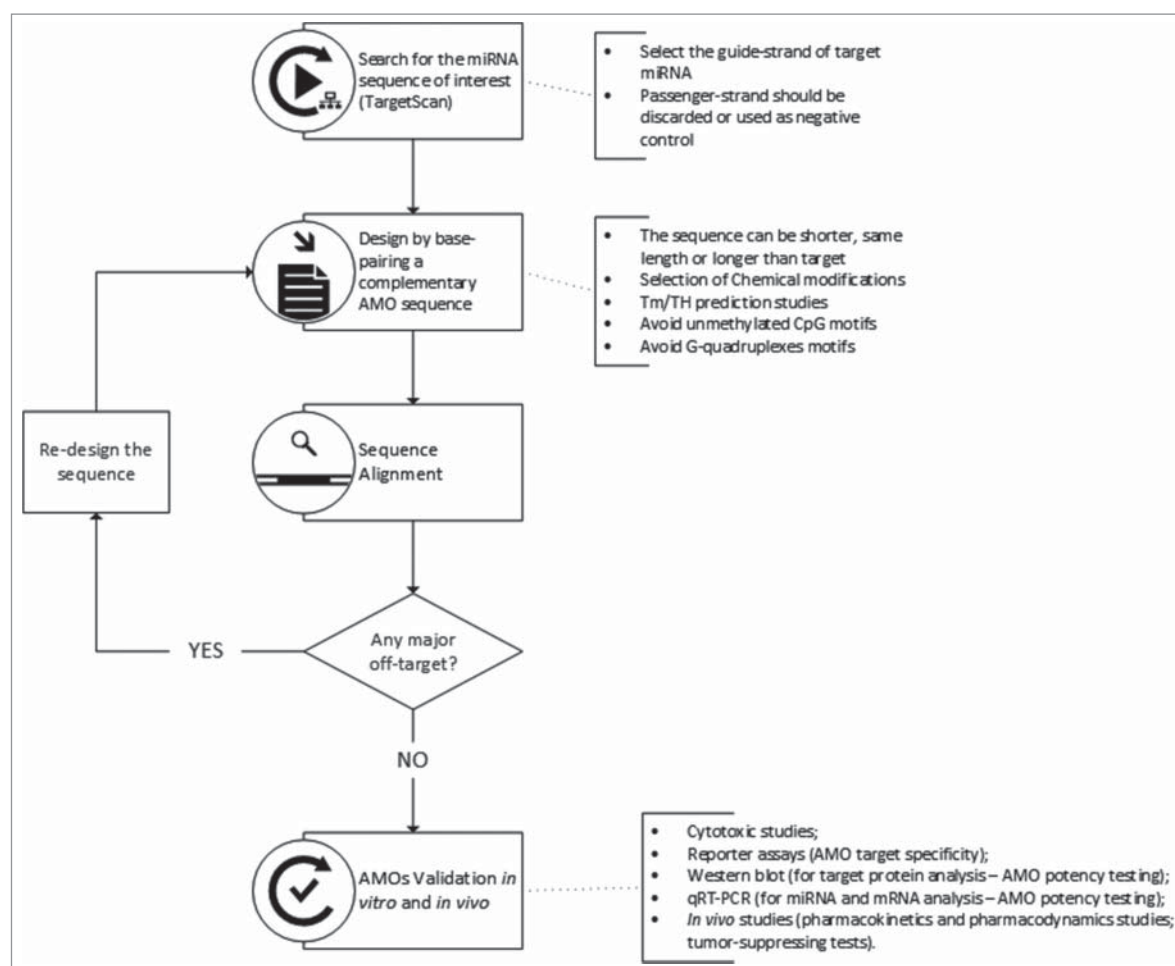


Figure 4. AMOs design flowchart: from the identification of target miRNA to the validation of anti-miRNA oligonucleotides.

ineffective inhibition of the miRNA activity, since they also serve as a passenger strand and thus trigger the dissociation mechanism responsible for miRNA passenger strand unwinding [64].

Overall, some of the most widely used chemical modifications in anti-miRNA technology are LNA, 2'-F-RNA, 2'-OME, PNA, and PMOs. Moreover, different combinations can be applied to further improve the performance of a certain AMO design. For instance, 2'-OME oligonucleotides with a PO backbone are easily degraded in the serum by exonucleases, while introducing a PS substitution linkages at each end of a 2'-OME-PO oligonucleotide improves nuclease resistance and is an effective AMOs substitution for a miRNA knockdown *in vivo* [105]. However, when applying PS substitutions in the entire sequence of a 2'-OME-AMO, the same outcome is not achieved and the miRNA levels remain unaffected. The reduction on the activity of a PS-AMOs, comparatively to a PO counterpart, can also be explained by the higher binding affinity of PS-AMOs to many proteins. Some of those are able to relocate to the nucleus, moving the PS-AMOs along with them. As a consequence, there is a decrease of available AMOs in the cytoplasm, which may contribute to the inhibition of anti-miRNA activity [106].

Another aspect to take into consideration when designing an anti-miR oligonucleotide is the temperature at which the hybridization occurs. The hybridization temperature (T_H) can be defined as the optimal temperature at which a full (or nearly full)

noncovalent binding between the AMO and the target miRNA forming a duplex is observed, [107] and at which the AMO does not hybridize with sequences that contain one or more mismatches. Up to date, there are no mathematical models that accurately predict the optimal T_H for a given oligonucleotide sequence. In order to overcome this and have an estimation of T_H for optimization studies, the melting temperature or mid-transition temperature (T_m) is usually determined as an indicator of T_H ¹⁰⁷. Thermodynamic parameters are well defined for DNA duplexes and in general, and the mathematical models to handle the thermodynamics of nucleic acid hybridization (and hence determine the T_m) are available and quite accurate. Different configurations (PNA: RNA; LNA: RNA, etc.) show different thermodynamic parameters and thus different models need to be adapted to predict the thermodynamic parameters of the hybridization [107].

As mentioned before, the PS linkage reduces binding affinity and a complete PS modification lowers the T_m to such extent that the AMO potency is compromised [105]. In spite of some exceptions, it is possible to establish a direct correlation between the increase of the binding affinity and the potency of the AMO to inhibit miRNA activity. However, chemical modifications presenting high T_m values, such as LNA or 2'-MOE/LNA mixmers, may result in an AMO with reduced potency, since they might show self-dimerization effects or form strong secondary structures within the oligonucleotide sequence, which can lower AMO potency. It is believed that there is a

Table 2. Properties of the different chemical modifications applied to the AMOs.

Type of modification	Advantages	Disadvantages	References
PO	<ul style="list-style-type: none"> ■ Natural backbone structure ■ Low-toxicity ■ Inexpensive 	<ul style="list-style-type: none"> ■ Poor stability <i>in vivo</i> – Low resistance to exonucleases 	[63]
PS	<ul style="list-style-type: none"> ■ Increased cellular uptake ■ High nuclease resistance 	<ul style="list-style-type: none"> ■ Non-specific binding to proteins ■ Slightly lower binding affinity compared with PO 	[64,88]
PACE/thio-Pace	<ul style="list-style-type: none"> ■ Improved and unassisted uptake ■ Combination with other modifications to improve thermal stability and binding affinity 	<ul style="list-style-type: none"> ■ Lower binding affinity when compared with other modifications (-1.3 °C/insert for PACE duplexes; -1.8 °C/insert for thio-PACE), but still higher than DNA-AMOs when combined with higher binding affinity modifications such as 2'-OMe 	[94,95]
PMO	<ul style="list-style-type: none"> ■ High nuclease resistance ■ Improved thermal stability ■ Uncharged – easy to conjugate with other molecules 	<ul style="list-style-type: none"> ■ Poor uptake/pharmacokinetic properties 	[97]
PNA	<ul style="list-style-type: none"> ■ High nuclease resistance ■ Improved thermal stability ■ Uncharged – easy to conjugate with other molecules 	<ul style="list-style-type: none"> ■ Poor uptake/pharmacokinetic properties 	[98,99]
2'-OMe	<ul style="list-style-type: none"> ■ Improved nuclease resistance ■ Improved thermal stability ■ Non-toxic 	<ul style="list-style-type: none"> ■ Relatively poor stability <i>in vivo</i>: 2'-OMe-PO AMOs are less resistance to exonucleases when compared with other modifications containing a PO backbone 	[64,71,73,148]
2'-MOE	<ul style="list-style-type: none"> ■ Improved nuclease resistance ■ Improved thermal stability ■ Non-toxic 	<ul style="list-style-type: none"> ■ When combined with other T_m-increasing chemical modifications (such as LNA) might form secondary structures within the AMO or present increased self-dimerization effect, lowering the potency of the AMO 	[64,65,73,76]
2'-F	<ul style="list-style-type: none"> ■ Improved thermal stability ■ Non-toxic ■ Lower cost compared to LNA substitution 	<ul style="list-style-type: none"> ■ 2'-F-PO show no resistance to exonucleases 	[73,80]
LNA	<ul style="list-style-type: none"> ■ High thermal stability ■ Increased nuclease resistance ■ Improved potency and specificity ■ Improved and unassisted uptake ■ Mismatch discrimination ■ Efficient to target miRNA families using tiny-LNA sequences 	<ul style="list-style-type: none"> ■ Low conformational flexibility ■ T_m-increasing chemical modifications might form secondary structures within the AMO or present increased self-dimerization effect, lowering the potency of the AMO 	[83,84,103,114,116,149,150]
UNA	<ul style="list-style-type: none"> ■ Great conformation flexibility ■ Mismatch discrimination within miRNA families 	<ul style="list-style-type: none"> ■ Might present <i>in vivo</i> toxicity ■ Poor thermal stability 	[124]

threshold in the thermal stability that directly promotes a good potency. After that point, any further affinity increases do not improve potency and higher are the chances of off-target hybridization [108].

Furthermore, other molecules can be applied in the sequence to improve uptake, delivery, or endurance of an AMO, especially when applied *in vivo*. Due to their polyanionic nature, these oligonucleotides do not easily diffuse across cell membranes, and most of the AMOs need to be associated with an efficient delivery system. Applying non-oligonucleotide molecules to the extremities of an AMO protects the sequence from degradation and thus increases the nuclease resistance of these designs [70,105]. Also, it has been reported that adding, for instance, a cholesterol group to the 3'-end facilitates the delivery *in vivo* of the 2'-OMe AMOs containing PS linkages [105,109]. Moreover, other molecules such as cell-penetrating peptides [98,110] can be applied in the extremities of an anti-miR oligonucleotide to promote cellular uptake of otherwise unattractive AMO designs for an *in vivo* delivery purpose. Although CPPs are an attractive way to promote the delivery of oligonucleotides, these molecules are typically conjugated with the non-charged PNA and PMOs [111].

Other technical features should be kept in mind when designing antisense oligonucleotides. Generally,

oligonucleotides containing unmethylated deoxycytidine-deoxyguanosine (CpG) dinucleotides motifs can trigger immune responses in mammalian systems by activating the toll-like receptor-9 gene (*TLR9*) expressed in immune system cells such as the Lymphocyte B or dendritic cells. For this reason they should be avoided, especially for *in vivo* applications [112,113]. The CG dinucleotide is more frequently found in viral and bacterial DNA than in the human genome, suggesting that it is a marker for the immune system to recognize infection. Nevertheless, AMOs containing these motifs can be applied in situations where it is desirable to induce an immune response for treating cancer, asthma and infectious diseases [113].

The development of more stable and specific oligonucleotides, targeting miRNAs is the investigation focus of many research groups that are continually improving AMOs design and finding new miRNAs that control specific pathways in cancer epigenetics. Improved AMOs could be used not only as a therapeutic tool but also for the detection of miRNAs in tissues.

5. miRNA detection using AMOs

A field breakthrough in the miRNAs technology might pass through the use of AMOs to profile and detect *in vivo*

miRNAs known to be involved in cancer processes. Most of these studies were validated *in vitro* and *in vivo*, yet to the best of our knowledge all the *in vivo* studies applying AMOs for the detection and identification of a target miRNA are performed analyzing the tissues *ex-vivo*. The progression of the AMOs technology allied to real-time *in vivo* imaging is, of course, determined by several factors, including the improved specificity and stability of the new generation of oligonucleotides, the optimization of efficient AMO delivery techniques and the advance of support instrumentation that allows the actual live detection of the molecules of interest.

Over the years, several studies have been reported to use LNA oligonucleotides to efficiently target miRNAs showing the potential of LNA-AMOs in different therapeutic and detection strategies [83,98,114-116]. In the detection field, Válóczy et al. [115] developed LNA oligonucleotides with improved sensitivity and high specificity to be applied in northern blot analysis for a highly efficient detection of miRNAs. With their work, they have opened the precedent to further explore the application of LNA oligonucleotides in other detection methods, like for instance to study the spatial expression/distribution of miRNAs in cells and tissues by *in situ* hybridization or to study the expression profile of miRNAs by LNA-microarrays.

In situ hybridization (ISH) analysis is a technique that applies antisense oligonucleotides complementary to a specific nucleic acid sequence to determine the precise location of miRNA within the cells. The main difference is that for detection purposes, the synthetic antisense oligonucleotides are linked either to a fluorophore, a radioactively-label or to an antigen-labeled bases so that they can be tracked respectively by fluorescence microscopy, autoradiography, or immunohistochemistry. Currently, the use of LNA-modified oligonucleotides constitute the most promising approach to visualize miRNA expression by *in situ* hybridization, [117-123] but as far as we are aware only analyzed the results *ex-vivo*. It is desirable that the molecular probes hybridize at normal body temperature (around 37 °C) to be prone to be used *in vivo* and push forward the miRNA detection methods in order to determine spatial and temporal miRNA accumulation in tissues. More recently, UNA modification has proven to be an interesting substitution for mismatch discrimination within miRNAs families. Robertson et al. [124]. demonstrated the properties of UNA-AMOs for detecting specifically overexpressed miR10b in metastatic breast cancer. UNA oligonucleotides showed increased specificity towards its target and were able to discriminate miR10b from miR10a (presenting 1 mismatch) and from miR10c (presenting 2 mismatches) more efficiently than its DNA counterparts.

There is already some instrumentation in the field of fluorescence microscopy that allows the detection of fluorescence signal by using *in vivo* fluorescent detectors. These detectors are basically labeled-oligonucleotides attached to sophisticated and expensive instrumentation, and a lot of effort is applied in the development of smaller, disposable and low-cost detector systems [125]. Another problematic relies on the sensitivity of the detection methods. Usually,

the fluorescence detection requires the high sensitivity devices that allow the measurement of very low levels of light emitted by the labeled probes. One example is a prototype developed by Stephen Bellis et al. [126] a hybrid platform consisting of a vertical cavity surface emitting laser (VCSEL) to provide the excitation and a photo-diode (APD) to detect the emitted fluorescence. The APD detectors combine the ability to count individual photons, being able to detect low levels of fluorescence, and as a plus are small in size.

Other technologies are now immersing to track and detect in real-time different molecules *in vivo* for biomedical research and clinical applications. It is the case of near-infrared fluorescence Imaging (NIR), a technology recently used in the field of cancer imaging. This technique uses high sensitivity molecular probes labeled with fluorophores in the near-infrared region wavelength (650 nm – 900 nm), that allows relatively deep photon penetration into tissue, minimal tissue auto-fluorescence and higher optical contrast. *In vivo* fluorescence imaging with near-infrared light holds enormous potential for a wide variety of molecular diagnostic and therapeutic applications [127]. A thorough review on imaging technology for *in vivo* uses is beyond the scope of this review, and a detailed analysis on this matter can be found on the literature [125].

Interestingly, using the same principle of the detection techniques stated above, AMOs could be further optimized to be stable *in vivo*, in order to allow miRNA detection in real-time by applying a fluorescence molecule at one end of the sequence. A real-time detection of miRNAs topically in any tissue holds a great promise towards an effective, safe and immediate detection of miRNAs in humans.

6. Concluding remarks

MiRNAs are small, single-stranded and noncoding RNAs that post-transcriptionally regulate gene expression. By complementarily binding to the 3'UTR of mRNAs, the miRNAs cells mainly cause translational repression and/or mRNA deadenylation. They are involved in the control of several biological pathways, and it comes with no surprise that alteration on miRNAs levels has an extensive impact on the synthesis of different proteins associated with different human pathologies, such as cancer.

The development of new technologies to study the function and the mechanisms of regulation of miRNA in human diseases is fundamental. Numerous works show the potential use of AMOs as specific diagnostic biomarkers and/or as a therapy to control aberrant miRNA. The use of AMOs in clinical practice for cancer treatment seems a promising alternative to silence undesirable miRNA function and gene activity. A fully comprehensive study of AMOs function using different *in vitro* techniques is mandatory. It is also important to associate the *in vitro* results to the *in vivo* studies to validate this strategy as an efficient therapeutic route in cancer treatment.

Up to now, no therapeutic strategy has been fully developed in order to modulate miRNA function. Clinical trials are already in place for experimental AMO-based drugs targeting specifically miR-122 (Miravirsin developed by

Santaris Pharma and RG-101 developed by Regulus®). Despite of the promising results, the use of anti-miRNA oligonucleotides as therapeutic agents still cares for further validation in terms of cytotoxic effects, specificity and stability of the hybridization between the oligonucleotide sequence and the target miRNA. Furthermore, allying the AMO technology to the emerging methodologies for real-time detection holds a great promise towards an effective, safe and immediate detection of miRNAs in humans. *In situ* detection of miRNA accumulation in tumor biopsies may provide a highly valuable approach for prognostic and diagnostic evaluation in a clinical setting.

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