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Small Molecules Targeting MicroRNA for Cancer Therapy: Promises and Obstacles

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Abstract

Aberrant expression of miRNAs is critically implicated in cancer initiation and progression. Therapeutic approaches focused on regulating miRNAs are therefore a promising approach for treating cancer. Antisense oligonucleotides, miRNA sponges, and CRISPR/Cas9 genome editing systems are being investigated as tools for regulating miRNAs. Despite the accruing insights in the use of these tools, delivery concerns have mitigated clinical application of such systems. In contrast, little attention has been given to the potential of small molecules to modulate miRNA expression for cancer therapy. In these years, many researches proved that small molecules targeting cancer-related miRNAs might have greater potential for cancer treatment. Small molecules targeting cancer related miRNAs showed significantly promising results in different cancer models. However, there are still several obstacles hindering the progress and clinical application in this area. This review discusses the development, mechanisms and application of small molecules for modulating oncogenic miRNAs (oncomiRs). Attention has also been given to screening technologies and perspectives aimed to facilitate clinical translation for small molecule-based miRNA therapeutics.

Graphical Abstract

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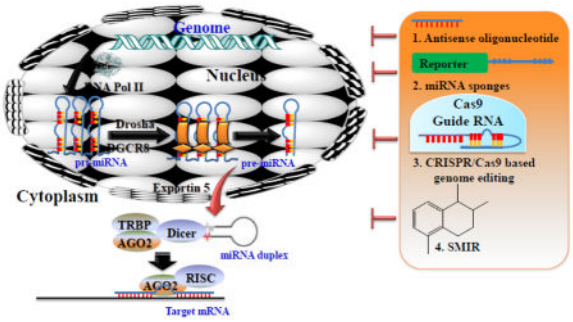

Strategies to modulate miRNA expression. i) antisense oligonucleotide (antagomir), ii) miRNA sponges, iii) CRISPR/Cas9-base genome editing, and iv) small molecule inhibitor of miRNA (SMIR)

Keywords

miRNA; small molecule miRNA inhibitors; oncomiR; cancer therapy

1. Introduction

MicroRNAs (miRNAs) are single stranded small non-coding RNAs (21–23 nucleotides) that have emerged as regulators of gene expression by hindering translation and triggering degradation of target mRNA post-transcriptionally.[1–3] miRNAs play a crucial role in the initiation and development of a variety of human cancers with numerous studies reporting aberrant miRNA expression as characteristic signatures.[4–6] miRNAs are not only deregulated in cancers, but are also acting as oncogenes or tumor suppressors. Oncogenic miRNAs (oncomiRs) function by either inhibiting tumor suppressor genes or genes responsible for promoting apoptosis or cell differentiation and are normally upregulated in cancer (Table 1). The corresponding antagomirs, also known as anti-miRNAs, are used to suppress these oncomiRs to inhibit tumor growth. In contrast, tumor suppressor miRNAs are downregulated in cancers.[5,7–9] These miRNAs function by inhibiting oncogenes or genes that hinder apoptosis or cell differentiation. For instance, miR-143 and miR-145 are both downregulated in colon cancer while miR-99 is overexpressed in pancreatic cancer.[10] The widespread involvement of miRNAs across human cancers suggests their utility as new ideal therapeutic targets. RNA inhibition agents such as antisense oligonucleotides and miRNA sponges have been used to restore miRNA balance in cancer networks by inhibiting overexpressed oncomiRs. Nonetheless, intrinsic challenges associated with these systems hinder their clinical translation. Obstacles include potential off-target effects, compromised tissue specific delivery, poor cellular uptake and *in vivo* instability. Although numerous delivery systems were developed for animal work, most of them including nanoparticles and liposomes have been proved to be ineffective or toxic for clinical use. In this regard, small molecule modulators of miRNA function are potentially better therapeutic candidates since they can be more easily delivered and more stable to overcome serum degradation.

First small molecule inhibitor of miRNA was developed by Gumireddy *et al.* for inhibition of miR-21.[11] In this study, a luciferase reporter plasmid was constructed for screening and

diazobenzene was finally selected as a potent compound. Since then, numbers of miRNA inhibitors targeting oncomiRs have been identified using high throughput screening or *in silico* sequence-based design.[12] Several potent oncomiRs have been selected as potent targets for small molecule miRNA inhibitor development. Moreover, fresh approaches such as the construction of small molecule-miRNA networks for a variety of cancers are being examined as alternative ways to fast track the drug discovery process.[13] Meanwhile, the mechanisms about miRNA inhibition by small molecules are elucidated. These promising findings are sparking a renaissance in developing small molecule modulators of miRNAs for cancer therapy.

In this review, we discuss the rationale and therapeutic strategies for targeting miRNAs responsible for cancer initiation and progression. We describe the development, mechanisms and applications of small molecules for modulating cancer related miRNAs. We also discuss the potential and pitfalls of small molecule modulators of miRNAs for treating cancer with emphasis on their delivery technologies to facilitate their clinical translation into cancer therapeutics.

2. miRNAs as valid Drug Targets in Cancer Therapy

Drug target selection remains a bottleneck in the quest for potent anticancer therapeutics. The current paradigm where drugs are designed to target proteins is flawed for several reasons. Since cancer is a complex process involving multiple factors and multistep processes, the efficacy of anticancer agents designed to target single therapeutic protein is often sub-optimal in cancer therapy. Although combination therapy, in which more than one targets are addressed, yields better therapeutic outcomes compared to single drug treatment, it is typically costly, associated with detrimental drug-drug interactions and involves complicated treatment regimens. Considering their dysregulated expression in cancer compared to normal tissues, miRNAs are regarded as high value drug targets for cancer therapy and targeting their expression can change cancer phenotype.[39–41] miRNA-based therapeutics are attractive alternatives to protein based cancer therapy since single oncomiR always downregulates multiple anticancer genes while tumor suppressor miRNA downregulates multiple oncogenes. For example, miR-221 has an oncogenic function by targeting Bmf, a proapoptotic BH3-only protein, to inhibit cell apoptosis.[42] In addition, miR-221 enhances cell migration and make cancer cell more aggressive by targeting PTEN and TIMP3.[43] Let-7g suppresses tumor cell proliferation by targeting c-Myc[44] and COL1A2.[45] Meanwhile, Bcl-xL, an anti-apoptotic member of the Bcl-2 family, is identified as a target of let-7g to induce cell apoptosis.[46] Consequently, miRNAs are highly efficient regulators of cellular processes pertinent for normal and malignant homeostasis.[47–49]

One rationale for miRNA-based therapeutics elegantly described by Garzon *et al.*, is the notion of cancer networks being miRNA wired.[49] The “miRNA wired cancer network” hypothesis suggests miRNAs to be the code that maintains required connection between all genes and protein networks in normal cells. As a result, normal tissues can be thoroughly characterized and miRNA expression patterns established as a coding blueprint. It might then be possible to compare this blueprint to miRNA expression patterns in cancerous tissue. Therapeutic approaches can be developed to “reboot” the cancerous tissue by restoring the

miRNA patterns to the default settings observed in the normal tissue. Clearly, such a therapeutic strategy involves targeting more than just a single miRNA, gene or protein. It may involve simultaneous inhibition and replacement of more than one miRNAs. From an implementation standpoint, it might be tempting to dispose the "miRNA wired cancer network" hypothesis due to its potential complexity. Another instructive argument might be that there is no universal miRNA blueprint for normal tissues. Nonetheless, since aberrant expression of single miRNA may affect hundreds of proteins,[50] reprogramming cancer network may be more feasible using miRNAs compared to proteins.

3. miRNA-based Therapeutic Strategies

For many cancers, oncogenic miRNAs are overexpressed while tumor suppressor miRNAs are downregulated. Therefore, two miRNA-based therapeutic strategies used are: (1) miRNA inhibition for addressing oncogenic miRNAs and (2) miRNA replenishment for tumor suppressor miRNAs. Similar therapeutic molecules such as oligonucleotides and small molecules may be employed in both approaches to either directly inhibit miRNAs or indirectly by targeting specific genes or transcription factors which modulate specific miRNA expression.

3.1. miRNA Inhibition

In contrast to the diminished levels of tumor suppressive miRNAs, a number of oncomiRs are overexpressed in cancer cells and directly target tumor suppressor genes. The current strategies (Fig. 1) to ablate oncomiRs include: (1) antisense oligonucleotide (ASOs, also known as antagomirs or antimiRs), which includes cholesterol-conjugated antimiRs, locked nucleic acid (LNA) antimiRs and tiny LNA antimiRs; (2) miRNA sponges which contain multiple tandem binding sites to target miRNA; (3) CRISPR/Cas9 based genome editing which modify the genome of cancer cells; and (4) small molecule inhibitors of miRNAs. ASOs are single stranded RNA molecules complementary to the target miRNAs that function as competitive inhibitors by obstructing their interaction with target miRNAs; LNA antimiRs, in which an extra methylene bridge connecting the 2′-O atom and the 4′-C atom "locks" the ribose, exhibit higher thermal stability and superior hybridization efficiency with their miRNA targets; tiny LNA antimiRs are 8nt long LNA-modified ASOs designed to target the 5′-seed region of miRNAs; miRNA sponges use transgenic overexpression of RNA molecules harboring complementary binding sites to a miRNA of interest to block the function of a given miRNA or a miRNA family; CRISPR/Cas9 is a novel technique for editing genomes by delivering Cas9 protein and guide RNAs into target cells; SMIRs are small molecule chemical compounds that interfere with miRNA biogenesis or maturation.

3.1.1 Antisense Oligonucleotides—The demonstration that oncomiRs are upregulated in cancer (Table 1) provides a rationale to investigate the use of antisense oligonucleotides to inhibit their expression. Antisense oligonucleotides work as competitive inhibitors of miRNAs, presumably by annealing to the mature miRNA guide strand and inducing degradation or stoichiometric duplex formation. However, the unmodified antisense oligonucleotides are degraded before reaching their targets. Thus, researchers introduced modifications to the chemical structure of oligonucleotides to increase stability, binding

affinity and specificity. Among these modifications, the introduction of 2′-O-methyl groups contributes to nuclease resistance and improved binding affinities to RNA.[51] Oligonucleotides with 2′-O-methyl groups have been proven to be effective inhibitors of miRNA expression in several cancer cell lines. As a proof of concept, Krutzfeldt *et al.* developed chemically modified (2-OMe-modified nucleotides, with a phosphorothioate linkage), cholesterol-conjugated single stranded RNA analogues (named 'antagomirs'), complementary to miR-122, which is abundant in the liver.[52] These antagomirs were injected into the tail vein of mice, and specific targeting of miR-122 in the liver was observed after 24 h. The silencing of endogenous miRNAs by this method was specific, efficient and long lasting, and the effects were observed even after 23 days post-injection. Gene expression and bioinformatics analysis of the whole transcriptome from antagomir-treated animals revealed that the 3′-UTRs of upregulated transcripts were strongly enriched in miR-122 recognition motifs, whereas downregulated genes were depleted of these motifs. To improve the binding affinity, LNA nucleotides were further developed. By "locking" the molecule with the methylene bridge, LNA oligonucleotides displayed unprecedented hybridization affinity toward complementary single stranded RNA and complementary single- or double stranded DNA.[53,54] In addition, they displayed excellent mismatch discrimination and high aqueous solubility. LNA antimiRs have been used successfully in several *in vitro* studies to knock down specific miRNA expression.[55,56] Studies in mice using LNA antimiR have shown the feasibility and high efficiency of this approach.[57,58] Elmen and colleagues examined whether combining LNA antimiR with phosphorothioate modifications could improve delivery of the compounds and silence miR-122 in mice without requiring additional chemical modifications.[59] This research suggests that LNA antimiRs are able to effectively silence their targets at much lower doses than cholesterol-based oligonucleotides. The simple systemic delivery of an unconjugated LNA antimiR-122 has also been shown to effectively antagonize liver-expressed miR-122 in non-human primates.[60]

3.1.2. miRNA Sponges—miRNA sponges are competitive inhibitors expressed from strong promoters, containing multiple, tandem binding sites to a miRNA of interest or miRNA family.61 The binding sites in these miRNA sponges are either perfectly antisense or contain mismatches in the middle position 9–12, which probably induce more stable interaction with miRNA, including miRNA complexed with Ago2. Normal miRNA sponge constructs contain four to ten binding sites separated by a few nucleotides as more binding sites increased the possibility of sponge RNA degradation.[62] In recent years, miRNA sponges have been proved effective in several cell lines, including non-small long cancer cell[63], embryonic neural stem cell[64] and B cell lymphoma.[65] To achieve stable miRNA sponge activity, several groups express the transgene from chromosomal integration and thereby perform long-term miRNA loss of function studies.[66–68] The applications of miRNA sponges are to elucidate the role of miRNA in differentiation pathways[69,70] and to mimic the downregulation of specific miRNA in certain diseases.[71,72] For *in vivo* studies, viral vectors are used to deliver sponge constructs to tissue in mice[73,74] while stable germline miRNA sponge expression was achieved in Drosophila using Gal4-UAS system.[75]

3.1.3. CRISPR/Cas9-based Genome Editing—Ishino *et al.* first discovered clustered regularly interspaced short palindromic repeats (CRISPR) in *Escherichia coli* more than 30 years ago.[76] CRISPR is characterized as cell immune system that confers resistance to foreign genetic elements such as plasmids by cleaving target DNA sequence via a short RNA molecule and the endonuclease Cas9.[77] Nowadays, CRISPR has been proven to be an efficient genome editing method, which includes a Cas9 protein and CRISPR RNA complex. CRISPR RNA guides the complex to a complementary sequence in the target DNA and destroys it to perform gene silencing. Several researches have been published about utilizing CRISPR/Cas9 system to perform genome editing in cultured mammalian cells.[78–80] A GFP-tagged CRISPR/Cas9 imaging system was also developed to monitor telomeres and coding genes in living cells.[81] As a novel gene silencing technique, researchers are concentrating to explore the application of CRISPR/Cas9 system for inhibiting miRNAs. For instance, *Zhao et al.* reported construction of sequence specific CRISPR/Cas9 based miRNA inhibitor to downregulate miR-17-92 cluster and miR-21, two typical oncomiRs, *in vitro*.[82] Xiao et al. co-injected Cas9 mRNA and guide RNA into one cell-stage zebrafish embryos and obtained chromosomal deletions and inversions.[83]

3.2. miRNA Replenishment

miRNA replenishment therapy involves restoring of downregulated tumor suppressor miRNAs in cancer cells. Loss of tumor suppressor miRNAs causes hyperactivation of multiple cancer pathways which facilitates cancer cell proliferation, inhibits apoptosis and promotes tumor-forming ability of cancer stem cells. Introducing miRNA mimics reestablishes tumor suppressor status in cancer cells thereby hindering tumorigenesis. miRNA mimics only need to be delivered to the cytoplasm to be active and available technologies for siRNA delivery are sufficient. Compared to miRNA inhibition therapy, miRNA replacement therapy is only now being explored and may at first appear counter intuitive. However, several advantages validate its application since miRNAs are generally downregulated in cancer[9,84] and miRNA mimics do not generate off-target effect once delivered.[85] For example, Sun *et al.* reported the role of miR-1280 in suppressing melanoma by regulating Src, which acts as proto-oncogenes by mediating tumor proliferation and invasion.[86] Intratumoral injection of miR-1280 complexed with the siPORT transfection reagent significantly suppressed melanoma progression *in vivo*. Tiwari et al. proved that oral squamous cell carcinoma cells with overexpressed miR-125a showed reduced proliferation and invasion since estrogen-related receptor α was significantly downregulated.[87] For combination therapy using an anti-tumor drug and a tumor suppressor miRNA, Mittal *et al.* reported that combining gemcitabine and miR-205 significantly inhibited tumor growth in a subcutaneous pancreatic cancer model.[88] Kumar *et al.* demonstrated that co-delivery of GDC-0449 and let-7b effectively decreased tumor cell proliferation with increased cell apoptosis by inhibiting hedgehog pathway.[89] Recently, several small molecule modulators of tumor suppressor miRNAs were reported to restore the levels of tumor suppressor miRNAs and inhibited tumor growth in animal models.

3.3. Challenges of Non-Small Molecule miRNA Therapeutics

Non-small molecule miRNA have the potential to be an efficient method for miRNA inhibition. Babar *et al.* demonstrated that systemic delivery of anti-miR-155 formulated in

nanoparticles resulted in rapid regression of lymphadenopathy.[90] Silencing of miR-21 through miRNA inhibitor markedly antagonized B-cell lymphoma tumor growth in mouse model.[91] As discussed before, LNA-modified miRNA inhibitor showed even higher efficiency of miRNA inhibition in mouse and non-human primates.[60] However, there are still several crucial obstacles that need to be overcome. Currently, 2588 mature human miRNA sequences are registered in miRBase, while around 200 of them have sufficiently high expression for targeting. As discussed before, most of antisense oligonucleotides are perfect complementary to their targets with chemical modifications to improve binding affinity and stability. However, these miRNA inhibitors may not distinguish between miRNAs within the same family, causing off-target effects.[92] Although the off-target effect of miRNA sponges are not reported yet, miRNA sponges always exhibit different degrees of inhibition in different contexts and it is still challenging to evaluate the degree of miRNA silencing under a sponge treatment.[62] Furthermore, chemical modifications have been observed to induce sequence-independent toxicity *in vivo*.[93] Although previously described phosphorothioate-modified antisense oligonucleotide showed significant miRNA silencing effect in non-human primate, it was shown to activate C5 complement and induce a transient decrease in peripheral white blood cell counts.[94] For LNA-modified oligonucleotides, Swayze *et al.* reported that they had significant hepatotoxicity as measured by serum transaminase activity as well as body weight during preclinical animal tests.[95] Compared to antisense oligonucleotides and miRNA sponges, CRISPR-Cas9 causes permanent genome alterations. However, its off-target effect has not been well-recognized and accurately profiled when applied in gene therapy, which significantly limits its clinical application. Moreover, this genome editing method still needs to be optimized since commonly used Cas9 gene derived from Streptococcus pyogenes is too big to be transduced, leading to less than 20% genome editing efficiency in vitro.[96]

Another issue which hinders the clinical development of non-small molecular miRNA inhibitors is the delivery-related concerns. Depending on the diseases and targets, people need to carefully consider and design the delivery systems to achieve optimized clinical effects. Organs, which are more accessible and responsible for metabolism and excretion including liver, kidney, and spleen, have shown exciting results for antisense oligonucleotide delivery. For example, Hatakeyama *et al*. encapsulated anti-miRNA oligonucleotides into pH-sensitive liposome and reduced the level of miR-122 in mouse liver.[97] Kriegel *et al.* demonstrated that intravenously delivered LNA modified anti-miR382 blocked miR-382 expression and significantly reduced kidney medullary fibrosis.[98] For hard-to-reach tissues such as solid tumors, people developed target delivery systems (less than 100 nm) including a target ligand, hydrophilic membrane, and positive charged and hydrophobic core to overcome off-target effects on normal tissues. Nevertheless, it is still difficult to ensure an effective dose reaching and entering the appropriate target cells.[99] For example, tumor vessels exhibit high permeability, high hydraulic conductivity, and high interstitial pressure that slow down the diffusion and convection of nanoparticles within the tissue.[100] Gilleron *et al.* recently reported that only 1–2% of siRNAs escaped from endosome degradation after delivering with lipid nanoparticles using an imaging-based fluorescence and electron microscopy,[101] suggesting the delivery system facilitating the release of oligonucleotides may considerably decrease the effective dose in vivo.

Theoretically, liposome or nanoparticle based non-viral delivery system can be used to deliver miRNA sponges or CRISPR/Cas9 based genome editing systems. However, these systems generally suffer from low gene delivery efficiency, especially for *in vivo* studies. Interestingly, the delivery of recombinant Cas9 protein instead of Cas9 gene can achieve a genome editing efficiency as high as 79%.[102] Currently, most miRNA sponges or CRISPR/Cas9 are using viral vectors for *in vitro* and *in vivo* gene delivery due to the high delivery efficacy, which probably explained why much of current efforts have been addressed on modifying viral vectors for safe and effective clinical applications.[103]

4. Small Molecule miRNA Therapeutic Agents

Due to the above challenges of non-small molecule miRNA inhibitors, it would be promising to develop small molecular weight drugs to target specific miRNAs and inhibit their activities (named SMIR). Actually, miRNAs have long been neglected as promising drug targets due to their structural flexibility and highly electronegative surface.[104] Furthermore, poor understanding of miRNA X-Ray crystallography or NMR structure as well as the limited availability of miRNA-Dicer or RISC complex structure makes the design of small molecule inhibitor of miRNA much more difficult.[105] These might be the reasons why the first reported SMIR by Gumireddy et al.[11] and most following designs were based on non-specific selection assay. For the first SMIR, they selected miR-21 as the target miRNA, which is overexpressed in various cancers including breast, ovarian, and lung cancer.[106,107] Lentiviral vector encoding complementary sequences of miR-21 and downstream luciferase reporter gene was constructed for HeLa cell transduction and non-specific compound selection. As a result, diazobenzene was selected for further modification since 251% increase of luciferase signal was detected relative to untreated cells. Currently, with the advancement of RNA (or RNA and protein complex) structure simulation software, high throughput virtual screening are performed to select SMIR according to RNA secondary and tertiary structures although vector based non-specific screening is still playing a crucial role for SMIR selection. On the other hand, several studies were carried out to elucidate the inhibitory mechanism of SMIRs. In the following sub-sections, we have first reviewed the current SMIR screening methods (Fig. 2), followed by discussion of their inhibitory mechanisms.

4.1 Luciferase (or GFP)-based Screening

Luciferase-based vectors, which contain a complementary sequence or control sequence of target miRNA linked with downstream luciferase reporter gene, are widely used for SMIR screening. After cloning into lentiviral vectors, they are transduced into culture cells in which target miRNA is highly expressed. These gene modified cells are thereby able to determine the efficacy of potent SMIRs. With the presence of effective SMIRs, less target miRNA is available for binding the complementary sequence and luciferase gene is expressed as a result. Thus, the more effective SMIR, the more luciferase signals will be detected. Young *et al.*[12] improved their previous screening methods[11] using psiCHECK-2 vector, which co-expressed both Renilla and firefly luciferase to normalize the signal. The potent inhibitors they found for miR-122 induced 773±38% and 1251±125% increase in the relative luciferase signal. Connelly *et al.*[108] used similar psiCHECK-miR-122 vector for the

same miRNA to screen SMIRs using Huh7 cell line and found benzothiazole was a potent SMIR for miR-122. Bose *et al.*[109] used a modified psiCHECK-2-prohibitin vector, where prohibitin was a miR-27a inhibitor, to select SMIR for miR-27a in MCF-7 cell line. They found luciferase signals were significantly increased under the treatment of amikacin, streptomycin, tobramycin, and neomycin at the concentration of 20 μM. Bose *et al.*[110] used another pEZX-MT01 plasmid, which co-express luciferase and PDCD4, a known target of miR-21, to screen SMIRs for miR-21 in MCF-7 cell line. Streptomycin was identified as the most potent target and was characterized as a direct miR-21 inhibitor docking with pre-miR-21 at a region close to the terminal loop. Tan *et al.* also used luciferase reporter system to prove and evaluate a specific SMIR for miR-1.[111] Luciferase reporter system resulting in an increased luciferase signal in the presence of an effective SMIR excludes false-positive caused by compound toxicity, which may occur in an assay based on a decreased signal. These studies need to be carried out using cell line, which is probably modified by lentiviral vectors containing reporter assay plasmid according to previous studies. Similarly, this luciferase reporter-based screening method can be used to select specific compounds promoting tumor suppressor miRNA activity. Xiao *et al.*[112] constructed miR-34a reporter vector using Huh7 cell line and found Rubone, which effectively inhibited luciferase activity, was a potent miR-34a promoter. Young *et al.*[12] reported an effective miR-122 modulator selected by luciferase assay and further demonstrated by RT-PCR. These miRNA promoting compounds were named small molecule modulator of miRNA (SMMR). Compared to SMIR selected by luciferase reporter system, small molecule modulator of miRNA needs further evaluation to exclude false-positive phenomenon caused by toxicity since this compound decrease luciferase activity.

Recently, another similar GFP-based screening was also developed for SMIR screening. To screen a general miRNA inhibitor, a cell line stably expressing lenti-GFP and lenti-shGFP was developed. A compound was considered potent SMIR if green fluorescence was increased. To screen a SMIR for specific miRNA, EGFP reporter gene expression was under the control of specific miRNA through its complementary sequence present at the 3′ UTR. Using this GFP based screening assay, Shum *et al.*[113] obtained 6 potent miR-21 inhibitors and 6-hydroxy-DL-DOPA was characterized as the most potent SMIR. This GFP-based screening is another method for screening SMMR as reported by Shan *et al.*[114] In their study, enoxacin, which decreased EGFP expression and enhanced GAPDH gene silencing, was selected as a small molecule modulator of miRNA. Melo *et al.*[115] further reported the anticancer activity of enoxacin, which acted by enhancing TAR RNA-binding protein 2-mediated miRNA processing.

4.2. Molecular Beacon-based Screening

Fluorescent beacons are usually hairpin shaped oligonucleotides which contain a 5′-fluorophore and a 3′-quencher, along with a miRNA targeting sequence (anti-miRNA sequence) in the loop. Davies *et al.*[116] first described the design of a fluorescent beacon and forecasted its potent application for screening SMIRs. In a hairpin shape, the base pair would bring the fluorophore and quencher closely, leading to quenching of the fluorescence. Thus, a Dicer-dependent increase in the fluorescence would be detected since mature miRNA is generated from Dicer-mediated hydrolysis, resulting in a dissociation of the

fluorophore and quencher, and an increase of fluorescence. In the presence of a Dicer inhibitor or ligand of pre-miRNA, Dicer activity would be inhibited and the beam showed a lack of fluorescence increase. Vo *et al.*[117] used this fluorescent beacon system to select Dicer inhibitor to inhibit biogenesis of oncogenic miR-372 and miR-373. In their study, Neomycin appeared to be the best aminoglycoside for Dicer inhibition and thereby be used for further modification. Bose *et al.*[109] modified this fluorescent beacon system since it was reported that the cleavage of miRNA by human Dicer depended on both 5′ and 3′-ends.[118,119] Therefore, the addition of quencher and fluorophore might affect the activity of Dicer and obscure the results. They overcame these drawbacks by using a DNA molecular beacon with a 5′ fluorophore and a 3′ quencher. This newly designed beacon was independent of pre-miRNA and the loop was complementary to mature target miRNA sequence after cleavage by Dicer. This beacon was further used for SMIR of miR-27a and neomycin was found the most potent compound, which was also demonstrated by luciferase-based vector screening. Recently, there were several other screening methods, such as fluorescence polarization screening assay reported by Tan *et al.*[120] and catalytic enzyme-linked click chemistry assay reported by Lorenz *et al.*[121] Molecular beacon-based screening does not need to perform under cell line and it is not widely used as luciferase-based screening method. Since SMIR causes a decrease of fluorescence in this molecular beacon system, further experiments need to be carried out to exclude false-positive caused by interfering with the fluorophore or quencher.

4.3. Structure-based Design

One difficulty that encountered in the drug development process is the high cost in the process of drug screening. With more accurate understanding of miRNA (or miRNA protein complex) structure and the simulation of binding affinity of SMIR to miRNA, *in silico* high-throughput screening is a promising technique to speed up the discovery of SMIRs and decrease the cost during the process. This computational approach is still challenging and needs further demonstration and recalibration to ensure the efficacy of screened compounds due to the flexible and complicated RNA tertiary structure. Shi *et al.*[122] reported AC1MMYR2 as an inhibitor of Dicer-mediated biogenesis of miR-21 using MC-Fold/MC-Sym pipeline for RNA secondary and tertiary structure prediction. In their studies, AC1MMYR2 was demonstrated a specific miR-21 inhibitor, which repressed pri-miR-21 expression by approximately 50% after 6 h and inhibited tumor growth in an orthotopic tumor model. Velagapudi *et al.*[123] reported a new method called Inforna for sequence-based design of SMIR to target pre-miRs. Inforna integrated a selection-based strategy (Two-Dimensional Combinatorial Screening; 2DCS),[124] a statistical approach (Structure-Activity Relationship through Sequencing; StARTS),[125,126] and the structural information about the RNA target of interest that identified RNA motifs that positively and negatively contributed to binding. After screening and optimization, they selected three compounds for miR-96 precursor, miR-210 precursor, and miR-182 precursor, respectively. The secondary structure was proved by enzymatic mapping assays and the downstream effect of miR-96 inhibitor was evaluated. Compared to traditional medicinal chemical approaches, Inforna provided a reliable prediction of SMIRs that was able to target specific miRNA.

4.4. Peptide or Peptoid Screening

Another category of SMIRs is peptides or peptoids, which are well reported for selective RNA binding. Herein, we introduce two peptide selection methods, peptoid microarrays and phage display selection. Chirayil *et al.*[127] performed peptoid microarrays to identify specific ligands for RNA hairpin precursor of miR-21. In their studies, they used peptoid microarrays as the foundation for a system of RNA ligand discovery to screen a library of 7680 N-substituted oligoglycines. Among them, two compounds were shown to have specific binding affinity to the secondary structure of miR-21 precursor hairpin. After identifying the functional groups contributing to the affinity and specificity, a compound with dissociation constant of 1.9 μM for miR-21 precursor hairpin was created. However, this compound did not show detectable binding to the targeted hairpin loop in the presence of Mg^{2+} which is required for microprocessor activity. Thus, they further modified their screening strategy and found higher affinity compounds inhibiting microprocessor activity *in vitro*.[128] The newly selected peptoid shows weaker binding affinity but has better selectivity of pri-miR-21 over pri-miR-16. A fusion phage is a filamentous virion displaying on its surface a foreign peptide fused to a coat protein.[129] In one study, the library of this fusion phage may represent up to billions of peptides.[130] If a phage displays a peptide which is a strong ligand of target miRNA, it can be eluted and the peptide sequences responsible for the binding are easily obtained by infecting the specific phage into bacteria and sequencing the relevant part of their viral DNAs.[131] Using this method, Bose *et al.*[132] reported that 'ALWPPNLHAWVP' was a potent peptide sequence for binding miR-21. After identifying the binding pocket of this peptide using a PEP-FOLD web server, they further demonstrated that this peptide suppressed tumor cell proliferation, invasion and migration by antiagonizing miR-21.

4.5. Mechanism of Small Molecule miRNA Therapeutic Agents

Deiters proposed three basic processing stages that present potent target for the activation or deactivation of miRNA function by small molecules: (1) the pre-transcriptional stage, (2) the transcription stage, and (3) the post-transcriptional stage.[133] For pre-transcriptional stage, the biogenesis of miRNA is affected by miRNA gene copy number, mutations in miRNA gene, or histone deacetylation and hypermethylation of miRNA promoters.[134,135] Scott *et al.* reported that 22 miRNAs were downregulated after treatment with LAQ824, a histone deacetylases inhibitor.[136] In transcriptional stage, the transcription factors regulating the expression of miRNAs are potent target for SMIR. Recently, novel c-Myc inhibitors, JQ1, were developed for the treatment of hematopoietic malignancies[137,138] and lymphoma.[139] Directly interacting with Drosha gene promoter, c-Myc activates the transcription of Drosha, which promotes the biogenesis of miRNAs.[140]

Compared to the previous two stages, much more SMIRs were developed targeting post-transcriptional process because SMIR targeting previous two stages mostly inhibited the biogenesis of several miRNAs, significantly decreasing the efficacy and specificity for oncomiRs. Bose *et al.* discovered that streptomycin can efficiently repress miR-21 by binding to its precursor and interfering with its downstream process by Dicer.[110] Velagapudi *et al.* used sequence-based design and found a SMIR specific for miR-96 by binding Dicer and Drosha.[123] Murata *et al.* reported that Xanthone derivatives could inhibit miR-29a by

targeting its secondary structure and suppressing Dicer binding.[141] Shi *et al.* reported that AC1MMYR2 probably blocked the Dicer binding site on pre-miR-21 to prevent the cleavage of pre-miR-21 to the mature miRNA.[122] The peptide miR-21 inhibitor developed by Bose *et al.* has a binding pocket for miR-21 and thus inhibit Dicer processing for miRNA maturation.[132] Nevertheless, there are still general SMIR that inhibits several miRNAs in post-transcriptional process. Watashi *et al.*[142] screened 530 compounds and discovered poly-L-lysine hydrobromide as a Dicer inhibitor and 3,6-diamino-10-methylacridinium chloride as an AGO2 inhibitor. Lünse *et al.* discovered an aptamer targeting the apical-loop domain of pri-miRNAs and modulating the maturation processing of miR-17, miR-18a, and miR-19.[143] Tan *et al.* discovered aurintricarboxylic acid as a RISC loading inhibitor after searching thousands of compounds using a novel method based on fluorescence polarization.[120] Enoxacin was demonstrated by Melo *et al.* to function by enhancing TAR RNA-binding protein 2-mediated miRNA processing, which was the only available mechanism study for SMMR.[115]

5. Promises and Challenges of Small Molecule miRNA Therapeutic Agents for treating Cancer

5.1. Therapeutic Potential of SMIR and Small Molecular Modulators of miRNA (SMMRs)

miRNAs are considered crucial factors in spectrums of human disease, especially cancer. In the past decade, various miRNAs have been reported to be associated with cancer development process. Drug discovery and development are always a time-consuming and expensive process, which significantly influences the therapeutic progress of cancer and other diseases, leading to the urgent need for new therapeutic alternatives. SMIRs and Small Molecular Modulators of miRNA (SMMRs) show another promising approach for the treatment of cancer due to its less time-consuming characteristic for drug development with reduced cost in the whole process. In addition, their exciting results as previously discussed further proved them to be an efficient tool for therapeutic use. JQ1, c-Myc inhibitor, significantly promoted differentiation, tumor regression, and improved survival in murine xenograft models of NUT midline cancinoma.[137] AC1MMYR2 inhibited tumorigenesis and invasiveness in an orthotopic U87 glioma intracranial model.[122] PLL, the Dicer inhibitor, and TPF, the AGO2 inhibitor, treatments suppressed tumorigenic activity of miR-93 over-expressed NIH3T3 cell lines when subcutaneously implanted into nude mice for tumor formation.[142] The replenishment of miR-34a reported by Xiao *et al.* significantly inhibited the growth of hepatocellular carcinoma in xenograft mouse model.[112]

To yields better therapeutic outcomes compared to single drug treatment, combination therapy can be performed to address more than one pivotal target. Qian *et al.* reported the synergistic inhibition of human glioma cell lines with the combination of temozolomide and antisense oligonucleotide miR-21 inhibitor.[144] Xu *et al.* reported that inhibition of miR-21 enhanced chemotherapeutic effect of cisplatin in non-small-cell lung cancer.[145] Theoretically, we can use previously discussed SMIRs to target miR-21 for combination therapy with another small molecule drug. Recently, Yu *et al.* developed mesoporous silica nanoparticles for combination therapy by co-delivering SMIR and antagomir against miR-122 in hepatocellular carcinoma cells.[146] In this research, they selected a previous

published SMIR against miR-122 selected by Young *et al.*[12] and miR-122 antagomir delivered by RGD-conjugated mesoporous silica nanoparticles. In vitro analysis showed that this SMIR and miR-122 antagomir significantly downregulated miR-122 expression in Huh 7 cells. To our knowledge, this is the only available combination therapy by using SMIR and antagomir for targeting cancer related miRNA. Compared to antisense oligonucleotide-based miRNA inhibitor, SMIR is more easily for systemic delivery using current drug delivery systems, including liposomes, micelles, and nanoparticles.

5.2. Pitfalls of Small Molecule miRNA Therapeutic Agents for treating Cancer

The challenges for development and application of SMIR and SMMR are searching for more potent compounds and the delivery issue. Based on previous researches, people are still far away from being able to efficiently design potent SMIRs and SMMRs with clear understanding of their inhibition mechanisms. According to recently developed SMIRs, we can conclude that what we did was only to discover the new application of previous drugs. Currently designed SMIRs were able to target only a small number of oncomiRs (Table 3). Furthermore, several crucial defects of the current screening strategies or structure-based design techniques cannot be ignored. For instance, molecular beacon-based screening needs further improvement to exclude the false positive which might be caused by the interaction with fluorophore and quencher. For structure based screening method, more powerful simulation software needs to be developed to accurately predict secondary and tertiary structure of target miRNA. In 2011, Paige reported RNA mimics of GFP and predicted its secondary structure using Mfold web-based software,[147] whereas the correct structure was discovered by Huang *et al.*[148] and Warner *et al.*[149] in 2014. Thus, more effective screening methods and accurate structural and thermodynamic simulation on the interaction between miRNA and SMIR or SMMR are in urgent need. Furthermore, drug interaction study needs to be performed if combination therapy is used to improve therapeutic efficiency. We also need to design efficient drug delivery systems for SMIR and SMMR delivery.

Since single miRNA may regulate several genes, the potent off-target effects are one of the major concerns for miRNA therapy. For example, miR-29 oligonucleotide mimics may act as anticancer drugs by targeting several oncogenic pathways including Mcl-1[150] and CDK6.[151] In contrast, miR-29 may promote tumor cell migration, invasion, and apoptotic resistance through direct targeting PTEN.[152] Meanwhile, it also regulates osteoblast differentiation[153] and immune inhibitory molecule expression.[154] Thus, an efficient target delivery system is always used to deliver oligonucleotide-based miRNA or anti-miRNA mimics. Except for tissue targeting related off-target effect, there is another kind of off-target effect of SMIR need to be solved since single SMIR may target multiple miRNAs. For example, streptomycin can target both miR-21 and miR-27a, whereas neomycin can target both miR-27a and miR-372/373 (Table 3). Thus, a miRNA profiling study might be crucial to evaluate the specificity of a certain SMIR.

6. Conclusions and Future Perspectives

OncomiRs are exciting targets for drug development and cancer treatment. In the past decade, SMIR has been proven a novel and effective method for inhibiting oncomiRs. Compared to non-small molecule miRNA therapeutics, SMIR and SMMR are more easily

systematically delivered. Furthermore, the development of non-small molecule miRNA therapeutic agents is always costly and the in vivo stability of these agents is another outstanding issue to be overcome. Thus, extensive work has been done to develop several promising methods for SMIR and SMMR discovery in recent years, including non-structure based screening and structure based design. There is significant improvement in the design of screening vectors and validating rational computational approaches. Moreover, peptide and peptoid based SMIRs were developed as another category for miRNA inhibition. However, we are still at the early stage of this area since outstanding challenges, including screening methods and simulation techniques, remain to be overcome. Overall, targeting miRNAs with SMIRs for cancer treatment constitutes a reasonable and evidence based strategy with strong potential and chance for success. The progress of screening techniques and computational stimulation may address bright future in this field.

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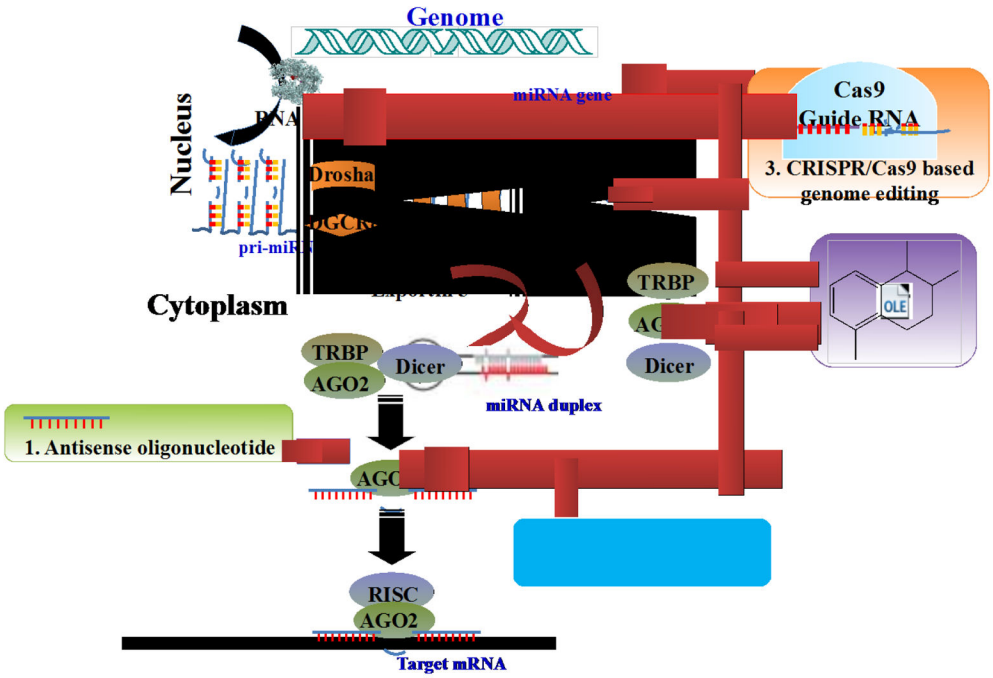

Figure 1.

Schemes of miRNA generation and the inhibition effect of antisense oligonucleotide, miRNA sponges, CRISPR/Cas 9 genome editing, and small molecule inhibitor of miRNA (SMIR). miRNA is transcribed by RNA polymerase II into primary transcripts pri-miRNA (~1 to 3 kb long). This pri-miRNA undergoes further processing by the ribonucleases Drosha and DiGeorge syndrome critical region gene 8 (DGCR8) complex in the nucleus, thereby resulting in a hairpin intermediate pre-miRNA (~70–100 nucleotides long) which then transported to the cytoplasm via exportin 5. In the cytoplasm, the pre-miRNA is processed by another ribonuclease, Dicer, into a mature double strand miRNA (~18–25 nucleotides long). After strand separation, the guide strand or mature miRNA is incorporated into an RNA-induced silencing complex (RISC) complex target the 3′-UTR region of mRNAs, resulting is a decreased level of targeted protein, while the passenger strand is commonly degraded. Antisense oligonucleotide and miRNA sponges work on mature miRNA while CRISPR/Cas 9 genome editing works on genome. SMIR works on almost every stage of miRNA biogenesis.

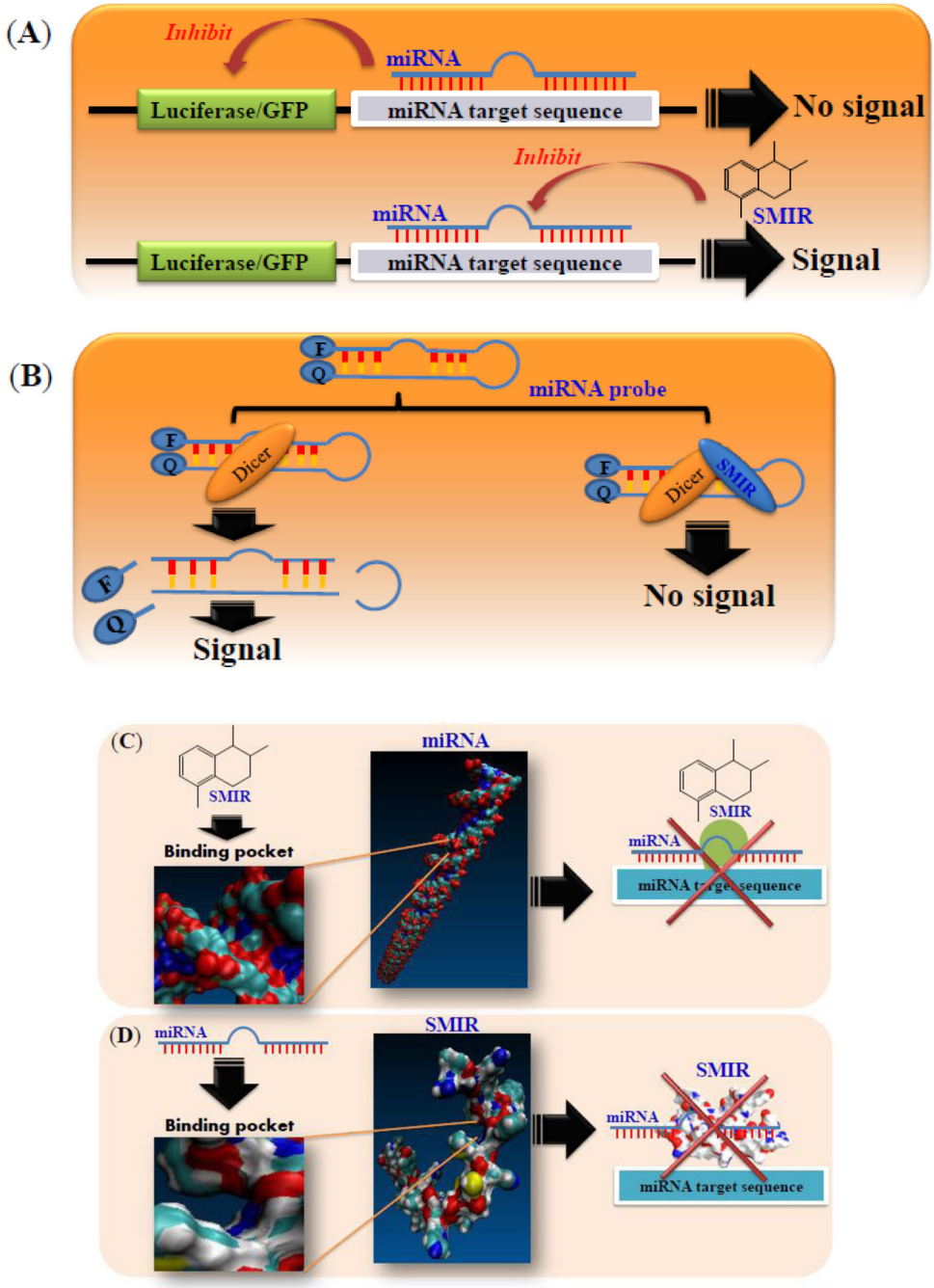

Figure 2.

Mechanisms of (A) luciferase/GFP based screening. Effective SMIR binds mature miRNA and further inhibit the binding of this miRNA to miRNA target sequence, leading to the expression of Luciferase/GFP. (B) Molecular beacon-based screening. Effective SMIR binds mature miRNA and further inhibit the activity of Dicer. The 5′-fluorophore (F) and 3′-quencher (Q) attach to each other and no signal can be detected. (C) structure-based design. Effective SMIR binds to the binding pocket of certain miRNA and further prevent this miRNA to bind miRNA target sequence. (D) Peptides or peptoids screening. Effective

SMIR (peptides or peptoids) binds specific miRNA and further prevent this miRNA to bind miRNA target sequence. SMIR, small molecule inhibitor of miRNA.

Table 1

Examples of oncomiRs for miRNA inhibition treatment

OncomiRs	Target genes	References
miR-21	PDCD4, PTEN, BCL2, TPM1, RECK,	14–18
miR-17-92 cluster	PTEN, Bim	19,20
miR-221/222	p27, TIMP2, DKK2	21–23
miR-155	DMTF1, annexin 7, LKB1, E2F2, GABA receptor	24–28
miR-223	PAX6, Stathmin1, FBXW7/hCdc4	29–31
miR-214	PTEN, p53	32,33
miR-191	C/EBPβ, checkpoint kinase 2	34,35
miR-25	CDKN1C, LATS2, RECK	36–38

Table 2

Different mechanisms of current SMIRs

Stage	SMIRs	Mechanism	Reference
Pre-transcription	LAQ824	Inhibiting histone deacetylases	136
Transcription	JQ1	Inhibiting c-Myc	137–139
Post-transcription	Streptomycin, xanthone derivatives, AC1MMYR2, poly-L-lysine hydrobromide, 3,6-diamino-10-methylacridinium chloride, aurintricarboxylic acid, Enoxacin	Binding pre-miRNA or pri-miRNA; binding Dicer, Drosha, or AGO2; inhibiting Dicer process or RISC loading, enhancing TAR RNA-binding protein 2-mediated miRNA processing	110,115,120,122,123,132,141–143

Table 3

Summary of current SMIRs for specific oncomiRs

OncomiR	SMIRs	References
miR-21	diazobenzene, streptomycin, 6-hydroxy-DL-DOPA, AC1MMYR2, peptoid and peptide as described in reference,	11,110,113,122,127,128,132
miR-122	Benzothiazole based compounds	108
miR-96, miR-210, miR-182	Compound 1, 2, 3 as described in reference	123
miR-27a	amikacin, streptomycin, tobramycin, and neomycin	109
miR-1	Compound 14 as described in the reference	111
miR-372/373	neomycin	117
miR-29a	Compound 5 as described in the reference	141
miR-17, miR-18a, and miR-19	Aptamer 7 as described in the reference	143