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miRNA-based therapies: Strategies and delivery platforms for oligonucleotide and non-oligonucleotide agents

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Abstract

The discovery of microRNAs as important regulatory agents for gene expression has expanded the therapeutic opportunities for oligonucleotides. In contrast to siRNA, miRNA-targeted therapy is able to influence not only a single gene, but entire cellular pathways or processes. It is possible to supplement down regulated or non-functional miRNAs by synthetic oligonucleotides, as well as alleviating effects caused by overexpression of malignant miRNAs through artificial antagonists, either oligonucleotides or small molecules. Chemical oligonucleotide modifications together with an efficient delivery system seem to be mandatory for successful therapeutic application. While miRNA-based therapy benefits from the decades of research spent on other therapeutic oligonucleotides, there are some specific challenges associated with miRNA therapy, mainly caused by the short target sequence. The current status and recent progress of miRNA-targeted therapeutics is described and future challenges and potential applications in treatment of cancer and viral infections are discussed.

Introduction

During the last decades the development of therapeutically applied oligonucleotides has experienced many ups and downs. Despite the huge promise, years of intense efforts, and generally promising preclinical data, only three oligonucleotides have ever won market approval, and only two of those drugs are on the market today, pegaptanib [1] and mipomersen [2]. Fomivirsen [3] is no longer available owing to a lack of demand for its indication, cytomegalovirus infections. These two examples prove the possibility of beneficial application of oligonucleotide agents in humans, but also highlight their current limitation to local administration or liver accumulation.

Rather than being driven by clinical data, innovation in the therapeutic oligonucleotide sector comes largely from basic science, specifically from the discovery of new biological roles of this compound class. Initial antisense development was ultimately hampered by poor pharmacokinetic properties and insufficient efficiency in clinical trials [4]. Although the introduction of chemical modifications such as phosphorothioates, 2′-methoxyethyl-nucleotides, and locked nucleic acids (LNA) brought some progress, the clinical outcome is overall still not satisfactory.

The discovery of an influence of oligonucleotides on splicing led to the development of splice-switching oligonucleotides, so far culminating in the clinical use of eteplirsen and

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drisapersen [5]. Preclinical data soon indicated promising results for the treatment of muscular dystrophy, and the ongoing clinical evaluation will show the full benefit of the technology.

Then, the advent of RNA interference [6] shifted much of the attention of therapeutic gene silencing from single stranded antisense compounds to the more potent double stranded siRNAs. However, because of their bigger size and the lack of tolerance of the RNAi mechanism for chemical modifications, packaging in (or conjugation to) efficient delivery systems is all but required [7]. A plethora of literature reports of the development and preclinical evaluation of particulate systems has so far not been translated to clinical applications [8-10]. Apart from the use of liposomal delivery vehicles [11] and GalNAc conjugates [12] for hepatocyte specific delivery, none of the approaches has yet reached the clinics.

During recent years, the discovery of the many regulatory roles of microRNA (miRNA) has sparked a booming interest of this compound class. In addition to the dissection of their relevance for cellular processes, miRNAs also show immense potential for diagnostic and therapeutic applications. Since the inception of the role of miRNAs in cancer in 2002, tens of thousands publications focusing on their biological basis, dysregulation in diseases, and their potential therapeutic value have been published. Circulating miRNAs in serum are attractive biomarker candidates. The presence and levels of a panel of miRNA could provide a disease fingerprint not only useful for non-invasive tumour diagnosis, but also offer prognostic values for a response against distinct chemotherapeutic treatments [13, 14]. Mirroring earlier developments of antisense, splice-switching and siRNA oligonucleotides, the initiation of the first clinical evaluation of an miRNA-targeted agent has taken only a short time. In 2012, miravirsen entered a phase II trial for the treatment of chronic hepatitis C virus infections [15, 16]. Miravirsen is a fully phosphorothioated oligonucleotide with several LNA nucleosides, which binds to miR-122, a human miRNA that is essential for HCV replication, and also inhibits its biogenesis. Early clinical data are encouraging, but the experience of the antisense development with similar chemical modifications has shown that careful evaluation of clinical efficiency and toxicity in a larger patient cohort undertaken in later stages are crucial for the success [4].

Within oncology, the first miRNA-based therapy approach, MRX34 [17] has entered clinical testing in 2013. Using a liposome-based formulation, MRX34 is a synthetic double stranded RNA oligonucleotide which can substitute depleted miR-34 and restore its activity on the p53/wnt cellular pathways. Increasing amounts of preclinical and clinical data for miRNA replacements and antagonists will become available during the next few years, and will determine the therapeutic future of this potentially very powerful technology.

miRNA biology

In 1993, Lee et al [18] found two small transcripts in Caenorhabditis elegans which would not code for a protein because their sizes were only 22 and 60 bp, respectively. In 2001, the term microRNA for these short non-coding sequences was used for the first time when Science published three accompanying publications on this topic [19, 20]. miRNAs belong

to the heterogenous family of small endogenous non coding RNAs that also include small nucleolar RNA (snoRNA) and small interfering RNA (siRNA). The coding regions for miRNAs lie usually in the regions historically called “junk DNA”. Continuous research with these seemingly useless DNA sequences revealed that the transcribed RNA is not genetic waste at all. [21] The localisation of miRNA sequences can be separated into intergenic and protein-coding intronic regions in the genome.

The miRNAs embedded in protein-coding and intergenic intronic regions are transcribed by RNA polymerase II (RNA Pol II) [22] and RNA polymerase III (Pol III) [23]. Promoter regions are involved in the initiation of miRNA transcription [24]. Recently, functional promoters for RNA Pol II and RNA Pol III were found upstream and downstream of miRNA sequences [25] shedding light on the transcriptional activation and regulatory network of miRNAs.

The primary long miRNA transcript (pri-miRNA) can be polycistronic, coding for several miRNAs, or monocistronic, coding for only one miRNA [26]. The pri-miRNA is processed into the pre-miRNA transcript, a 60 to 70 nucleotides long hairpin structure by the Drosha (nuclear RNAse III) - DGCR8 complex [27]. After this first process step the pre-miRNA hairpin translocates from the nucleus to the cytoplasm through binding to exportin 5, a member of the Ran-dependent nuclear transport receptor family [28, 29]. The exported pre-miRNA is subsequently cut by the cytoplasmic RNase III Dicer into a 19 to 23 nucleotides long mature double stranded miRNA [30-32]. This mature miRNA duplex is then incorporated into the RNA inducing silencing complex (RISC) in a process mediated by TRBP (HIV transactivating response RNA-binding protein) [33]. The stable associated guide strand remains in the RISC, while the passenger strand is generally released and cleaved [34].

When the active miRNA loaded RISC binds to a target mRNA, two possible silencing pathways can be initiated, depending on the extent of base complementarity between miRNA and mRNA (Figure 1). If there is a near-perfect base pairing, Ago2 [35] induces a slicer dependent mRNA decay where deadenylation of the mRNA is the first step followed by one of two distinct degradation processes: One decay process is the cleavage from 3′ to 5′ in exosome compartments, the second one employs decapping enzymes Dcp1, Dcp2 and a 5′ to 3′ cleavage by the exoribonuclease Xrn1p [36]. Protrusions between miRNA and mRNA are the result of limited base pairing, leading to a slicer independent mRNA storage or decay mechanism. For this silencing mechanism the binding of the seed sequence, the 7 to 8 base pair part near the 3′-end of the miRNA guide strand, in the 3′ UTR of the target mRNA is fundamental [37]. The fact that a complementarity of 7 to 8 base pairs in the 3′ UTRs of mRNAs is enough to directly repress translation increases the amount of matching seed regions up to several hundred hits for one miRNA [38, 39]. On the opposite, a single mRNA can be regulated by several miRNAs due to the number of seed regions in its 3′-untranslated region (UTR). It is known that miRNAs which induce a slicer independent translational repression interact at the initiation and elongation of translation [37, 39]. How this selection between mRNA decay or storage in P-bodies takes place is not fully understood.

These miRNA binding properties and the currently incomplete understanding of sequence recognition patterns complicate the use of algorithms for the sequence-based prediction of mRNA targets for a certain miRNA. All available computer programs list and sort the prospective mRNA targets by scanning for seed region complementarities and its conservation. The available software tools [40] often end in significantly differently ranked hitlists. Thus, comparison of several programs for high confidence prediction of the target mRNAs is advisable and experimental validation indispensable. To this end, luciferase assays for verification of miRNA binding to the 3′-UTR of putatitve target genes are a valuable model. After inserting the respective 3′-UTR into a vector encoding luciferase to express a fusion gene, the plasmid is co-transfected together with the miRNA. Luciferase expression levels are reduced through miRNA functions, and can easily be assays via luminescence.

Today more than one thousand miRNAs are annotated in the human genome, but it is reasonable that more will be discovered. It is assumed that these miRNAs can regulate or influence around 30-60% of protein-coding mRNAs by translational repression [41]. The extent of changes in protein expression of the miRNA-targeted genes is subtle, usually in the range of around 20 %. These features underline the relevance of miRNAs as fine-tuning agents of gene expression. The known diseases in which a deregulation of miRNAs has been detected spread from viral infections [42] to metabolic disorders [43], cardiovascular diseases [15] and cancer, which is the most investigated disease in this area [44-47].

The analysis of global miRNA expression in diseases employs miRNA micro arrays, miRNA PCR arrays and miRNA sequencing. The last technique emerged from next generation sequencing. Reliable data output for comparing different samples (healthy tissue vs. tumour tissue, drug treated vs. untreated) by these two techniques as well as by RT-qPCR need a well-considered RNA input and normalisation strategy [48]. The quality of isolated RNA is routinely characterized with the RNA integrity number (RIN) [49] but its significance is limited because mainly ribosomal RNA is taken into consideration. Concentration measurement at 260 nm also represents mainly the ribosomal RNA fraction and is prone to other contaminants as well [50]. In particular, the comparion of healthy tissues to tumours often shows an intrinisic bias between the miRNA and mRNA fractions whereas the ribosomal fraction, which is used for concentration and quality measurement, stays unaffected [51].

In expression experiments this issue should be considered to reduce the likelihood for false positive results, especially when expression profiles of healthy and tumour tissue samples are compared. After choosing the correct RNA fraction (isolation of miRNA only, or the use of total RNA or RNA void from the ribosomal fraction) [52, 53], an adequate normalisation needs to be applied to avoid over- or underestimation of specific miRNAs [48, 54]. For deregulated miRNAs, analysis by stem-loop RT-qPCR is the technique of choice for verification of the expression level with high reliability [55-57]. The MIQE guidelines for RT-qPCR experiments recommend a selection of several reference genes for normalisation. Providing sufficient and transparent information of experimental details is a prerequisite to ensure high qualitative reproducible RT-qPCR results [58].

miRNA in cancer

Oncogenic miRNAs

The implication that miRNAs are involved in carcinogenesis was first proven by identifying genomic alterations of the miR-15a/16 cluster in CLL [59]. In cancer research, two fields of miRNA research are in focus, the identification of circulating miRNAs as biomarkers for classification of cancers [60] and the search for carcinogenesis associated miRNAs [61]. miRNA microarray profiling resulted in a more exact classification of tumours and allowed better sub classification of a cancer type compared to mRNA profiling [62]. The results of genome wide transcriptional experiments with cancer cell lines and patient samples proved that miRNAs are driving increased cell growth, higher apoptosis resistance and metastasis [63-68]. An aberrant miRNA expression profile is also implicated in cancer stem cells, which promote tumour genesis and progression [69, 70]. Carcinogenesis associated miRNAs can be split into two distinct subpopulations, tumour suppressor miRNAs, which are silenced, and oncogenic miRNAs, also called oncomiRs, which are characterized by a gain of function [61, 71]. Interestingly, the total miRNA expression has been found to be down regulated in tumours, indicating a higher share of tumour suppressor miRNAs.

Contrary to oncogenes and tumour suppressor genes, the affiliation of a miRNA to these two groups can differ between distinct cancer types. If a certain miRNA functions as an oncomiR, it can have no implication in another type of cancer, even when these two are closely related (Table 1). Similar to tumour suppressor genes, tumour suppressor miRNAs [59, 72-74] are often located in fragile sites of the genome [75]; these are unstable genomic regions where DNA arrangement like deletions or heterochromatin formation is very common during carcinogenesis. This often results in an inactivation or deletion of whole cluster of tumour suppressor miRNAs, like the miR-15a/16 cluster in CLL.

The epigenetic analysis of genomic regions of known tumour suppressor and oncomiR clusters showed that the methylation frequency increases up to ten fold in these miRNA areas [76]. In protein-encoding regions only a slight increase of methylation occurs [77, 78]. These data provide the first evidence that miRNAs are located in epigenetic hot spots on chromosome 1q, 7q, 11q, 14q and 19q [79]. Due to the fact that the majority of miRNAs is transcribed by RNA Pol II, the result that DNA methylation is controlling the miRNA expression is all but expected, but the extent of methylation is nonetheless surprising. Experiments with 5-aza-2′-deoxycytidine identified miR-127 as epigenetically regulated [80, 81]. In addition, the inhibition of histone deacetylases (HDACs) in pharmacological or knock out experiments showed a severe alteration in global miRNA expression, which proves that DNA methylation and histone modification have a major control function in miRNA transcription [82].

A meta-analysis of published data from 2006 till 2010 analyzing the epigenetic functions and control of miRNA [79] revealed that 20% of epigenetic miRNAs have a CpG island within 5 kb upstream of their transcription start point. In addition 14% of epigenetically regulated miRNAs reside in a CpG island, resulting in a total of 1/3 of the known epigenetically regulated miRNAs associated with a CpG island.

Tumor suppressor miRNAs

MiR-145 is a well studied tumour suppressor miRNA which is commonly down regulated in epithelial malignancies [83, 84]. In hepatocellular carcinoma (HCC), re-expressing miR-145 resulted in inhibition of cell viability, proliferation and a G2-M cell cycle arrest [85]. The miR-145 mimic down regulated the predicted target genes in the insulin-like growth factor signalling pathway. The direct interaction of miR-145 with the 3′-untranslated region of Insulin substrate (IRS)-1, IRS-2, and insulin-like growth factor 1 receptor (IGF1R) was responsible for the harmful effects on HCC cells. A retrospective analysis of miR-145 levels in patient tumours indicated that a low miR-145 level was associated with a shorter disease-free survival. Contrary, a high miR-145 level accounts for a longer disease-free survival which is in line with the *in vitro* results [85]. A recent study showed that up regulation of miR-145 or silencing of IRS1 reduced the phosphorylation of Akt and FOXO1, leading to a decreased cyclin D1 expression which causes cell cycle arrest [86]. New insights in the field of non-coding RNAs, especially circular RNAs [87], and transcribed pseudogenes [88] present them as new group of miRNA regulators in the cell. In the case of miR-145, the pseudogene OCT4-pg4 functions as a sponge for miR-145 and high expression of OCT4-pg4 correlates with poor prognosis in HCC patients [89]. In gastric cancer, miR-145 has an anti-metastatic function due to the posttranscriptional inhibition of the v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS1) gene [90].

Many miRNAs show an ambivalent character, like miR-149. Depending on the analysed cancer type, miR-149 can function as oncomiR [91], tumour suppressor miRNA [92] and metastasis inhibitor [93]. MiRNA-149 has two mature product, mir-149-5p, and mir-149-3p, originating from one end of the hairpin strands, respectively. MiR-149-5p has been shown to be down regulated in highly metastatic breast cancer cell lines which results in increased GIT1 expression and elevated integrin signalling [93]. *In vitro* the up-regulation of pre-miR-149 resulted in a reduced invasion and migration activity. *In vivo*, miR-149 overexpression reduced the metastatic ability of invasive cancer cells, caused by impaired lung targeting of circulating cells. The reduced miR-149 expression leads to elevated levels of GIT1 which on the other hand activates and stabilises paxillin, α5 and β1-integrin complexes. Biopsy analyses of patient verified this negative correlation of miR-149 and GIT1 in primary breast tumours and metastases. MiR-149 has also been shown to act on forkhead box M1 (FOXM1) in non-small-cell lung cancer, and a negative correlation between miR-149 levels and invasiveness and epithelial-to-mesenchymal transition was found [94]. In gastric cancer, reduced miR-149 expression is responsible for an increased ZBTB2 level which enhances cell cycle progression and reduces apoptosis [92]. MiR-149 expression was higher in patient samples with stage I/II disease without lymph node metastasis. ZBTB2 is a repressor of the p53 pathway and an miR-149 mimic restored the signalling pathway by decreasing ZBTB2 and HDM2 levels and increasing ARF, p53 and p21. The miR-149 mimic reduced the proliferation rate and induced a G0/G1 cell cycle arrest.

In melanoma, miR-149-3p acts as an oncomir, induced by ER stress and increased p53 expression [91]. Increased levels of miR-149-3p up regulate MCL1, which is responsible for an anti-apoptotic effect in melanoma. *In vivo* experiments with anti-miR-149-3p-transfected

melanoma cells showed a retardation of tumour size in xenografts. Clinical samples of 60 fresh isolated melanoma showed that high levels of miR-149 correlated with low levels of p53 and MCL1. Thus, instead of being a functionally active tumor suppressor, p53 mediates a prosurvival pathway through up-regulation of miR-149 in melanoma.

Many other distinct miRNAs (Table 1), including the let-7 family, miR-10b, miR-17, miR-19, miR-29, miR-34, and the cluster of miR-143 and 145, have been linked to a role in cancer. Several recent reviews describing the elucidation of their respective roles and function in tumour biology are available [13, 68, 95-97]. Although it is not fully clear whether the dysregulation of miRNA expression in tumours is a cause or a consequence of carcinogenesis, there is hardly a doubt that the modulation of miRNAs is an attractive strategy for the development of novel cancer therapeutics.

In contrast to cancer, the investigations for the roles of miRNAs in other diseases including diabetes, cardiovascular diseases and mental disorders have just started. Some miRNAs involved in carcinogenesis (miR-15a, miR-16, miR-145 and miR-124a) also play a role in pancreatic β cells and glucose metabolism [98]. MiR-7 is a negative regulator of glucose-stimulated insulin secretion [99]. Transgenic mice with an overexpression of miR-7a in β-cells developed diabetes because of reduced insulin secretion. Expression of miR-7a is decreased in obese and diabetic mouse models and human samples from respective patients, supporting a regulatory role in diabetes.

A deregulation of distinct miRNAs has also been found in cardiovascular diseases [100] (mir-15 family, miR-33, and others), schizophrenia [101] (miR-137, and others), Alzheimer's disease [102] (miR-107, and others), Huntington's disease [103] (miR-34b), and multiple sclerosis [104] (miR-92, and others) to just name a few. For all those and more disorders, miRNAs show a potential value for diagnosis and as targets for treatment. The role of miR-122 in hepatitis viral replication has already been translated to the clinical evaluation [42].

Strategies for therapeutic intervention

There are two distinct possibilities to exploit miRNAs for therapeutic interventions. Isosequential, exogenous miRNAs can be applied to substitute for endogenously expressed miRNA [105], and oligonucleotides [106] or small molecule antagonists [107] can be used to lower the gene regulatory effect of natural miRNAs. The latter approach allows the use of synthetic oligonucleotides for enhancing instead of silencing gene expression instead of down regulation like antisense and siRNA.

miRNA Mimics - Substitution of Depleted miRNAs

Synthetic miRNA mimics can assume the regulatory role of natural miRNAs. Like siRNA, double stranded RNA oligonucleotides with minimal chemical modifications are suitable miRNA replacements *in vitro* and *in vivo*. Since the synthetic oligonucleotides need to be incorporated into the RNAi machinery, phosphorothioate backbone modifications as well as 2′-alkylated nucleotides are tolerated only up to a small percentage without considerable loss of activity [108]. The lack of making use of the medicinal chemistry toolbox to a higher

degree comes at the expense of enzymatic stability and the pharmacokinetic properties. Unformulated dsRNA is degraded in biological fluids within minutes, and thus delivery systems seem to be a prerequisite for successful development of therapeutic miRNA replacements [109]. Borrowing from siRNA technology, the currently prevailing approaches in preclinical development rely primarily of optimized liposomal formulation (SNALPs, stable nucleic acid lipid particles), viral particles, and cationic polymers such polyethylene imine (PEI). These advanced formulations successfully shield RNA oligonucleotides from degrading enzymes, and can deliver their cargo to organs such as liver and kidney. Efficient cellular uptake and intracellular re-release of the cargo, as well as penetrating other organs and tissues, are not satisfactorily solved to date.

The standard design consists of two RNA oligonucleotides with one-two terminal phosphorothioate linkages and several, up to 50 % 2′-methylated nucleosides. The passenger strand, which tolerates a higher degree of derivatization, has usually a higher number of these modifications. Recently, a three stranded, nicked design was introduced to siRNA and miRNA agents (Figure 2). By using two shorter oligonucleotides as passenger strands, the incidence of off-target effects caused by inadvertent recruitment of the ‘wrong’ component as guiding strand is aimed to be reduced.

MRX34, a miR-34a replacement, was the first to enter clinical testing [110]. miR-34 is a well-defined miRNA tumour suppressor. It acts on several cancer relevant cellular pathways, including the p53 and wnt/β-catenin pathways. A loss of expression has been found in many tumour types, including lung, liver, breast, and colon carcinoma, and the restoration of its function through exogenous miR-34 mimetics has been shown to inhibit tumour growth and progression. The miR-34 family consists of miR-34a, miR-34b, and miR-34c, which share a high degree of sequence homology, including the seed region. Thus, all three influence largely the same set of genes and are functionally redundant. MRX34 is an RNA oligonucleotide isosequential to miR-34a, the most abundant one in human cells.

Similar to pre-miRNA is short hairpin RNA (shRNA), a research tool and potential therapeutic agent in form of a plasmid vector equipped with RNA Pol III promoter sequences for knocking down any gene of interest. The accordance between the size, structure and energetic properties of pre-miRNA and shRNA enable the use of the same cellular processing mechanisms for shRNA like for pre-miRNA and result in effective gene silencing. However, using shRNA overexpression models, the available exportin 5 transporters and the RNAi (RISC) machinery can become saturated, having influence on the endogenous miRNA export and potentially causing side effects by inhibiting unrelated endogenous miRNA functions [111, 112].

miRNA Antagonists - Interception of Overexpressed miRNAs

On the other hand, antagonistic oligonucleotides (antagomirs, antimirs) targeting oncomirs are a promising strategy for treatment of cancer and other diseases. Single-stranded antisense-like compounds can be applied to intercept and degrade mature miRNAs. Molecules with higher affinity to the miRNA or with higher abundance than the mRNA target will prevent the functional miRNA effect, that is silencing of the respective target genes [113]. Standard chemical modifications of the antisense research field, including 2′-O-

methyl or methoxyethyl nucleotides, 2′-F nucleotides and phosphorothioate backbone modified oligonucleotides have been shown to successfully interfere with miRNA effects [106, 108]. Full 2′-alkyation will prevent the activation of RNase H, an enzyme recognizing DNA/RNA duplexes, and thus a pronounced antisense effect of corresponding mRNAs [114]. Because of the short length of the targeted RNA, oligonucleotides with high intrinsic target affinity are particularly useful. Therefore, the incorporation of locked nucleic acid (LNA) building blocks, one of only few nucleotide derivatives with higher Watson-Crick base pairing affinity compared to wild-type nucleotides, is an attractive option [115]. A methylene bridge between the 2′-oxygen and the 4′-carbon atoms provide higher structural rigidity and increased selective affinity to the RNA counter strand [116]. In contrast to mRNA-targeted oligonucleotides, the available miRNA sequence is drastically limited. Poor sequence design can result in partial hybridization to similar sequences which in turn causes off-target effects [117].

Because of their chemical modifications, the pharmacokinetic properties of antagomirs are more favourable that those of miRNA mimetics [4]. Phosphorothioate backbone modifications adequately stabilize the oligonucleotides against degradation, and result in a high degree of binding to plasma proteins, which reduces rapid elimination [118]. Phosphorothioates are distributed to nearly all organs and tissues (a notable exception being the brain), but show a preference for liver and kidney. Additional 2′-methoxy or methoxyethylene modifications increase the stability and allow for lower doses.

The first report of using miRNA-antogonistic oligonucleotides *in vivo* employed a 2′-methoxy phosphorothioate oligonucleotide conjugated to cholesterol [119]. miRNA-122, found abundantly in hepatocytes, was silenced effectively through accumulation of cholesterol-conjugates in the liver. Although cholesterol induces liver accumulation and facilitates cellular uptake into hepatocytes, those conjugates need a relatively high dose, and are ultimately not suited for therapeutic applications. Further structural optimization made use of LNA, leading to the development of miravirsen, a partial LNA oligonucleotide likewise targeted at miR-122.

Taking advantage of the superior thermodynamic binding properties of LNA, short phosphorothioate, fully LNA-modified oligonucleotides were designed to target the seed region of miR-122 and closely related miRNAs [115]. The so-called ‘tiny LNAs’ simultaneously antagonize many different miRNAs (those with identical seed regions). Phenotypical changes were very similar to those after treatment with a full-length miR-122 antagomir. Using these shorter sequences, it may be possible to deplete the function of whole miRNA-clusters, instead of targeting single miRNAs. Although miRNAs targeting identical seed regions often have similar effects, this approach potentially results in off-target effects on other either short or long RNA transcripts.

Other chemical modifications that have been employed for miRNA antagomirs include peptide nucleic acids (PNA) [120], with non-nucleoside synthons linked by a peptide bond, and phosphoroamidate morpholino oligomers (PMO), with morpholino heterocyclic building blocks. Both structures suffer from poor cellular uptake, and are thus not as widely employed as 2′-modifications.

In recent years antagomirs have also been developed, among others, for potential treatment of cardiovascular diseases (anti-miR-21) [121], status eplilepticus (anti-miR-132 and anti-miR-134) [122], rheumatoid arthritis (anti-miR-155) [123], autoimmune diseases (anti-miR-21) [124], hypercholesterolemia (anti-miR-122, anti miR-33) [125], and for prevention of stroke [126].

Similiar to antagomirs, miRNA sponges or decoys aim to inibit miRNA functions by preventing stable binding to their targets. Instead of short oligonucleotide strands, these agents are longer nucleic acids, usually DNA plasmids or transcribed RNA, with several miRNA binding motives [127]. Due to the higher abundance, they inhibit the downstream effects of the corresponding miRNA. This has been a useful approach for studying miRNA functions *in vitro*. For therapeutic applications, however, it seems less likely to succeed because of safety concerns and off-target effects caused by highly abundant exogenous nucleic acids.

Besides oligonucleotides, miRNA functions can also be modulated by traditional pharmacologic agents with low molecular sizes. Compared to protein binding sites, nucleic acids are much poorer pharmacological targets for small molecules because of their lower structural diversity both with regard to the sequence and secondary structures. Nevertheless, successful and selective inhibition of miRNAs with small molecules has been reported [128-130]. In 2008, a large compound library was screened for inhibition of miR-21, and after structural lead optimization a diazobenzene compound (**1**, Figure 3) was developed which showed good inhibition of miR-21 in concentration of 2 to 10 μM assayed through a luciferase reporter construct [128]. Since the compound (**1**) did not influence the expressional levels of two other miRNAs, it is considered to have at least some target specificity for miRNA-21, however, the exact cellular target is unknown [129]. Since then, compound screenings have shown effective small molecules for other miRNA targets [131], and methods for rational structure design have been refined [130, 132]. These include *in silico* technologies for analyzing putative miRNA binding motivs and selectivity of lead structures. Drosha- or dicer binding sites were chosen as targets for the anti-miRNA compounds, aiming at inhibition of miRNA biogenesis by cleavage from pri-miRNAs. Lead molecule prediction was used to discovery compounds (including **2**, **3**, and **4**, Figure 3) reducing miR-96, miR-182, and miR-183, respectively, by at least 40 % [130]. The selectivity for this set of miRNAs was found to be remarkably high, and well comparable to what can be achieved with oligonucleotides. Still, the very close structural and sequential similarities between distinct miRNAs cast doubts about the applicability of this approach for a wider range of miRNAs.

A number of fluoroquinolone antibiotics were shown to enhance the effect of siRNAs [133] and miRNAs [134] through interaction with the RNAi machinery (Figure 3). The most potent compound, enoxacin, was further studied for the molecular mechanism of this effect. Enoxacin was found to increase the binding affinity of TRPB, an integral component of the RISC, to miRNA precursors. As such, there is of course no specificity for certain miRNA sequences. Because the global miRNA expression is significantly lowered in tumours, a general increase of miRNA activity may nevertheless be an attractive option in oncology. Indeed, enoxacin reduced cell viability in cancer cell lines [134].

Although lacking most of the intrinsic specificity of oligonucleotide agents, small molecule-mediated miRNA modulation may be a viable way for drug development. They do not suffer from the problems that have so far prevented the widespread clinical application of oligonucleotides, namely their insufficient drug-like properties and poor pharmacokinetics and cellular uptake. A more appropriate biodistribution pattern would nearly instantly allow treatment of all tissues and organs, including the brain. Recently it was reported that enoxacin successfully elevated miRNA levels in the frontal cortex in rats [135]. Thus, miRNA are attractive targets not only for biotherapeutics, but also for small molecules. However, both approaches need to overcome their distinct obstacles before being widely used in clinical applications.

Delivery vehicles

As indicated above, the poor tolerance for optimizing the chemical structure prevents adequate stabilization against degradation and improved cellular penetrations of miRNA mimics through chemical engineering. Being nearly identical to siRNAs, the same pharmaceutical formulations, encompassing liposomes, polymeric nanoparticles, and viral systems, can be applied to increase stability and enhance the pharmacokinetic behavior of miRNA oligonucleotides. Administration of an artificial miRNA replacing miR-26a with adenovirus-associated virus was effective against progression of hepatocellular carcinoma in a mouse model [136]. Safety concerns of viral delivery systems limit the clinical perspective for the moment, and non-viral approaches seem to be more promising.

Liposomes

Among those, lipid-based delivery is by far the most popular approach [109]. The classical approach consists of a mixture of lipids with cationic head groups and helper lipids, including some with polyethylene glycol chains for masking of the surface charge. Polyanionic nucleic acids are electrostatically complexed to the cationic lipid, yielding lipoplexes [137, 138]. A high degree of optimization of those formulations, both in terms of structures and multi-component compositions, has been achieved, and loading capacity and delivery efficiency have been increased to considerably lower the dose necessary for functional effects [139, 140]. However, they still suffer from inherent toxicity, which is, like uptake and re-release efficiency, closely linked to the cationic surface charge [109]. Examples of the *in vivo* use of lipoplexes include the delivery of miR-133b and miR-29b with a mixture of DOTMA, cholesterol and a PEG lipid [141, 142], pre-miR-107 with DDAB, cholesterol and PEG lipids [137], and the use of solid lipid nanoparticles consisting of DDAB, cholesterol and other components for delivery of miR-34a [143].

Grafting a targeting molecule on the surface of lipoplexes may constitute a viable strategy for achieving accumulation in certain tissues or tumours. Maleimide tethers were employed as anchoring points for a scFv antibody fragment on lipoplexes made up from protamine, DOTAP, cholesterol and PEG-DPSE. The resulting system successfully delivered miRNA to lung metastases and resulted in inhibition of tumour growth in mice [144]. Compared with free siRNA, accumulation in the lung/tumour was doubled, but the largest part of the dose was detected in liver tissue.

To alleviate the adverse effects and non-specific interactions of cationic particles, neutral lipid delivery systems were developed. A neutral lipid emulsion was combined with synthetic miRNA-34a and let-7 and tested for efficiency in a non small cell lung cancer (NSCLC) mouse model [145]. Unlike cationic lipoplexes, neutral lipid particles show less accumulation in liver after systemic administration, and are distributed more evenly to other tissues, including the lung. The treatment with the miRNA mimics resulted in reduced tumour size with a concomitant increase in tissue levels of miR-34a.

Polymers

The cationic polymer polyethylene imine (PEI) is the most widely used polymeric delivery system for plasmid DNA and siRNA [146, 147]. Efficient packaging and a net cationic charge ensure adequate shielding and sufficient interaction with anionic polysaccharides on cell membranes. PEI is thought to increase endosomal escape by the proton sponge effect, an influx of hydrogen ions into acidic endosomes, resulting in swelling and disruption of the intracellular vesicles. Thus, PEI has quickly been adopted for delivery of miRNA mimics. Using a polyurethane-polyethylene imine copolymer (PU-PEI), miR-145 was delivered to lung adenocarcinoma xenografts derived from cancer stem cells [148]. Synergistic effects on tumour growth were found in combination with radiation and cisplatin, while the same treatment in absence of miR-145 was ineffective. Tumourigenesis of cancer stem cell derived glioblastoma was similarly reduced after intracranially delivered miR-145 with PU-PEI, and synergistic effects in conjunction with radiotherapy and chemotherapy were detected [149].

PEI can also be used as carrier system for targeted delivery by attaching specific ligands to the polymer. For an miRNA application, the rabies peptide RVG was attached to PEI for transport to and across the blood-brain barrier [150]. In mice, fluorescently tagged miR-124a was found in higher accumulation in the brain compared to underivatized PEI. However, no functional effects were reported, and mannitol was necessary for sufficient blood-brain barrier permeabilization, which limits the therapeutic utility.

Poly(lactic-co-glycolic acid) (PLGA) is a polymer that has been utilized for antisense and siRNA delivery [151]. PLGA needs to be coated or functionalized for efficient oligonucleotide delivery, but affords long-term dissociation from the carrier for a prolonged effect. While no studies with miRNA mimics delivered with PLGA have been reported, oligonucleotides for antagonizing miRNAs have been successfully delivered to tumours [152, 153]. Coating with cationic peptides nona-arginine or penetratin afforded passive tumour accumulation of an antisense peptide nucleic acid targeted at miR-155 in lymphoma *in vivo*.

The use of targeted silica nanoparticles for miRNA delivery resulted in reduction of neuroblastoma growth [154]. The oligonucleotide cargo is noncovalently entrapped in the silica matrix, and dissociates upon hydrolysis of the matrix. Since this anorganic carrier will not be taken up into cells on its own, a receptor-targeting ligand is necessary to achieve intracellular delivery. Grafting an antibody against the cell surface antigen disialoganglioside GD2 afforded successful delivery of miR-34a into neuroblastoma cells in a murine xenograft model [154].

Conjugates

Conjugation of lipids or receptor-binding molecules directly to the nucleic acid is a promising way to increase cellular uptake of siRNA, and has also been explored for miRNA applications. By attaching cholesterol to the 3′-end of the passenger strand, an accumulation in liver tissue can be achieved [155]. The restriction to liver targeting and the high doses needed for sufficient delivery limit the use of cholesterol- and similar conjugates to scientific rather than therapeutic applications.

The asialoglycoprotein receptor ligand N-acetylgalactosamine (GalNAc) has been intensively used for targeting siRNA and antisense oligonucleotides to hepatocytes [12], with several agents already advanced to clinical evaluation. For achieving hepatocyte delivery of an anti-miR-122, a covalent conjugate with GalNAc has been developed. The respective compound RG-101, developed for treatment of HCV infections, has recently begun testing in human volunteers [156]. In preclinical animal models, RG-101 showed efficient reduction of viral titers, and a good safety profile [42].

Exosomes and Bacteriophages

Circulating miRNAs are found in body fluids (plasma, saliva, etc.) and are exchanged between cells despite the abundance of nucleases throughout the body [157]. Natural shielding of endogenous miRNAs is afforded through extracellular vesicles, called exosomes. They are small membrane vesicles (up to 100 nm), and are produced by many cell types, including epithelial, dendritic, and immune cells [158]. Lately, exosomes have been used to encapsule and deliver synthetic or endogenously expressed siRNAs [159] and miRNAs [160] *in vivo*. The oligonucleotide cargo can be introduced by transfection of corresponding plasmid into exosome-producing cells, or synthetic oligonucleotides can be inferred through electroporation of the mature exosomes. For miRNA delivey, transfection of exosome-producing HEK293 cells with synthetic let-7 was employed to produce miRNA containing exosomes. A peptide binding to the EGF-receptor was introduced by means of a peptide-encoding plasmid [160]. Exosomes distribute preferentially to the reticuloendothelial system (RES), and thus the spleen and liver, but are also found in other organs. Targeting can be achieved through specific ligands, which can be expressed through genetic engineering methodology. Using the RVG peptide, siRNA-containing exosomes were even shown to cross the blood-brain barrier [159]. Targeting and biodistribution of exosomes is also dependent on the cell type they are isolated from. Exosomes are similar to liposomes in terms of consisting of bilayered phospholipids, but the biogenesis of exosomes ensures their biocompatibility and low toxicity. It also significantly complicates pharmaceutical development, production and safety profiling (immunogenicity, and potential biological impurities).

Bacteriophages have been used to develop virus-like particles for oligonucleotide and drug delivery. The MS2 bacteriophage was modified to produce particles with covalent linkage to pre-miR-146a. After grafting the HIV-TAT peptide to the particles, the system induced a two-fold higher expression of miR-146a *in vivo* [161]. A similar approach used RNA from bacteriophage Phi29 for packaging miRNAs targeted at coxsackievirus B3. Conjugation of folic acid was used for folate-specific cellular uptake to result in reduction of viral

replication in an *in vitro* model [162]. Similar to exosomes, concerns of immune responses triggered by virus-like particles need to be addressed before further development of bacteriophage-derived particles into clinical evaluation.

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Defined key terms

oligonucleotide therapeutics	Short synthetic nucleic acids for medical use comprise antisense, siRNA, aptamers, splice-switching oligonucleotides, decoys, miRNA mimics, antagomirs, and others. They are categorized by their differing mechanism of action, and share structural characteristics and chemical modifications to a large extent. Therapeutic development has been hampered by poor pharmacokinetic properties, and some adverse effects encountered in clinical evaluations.
phosphorothioate	This chemical modification consists of a substitution of a non-bridging oxygen of the phosphate linkage for a sulfur atom. The main virtues are stabilization against degrading nucleases, binding to plasma proteins, and the ability to be a substrate for RNase H when used with 2′-desoxynucleotides. On the downside, induce sequence-independent effects, including cell stress and death.
RNA induced silencing complex (RISC)	This protein complex is constituted in humans upon recruitment of the argonaute protein Ago2 by the complex of siRNA or miRNA with Dicer and TRBP. While one passenger strand of the short RNA duplex is degraded, the remaining part acts as guide strand for the complementary RNA. Upon hybridization, Ago2 cleaves the mRNA and provokes gene silencing.
stem-loop qPCR	Quantitative PCR is the method of choice for nucleic acid quantification. For short RNA transcripts, a specific stem-loop primer hybridizes and is reverse transcribed. Using a specific forward and a universal reverse primer, which binds to the sequence derived from the stem-loop primer, the RT product is amplified and quantified in a real-time cycler.
fluoroquinolones	This class of broad-spectrum antibiotics is used to treat severe bacterial infections. Some fluoroquinolones inhibit topoisomerases II (gyrase) and IV, and result in suppression of bacterial DNA replication. Adverse effects include tendon problems and effects

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	on the central nervous system, attributed to interaction with GABA receptors. Enoxacin is a second-generation fluoroquinolone for oral application.
DNA methylation	5′-Methylation of cytidine in DNA is an epigenetic mechanism for controlling gene expression. Methylated cytidines are usually adjacent to a guanosine, forming a CpG dinucleotide. Sequences with multiple CpGs are called CpG islands, and tend to be near transcription initiation sites. 5′-Methylcytidine nucleic acids are not transcribed, so methylations are locking genes permanently in the ‘off’ state.
Stable nucleic acid lipid particles (SNALPs)	are lipids consisting of cationic, fusogenic, and PEGylated lipids and encapsulate nucleic acid cargos. They have a diameter of around 100 nm, and have been applied for rather efficient delivery *in vitro* and *in vivo*. Many clinical programs of siRNA use SNALPs.

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Future perspective

Because cancer involves deregulation of multiple genes and miRNAs, the focus on single targets is generally realized to be insufficient for an efficacious treatment. In this area, the dogma of high target specificity has been displaced by multi-specificity. In that regard, miRNA-based therapies are an evolution upon siRNA agents, in that they influence a regulatory sequence often acting on a whole pathway or even multiple pathways, and not a single gene. Instead of antagonizing or amplifying a single miRNA, applying a systems biology view with concurrent influence on multiple target sequences promises to be an even superior approach for oncology. During the next few years, basic research will further define and validate potential miRNA as therapeutic targets. Special attention needs to be paid to the issue whether the influence on gene regulation of a single miRNA or an miRNA cluster is sufficient for phenotypic effects. An increasing perception of the incredibly high complexity of biological gene regulatory systems will be essential for realistic evaluation of the therapeutic value of miRNA targets. It seems likely that miRNA mimics and antagonists will not be highly potent cancer therapeutics *per se*, but they are expected to become valuable agents for modulation of certain oncogenic characteristics such as chemoresistance, or metastasis, and could be beneficially applied as part of combination therapy together with cytostatic compounds.

Much more than target identification and validation, progress on the delivery issue will be decisive for successful miRNA-based therapy with oligonucleotides. During the next years, an increasing number of therapeutic miRNA modalities can be expected. Drug development will initially focus on oligonucleotide compounds for both substitution and antagonism of disease-modifying miRNAs. Development programs will mainly be driven by biotech companies, and predominantly stimulated by discoveries of roles of new miRNA targets. However, ultimately success depends primarily on solving the pharmacokinetic and delivery hurdles tightly associated with any kind of oligonucleotide therapy. Currently available solutions are insufficiently active (with maybe the exception of hepatocyte delivery). A much desired major breakthrough in this area would certainly pave the way for novel therapeutic modalities by miRNA targeting. Considering the long-standing experience with oligonucleotide therapeutics, similar changes of sentiment of scientists and industry can be expected for miRNA targeted agents: An initial period of enthusiasm with strong investments into early stage programs, followed by disappointment caused by slower than expected advances, and finally more realistic development strategies focusing on appropriate diseases and delivery systems and well-characterized data.

Today, it is difficult to judge the full potential of miRNA-targeted small molecules. Recent data indicates the possibility to modulate miRNA expressions with a certain degree of specificity. While it seems doubtful that full sequence specificity can be achieved with small molecules - given the enormous number of distinct nucleic acids present in every single cell - it appears plausible that groups of closely related miRNAs can indeed be modulated through carefully designed and optimized pharmacophores. Considering the aforementioned system biology view, this concurrent targeting of several

small RNAs could even be superior than a highly sequence specific oligonucleotide therapy.

Both the characterization of specific miRNA roles and the development of corresponding therapeutics are still in very early phases, and several years of research is required to rightfully judge the therapeutic potential.

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Executive summary

miRNA in Cancer

- The expanding knowledge of the roles of microRNA opens new avenues for therapeutic intervention in cancer and other diseases. Dissection of the gene targets and the impact on cellular pathways is essential for the design of miRNA-based therapies.

Strategies for Therapeutic Intervention – Oligonucleotides and Delivery Systems

- The development of miRNA mimetics is an attractive option for substitution and functional restoration of depleted miRNAs. For efficient application, the delivery hurdle needs to be solved. Current approaches rely primarily on liposomal and polymer-based delivery systems, but need to be improved.
- miRNA effects can be antagonized through application of oligonucleotides complementary to the guiding strand. By derivatization with phosphorothioate linkages and 2′-O-methyl or LNA nucleosides, enzymatic degradation is delayed and pharmacokinetics are enhanced.

Non-Oligonucleotide Agents

- An interaction of small molecules with miRNA precursors or effector proteins may off an alternative option for therapy. Inhibitors have been designed to bind to specific miRNA precursors on binding sites for processing nucleases, and the fluoroquinolone antibiotic enoxacin is an enhancer of the miRNA/siRNA machinery, resulting in increased gene silencing.

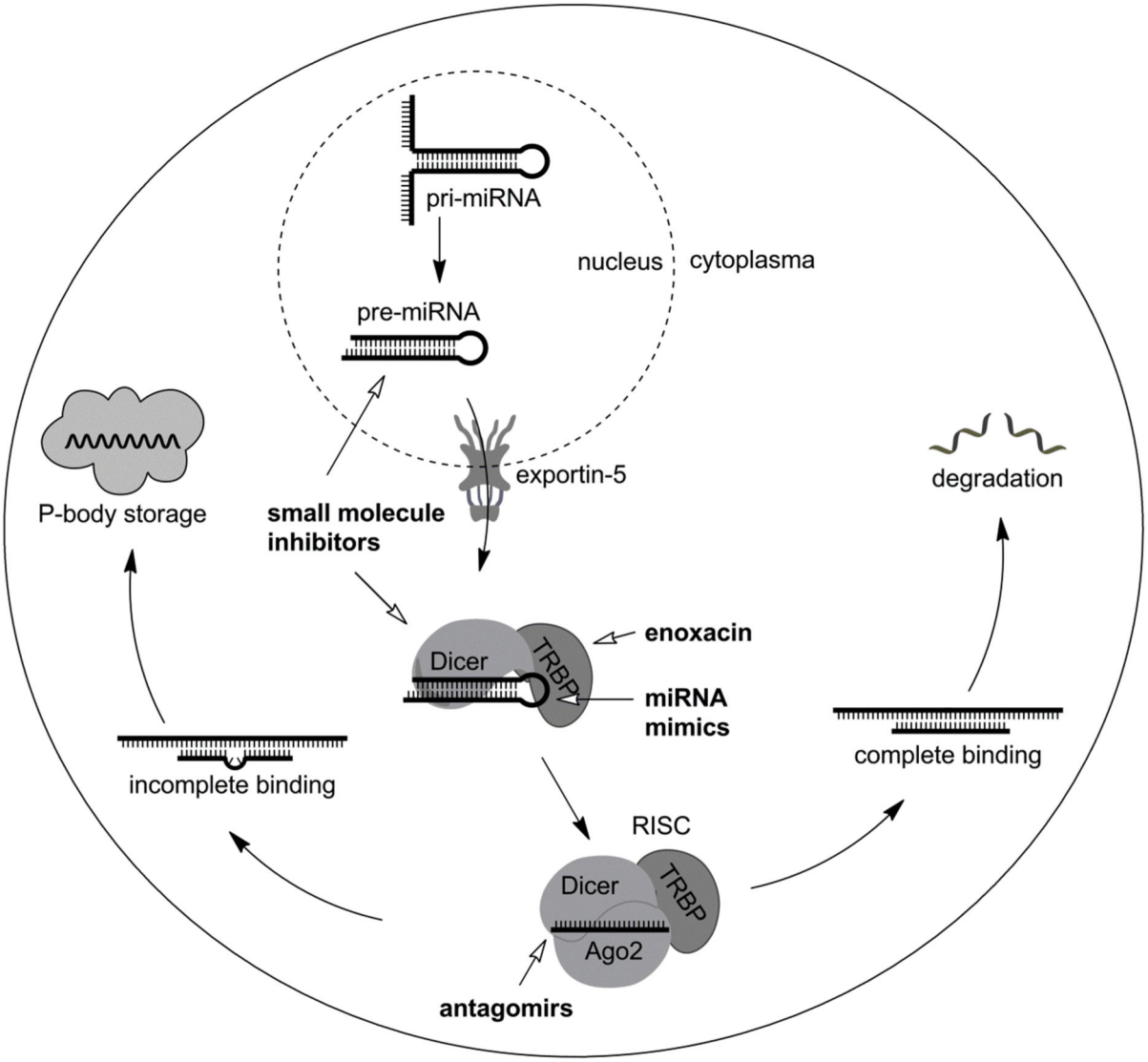

Figure 1. miRNA biogenesis, mechanisms of action and the use of therapeutic agents for modulation of miRNA functions

MicroRNA is transcribed initially as the pri-miRNA, which is processed by the nuclear nuclease Drosha to give the characteristic pre-miRNA stem loop structure. The pre-miRNA is transported from the nucleus to the cytoplama by exportin-5 and Ran-GTP. Further processing by Dicer with TRBP is essential for generation and selection of the guide strand, which is incorporated into the mature RISC, assembled by recruitment of the Ago2 nuclease. Depending on the binding sites (3′-UTR, ORF or 5′-UTR), and perfect or incomplete hybridization to the mRNA, the result is degradation of the target mRNA, storage in P-bodies, or translational repression. Therapeutic intervention is mainly focused on the use of oligonucleotides as either substitutes (mimics) of depleted endogenous miRNA, or as complementary strands with the aim of intercepting the miRNA guide strand (antagomirs, miRNA sponges). Other approaches with small molecules can inhibit the miRNA processing

by inhibiting the binding of Drosha and Dicer, or unspecifically promote miRNA biogenesis by promotion of the interaction with TRBP.

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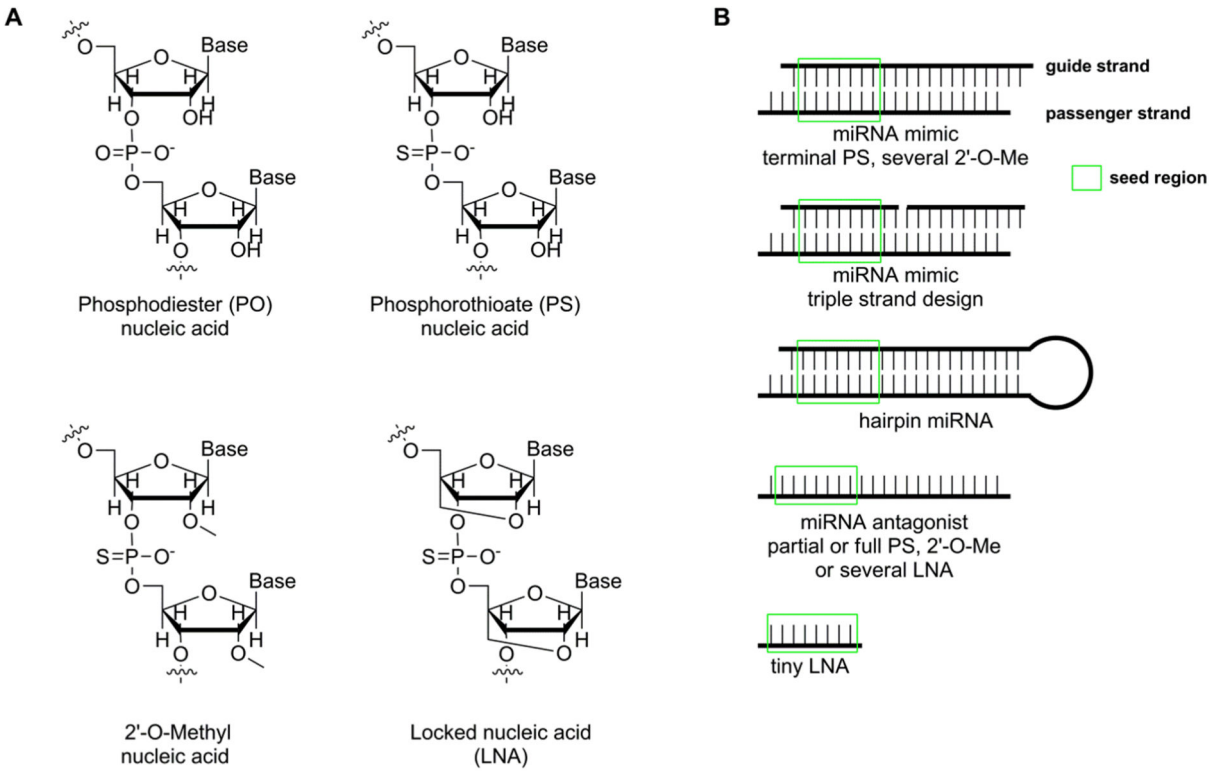

Figure 2. Chemical oligonucleotide modification patterns for miRNA-based therapeutic oligonucleotides

For effective treatment with oligonucleotides, chemical modifications are essential to prevent rapid degradation and promote cellular uptake (**A**). For miRNA-based applications, phosphorothioates and 2′-O-methyl modified sequences are most commonly employed. While miRNA mimics only exhibit a small degree of modification, antagomirs can be fully modified. LNA nucleosides stabilize oligonucleotides and increase binding affinity, and are thus particularly useful for the inhibition of miRNA through seed sequence binding, and enable the simultaneous depletion of several miRNA with the same seed region motive. The derivatization usually does not cover the entire sequence, but is restricted to partial regions with the seed region often omitted. Besides structural modification, sequence length and format can also be tailored to show higher stability or selectivity (**B**). Possibilities include single or double strand formats, nicks, and connections by hairpins.

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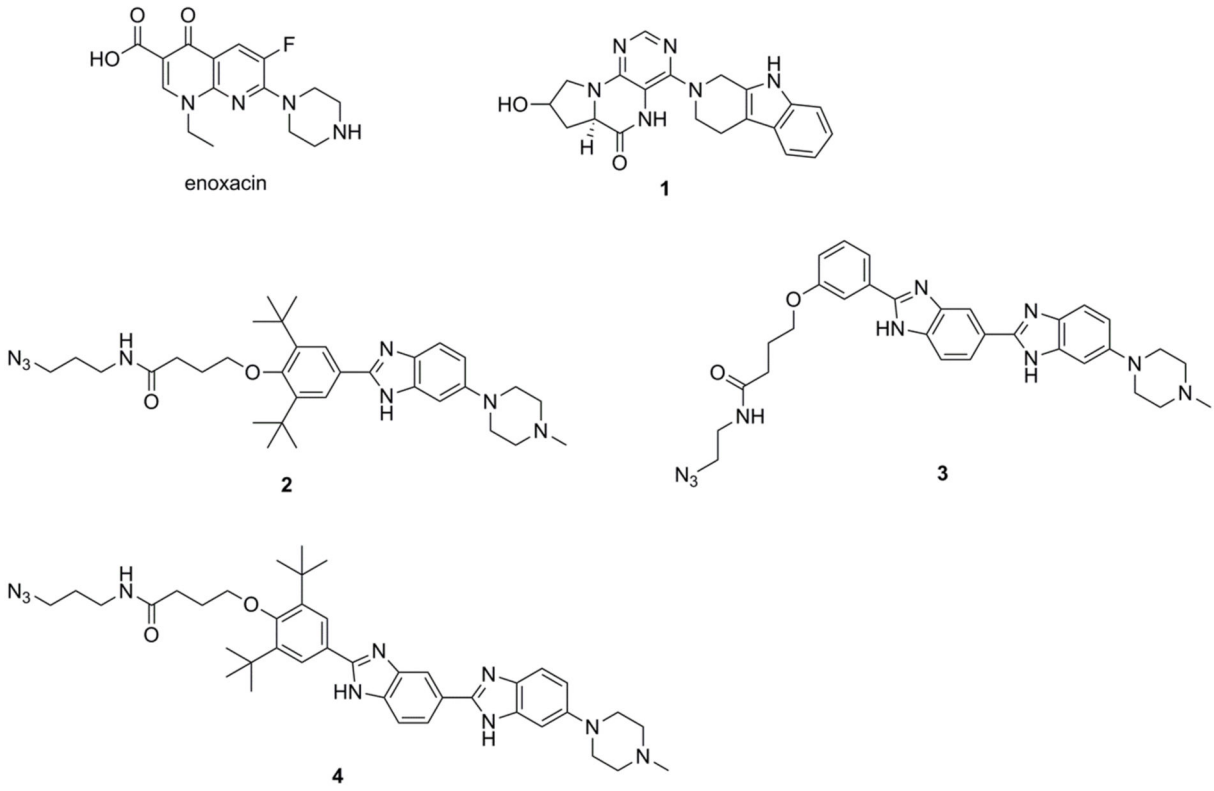

Figure 3. Small molecules for regulating miRNA expression and functions

The fluoroquinolone enoxacin has been identified as an enhancer of siRNA and miRNA effects. It promotes the interaction of nucleic acids with the HIV-1 TAR RNA binding protein (TRBP), an important co-factor of Dicer and integral component of the RNAi silencing complex (RISC). Several specific miRNA inhibitors have been described, and examples of structures (1 [129], 2, 3, and 4 [130]) are depicted.

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Table 1

Selection of miRNAs as potential targets for tumour therapy

miRNA	role in cancer	up / down regulated	known gene targets	references
let 7a	suppression of metastasis, cell cycle control, apoptosis regulation	↓	RAS genes, HMGA2, MMP11, CDK genes, BCL2, CASP3, etc.	[74, 163]
miR-15a	inhibition of proliferation	↓	BCL2, CAPZA2, CXCR4, COPA, PIR121, GAP43, etc.	[59, 73]
miR-16-1	inhibition of proliferation	↓	BCL2, RARS, ATP5E, BPI, DC2, EPC1, IGF1, KRT18, TPX1, etc.	[59, 73]
miR-21	oncomir	↑	PTEN, TRPs, BCL2, PDCD4, etc.	[164]
mir-34a	tumour suppressor	↓	BCL2, LDHA, CDK4, etc.	[110]
miR-137	tumour suppressor, cell cycle control	↓	AEG-1 (ovarian cancer), CDC42, CDK6 (lung cancer)	[165, 166]
miR-145	tumour suppressor	↓	ADAM17, IRS1, IRS2, IGF1R, OCT4, SOX2, ETS1, pAKT, pFOXO1, etc.	[85, 86, 90]
miR-149-5p	metastasis suppressor	↓	GIT1, ZBTB2	[93]
miR-149-3p	tumour suppressor	↓	E2F1, AKT1	[91]
miR-181a	cervical cancer – oncomir chemoresistance	↑	PRKCD	[167]
miR-181a	tongue cancer – tumour suppressor	↓	TWIST1	[168]
miR-210	apoptosis regulation, radioresistance	↑	HIF-1α	[169]
miR-218	tumour suppressor	↓	ROBO1, BIRC5, GJA1	[170]
miR-330	glioblastoma - oncomir	↑	SH3GL2	[171]
miR-330	colorectal cancer – tumour suppressor	↓	CDC42	[172]
miR-338	tumour suppressor, cell differentiation	↓	NRP1	[173]
miR-363	tumour suppressor, metastasis suppression	↓	S1PR1	[174]
miR-4284	glioblastoma - tumour suppressor	↓		[175]