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Mini Review

Small Molecule Modulators of Micro RNA and Their Role in Cancer Therapy

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Submitted: 30 August 2016
Accepted: 24 November 2016
Published: 01 December 2016
ISSN: 2333-7117

Abstract

Micro-RNAs (miRNAs) are endogenous, evolutionarily conserved, non-coding, RNAs of about 20-25 nucleotides in length. They control cell fate via cell proliferation, differentiation, apoptosis and stress response. They regulate gene expression by primarily disrupting mRNA translation and stability, or by modulating the transcription of target messenger RNAs. The expression of miRNA has been shown to be de-regulated in various human diseases, thus making them novel diagnostic biomarkers and therapeutic targets. It is challenging to target miRNAs with small molecules; different groups have identified small molecule modulators of miRNA by targeting various pathways. In the present review, we discuss about the role of micro-RNAs in cancer, and strategies for modulating their expression, which can be used to achieve therapeutic outcomes.

INTRODUCTION

MiRNAs are short, non-coding RNAs that regulate gene expression at post-transcriptional level [1-3]. They originate via transcription of a miRNA gene by RNA polymerase II. Subsequently they undergo a dual processing step, where an initial nascent transcript folds over itself, forming a secondary hairpin structure, called long primary miRNA (pri-miRNA) [4]. This is then cleaved by the RNase endonuclease III, Drosha, along with a microprocessor part, DGCR8, forming a precursor sequence, called pre-miRNA, of about 70 nucleotides in length [5-7]. The pre-miRNA is transported to the cytoplasm via Exportin 5 and RanGTP [8,9]. A double stranded RNA-specific cytoplasmic nuclease, called Dicer causes a cleavage to form a 22 nucleotide-long double stranded RNA transcript (dsRNA), following which, the guide strand, involving the RNA-induced silencing complex (RISC) targets the 3′-untranslated region (UTR) of messenger RNAs [10]. The final result is decrease in target protein levels.

Imbalance in the levels of miRNA can destabilize the entire cellular machinery, as a single miRNA can regulate hundreds of mRNA at the same time. Many genes can be regulated simultaneously as their nucleotide pairing with miRNAs is imperfect [11]. In case of human cancer, these genes are responsible for various cancer associated pathways, such as tumor initiation, development, invasion and metastasis. Owing to their aberrant expression, modulation of miRNA levels is of therapeutic potential. miRNAs can be divided into two main groups based on their expression levels: tumor suppressive miRNAs (ts-miRNAs) and oncomiRs. Ts-miRNAs are down regulated and target tumors suppressor genes and oncomiRs have increased expression, targeting oncogenes (Figure 1).

In addition to their traditional roles, miRNAs also cause an increase in translation during various cellular processes. For example, human microRNA, miR-369-3 is involved in the association of argonaute (AGO) and fragile X mental retardation-related protein 1(FXR1), [proteins which are associated with micro-ribonucleoproteins (microRNPs)] with AREs (AU-rich elements) to activate translation. Additionally, microRNAs Let-7 and synthetic microRNA miRcxcr4 induce translation upregulation of target mRNA upon cell cycle arrest; however they repress translation in proliferating cells [12]. miR-15b represses WEE1 (a key mammalian cell cycle regulator) protein during G1 and S phase. Regulatory factors such as CPEB1 (cytoplasmic polyadenylation element-binding protein) also bind to mRNA 3′UTR during post transcriptional repression. Intriguingly, both factors lose their inhibitory activity at G2/M transition, when WEE1 expression is maximal and activate WEE1 translation in a synergistic manner [13]. Another miRNA, miR-276 is involved in the upregulation of transcription coactivator gene (brm), through a process that depends on secondary structure of brm, involving a stem-loop around the binding site of miR-276 [14]. miR-328 upregulates CEBPA (CCAAT-enhancer binding protein alpha) by releasing it from heterogenous ribonucleo protein mediated

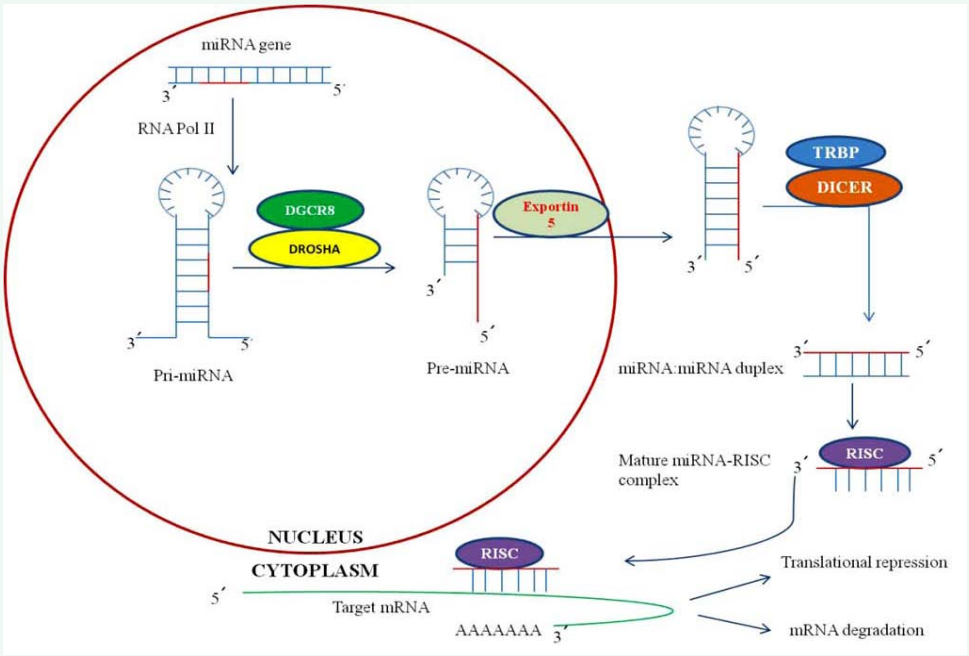

Figure 1 The miRNA processing pathway involves the production of pri-miRNA by RNA polymerase II. This is followed by cleavage by Drosha-DGCR8, to form pre-miRNA. Then the pre-miRNA is transported to cytoplasm by Exportin 5 and RanGTP and cleaved by Dicer to form a double stranded RNA transcript. The functional strand is loaded onto the RNA Induced Silencing Complex (RISC), thus guiding RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand is degraded.

translation inhibition [15]. miR-373 induces transcription of E-cadherin by targeting promoter sequences [16]. It has also been reported that nuclear entry of miRNAs are necessary for upregulation of genes in some cases [17]. miRNA was also found to function endogenously, activating Cyclin B1 (Ccnb1) expression in mouse cells, thus manipulating *in vivo* tumor development and growth. Three more miRNAs were found, miR-744, miR-1186 and miR-466d-3p that induce Ccnb1 expression in mouse cell lines [18].

MicroRNAs in cancer

Cancer is a complex disease involving changes in gene expression. In recent years there has been tremendous interest in investigating the role of miRNAs in cancer. miRNA expression profiling has been extensively studied and taken into cancer clinics, to be used as a diagnostic and prognostic biomarker, in order to monitor tumor initiation, progression and response to treatment [19]. The roles played by miRNAs have been reported in different types of cancers, such as breast, colon, gastric, lung, prostate and thyroid cancers [20-24]. The role of miRNA in cancer has been extensively studied, as evidenced by peer-reviewed scientific literature covering nearly 23,467 hits on Pubmed, as of November 2016.

miRNAs have either oncogenic or tumor suppressor function and their expression is globally suppressed in tumor cells, compared to normal tissues. miRNA dysregulation in cancer was first reported in CLL (chronic lymphocytic leukemia) in 2002. A cluster of two miRNAs, namely miR-15 and miR-16 were identified at 13q14.3 chromosomal region, which is frequently deleted. This deletion was partly responsible for higher expression of miR-15/16 anti-apoptotic target, B cell lymphoma 2 (BCL2). miRNAs can act as tumor suppressors, such as let-7 family of miRNAs that target critical oncogenes like RAS family members (HRAS, KRAS and NRAS) and MYC. The differential pattern of miRNA expression in cancer allows their use as diagnostic markers, which correlates with disease progression.

Another miRNA, miR-34 consists of a family of three miRs, namely miR-34a, miR-34b, and miR-34c, which have presumably tissue-specific functions, even though they are direct transcriptional targets of the onco-suppressor p53, whose expression is greatly affected by DNA damage and oncogenic stress. Thus miR-34 family contributes to arrest of cell proliferation and induction of apoptosis, by targeting c-MYC, CDK6 and c-MET [25]. Di Martino et al., have reported the molecular effects induced by enforced expression of miR-34a on multiple myeloma (MM) cells, showing time-dependent modulation of several signalling pathways, controlling cell proliferation and apoptosis. This is the first group to have reported the role of miR-34a in the pathogenesis of MM, through its downregulation in a wide series of MM samples [26,27]. The most affected pathway was the Erk/Akt dependent pathway [27]. The researchers showed that miR-34a induces sequential down modulation of both Erk and Akt activity, followed by pro-caspase-6 and -3 cleavage and apoptosis induction in MM cells. Previously, the same scientists have also shown the potential of miR-34a treatment in downregulation of Bcl-2 and NOTCH1, and in induction of apoptosis, both *in vitro* and in a xenograft mouse model [26]. Di Martino et al., tested the efficiency of stable nucleic acid lipid particles (SNALPs) in delivering miR-34a *in vivo* [27]. SNALPs have high serum stability and are an attractive option to translate miR based therapies in

the clinic. According to these findings, a liposome-carried form of miR-34a is under investigation in Phase I clinical trial in patients affected by several malignancies, including MM (clinicaltrials.gov: A multicenter phase I study of MRX34, miRNA miR-RX34 liposomal injection NCT01829971). Other recent studies explore the blocking of Bcl-2 by vaccinia virus-miR-34a, which increases release of cytochrome C from mitochondria and synergistically amplifies the antitumor effects of Smac-induced cell apoptosis. This is the first study to utilize oncolytic vaccinia virus as vector for miR-34a or Smac expression for treating MM, and lays groundwork for future clinical therapy [28]. Finally, miR-34a has also been associated with regulation of cancer stem cells function in various cancer types, including prostate cancer [29], pancreatic cancer [30], breast cancer [31], and glioblastoma [32].

miRNAs can also function as oncogenes. An oncogenic cluster of miRNAs, the polycistron, miR-17-92, comprising of a cluster of seven miRNAs: miR-17-5p, miR-17-3p, miR18a, miR-19a, miR-19b-1, miR-20, and miR-92-1, was found to be overexpressed in many lymphoma samples compared to normal tissues [33]. miR-21 was found to be strongly overexpressed in highly malignant glioblastoma tumor tissues [34]. A knockdown of miR-21 in cultured glioblastoma cells activated caspases and led to cell death via an apoptotic pathway. Further, a comprehensive analysis of miR-21 in solid tumors showed overexpression, that is common to six types of cancer that were studied, namely breast, colon, lung, pancreas, prostate, and stomach [35].

Other studies have shown the aberrant expression of miRNAs in brain tumors. The analysis of 245 miRNAs in glioblastoma multiforme showed that miR-221 was highly upregulated. Reports showed that miR-221 and miR-222 are highly expressed in glioblastoma and directly target $p27^{Kip1,}$ a negative regulator of the cell cycle [36]. miRNAs 221/222 are of interest as they are strongly upregulated in a variety of solid and hematologic malignancies. In case of Breast Cancer (BC) miR-221 mainly supports tumor growth and progression. In basal-like BC, miR-221/222 is highly expressed and promotes S-phase entry, cell migration and invasion through the inhibition of suppressor of cytokine signaling 1 (SOCS1) and cyclin-dependent kinase inhibitor 1B (CDKN1B) [37]. Upregulation of miR-221/222 is also relevant in triple negative BC, via cell cycle regulation and inhibition of apoptosis [38]. These data suggest that targeting miR-221 may be of value for the therapy of BC. Tanaka R et al. [39**],** have recently shown that Metformin, an anti-diabetic drug causes G1 phase arrest by downregulating miR-221, followed by rescue of p27 checkpoint and enhancement of TRAIL sensitivity through DR5 upregulation in pancreatic cancer cells. In case of transgenic mice, liver tumors ranging from typical adenomas to HCC (hepatocellular carcinomas) - like lesions, develop with selective upregulation of miR-221. In these tumors, targets of miR-221 such as cell cycle inhibitors, p27, p57 and pro-apoptotic proteins were found to be downregulated. When transgenic mice were intravenously treated with 2′-O-methyl modified oligonucleotides targeting miR-221, tumor growth was inhibited and no systemic toxicity was observed [40**]**. miR-221/222 also showed tumor suppressor activity in Gastrointestinal Stromal Tumor (GIST) cell lines, where miR-221/222 transfection promoted apoptosis through inhibition of KIT expression and activation of caspase 3 and 7 [41]. In MM cells highly expressing miR-221/222, enforced expression of miR-221/222 inhibitors triggered *in vitro* anti-proliferative effects, along with upregulation of miR-221/222 targets such as p27Kip1, PUMA, PTEN and p57Kip2. Contrarily, in MM with low basal miR-221/222, transfection of miR-221/222 mimics increased S phase and downregulated p27Kip1 protein expression. miR-221/222 inhibitors were also evaluated in MM Xenografts in SCID/NOD mice. Significant anti-tumor activity was observed along with upregulation of established protein targets in tumors retrieved from animals [42].

Modulation of microRNA expression

MicroRNAs regulate the stability and translation of messenger RNA (mRNA), consequently controlling protein synthesis and gene expression. They control these processes at post-transcriptional level by complementary binding to the sequences in the 3'-untranslated region of mRNA, and play a pivotal role in cell fate. Changes in miRNA expression are associated with several human diseases, including cancer, thus making them attractive therapeutic targets. However there are challenges to achieve therapeutic effects, using miRNA. Identification of molecules that regulate loss or gain of function of miRNA and their efficient delivery into the cell are two of the major challenges.

OncomiRs are known to have links to the pathogenesis and aggressiveness of cancer. Large-expression screens comparing tumor versus normal tissues are useful for identifying unique miRNA signatures and can be used as attractive anti-cancer therapeutic targets [43-45]. The approach to miRNA based therapeutics involves inhibition of potent cellular targets. For example, miR-21 targets PTEN and PDCD4 [46-49]. Likewise, miR-155 blocks the translation of CEBPβ, IL17RB, PCCD4, TCF12, ZNF652, which are tumor suppressor genes [35,50]. Additionally, oncogenic miRNA targets have been validated in similar patient populations. The major obstacle in this area is finding an effective delivery mechanism, such as nano-particles, liposomes and peptides, majority of which have proven to be ineffective or toxic [51,52].

miRNA and cancer therapy

For over a decade, miRNAs have been hallmarks of cancer. OncomiRS have been profiled and shown to be causative factors in the activation of oncogenic pathways leading to cancer. Hence it is important to develop strategies to efficiently inhibit their expression. Use of small molecule inhibitors (SMIRs) presents an effective strategy to target specific miRNAs. Other methods of miRNA inhibition are based on antisense oligonucletides (antimiRs), locked nucleic acids (LNA), LNA-antimiR constructs, antagomirs, miRNA sponges, ribozymes/DNAzymes, small interfering RNAs (siRNAs) and short hairpin RNAs (shRNA)**.** The discovery and usage of these strategies for cancer therapy were found to be exciting. However, there are challenges in delivery of these small-molecules, besides the pharmacodynamic and pharmacokinetic properties. Due to challenges in the delivery of these molecules, new approaches to target oncomiRs have to be discovered. The first miRNA molecule to reach clinical trials is Miravirsen, developed by Santaris Pharma A/S, a locked nucleic acid targeting miR-122. It is useful for the treatment of HCV (hepatitis C) infection, which increases the chance of patients

developing Hepatocellular carcinoma. Miravirsen completed a phase 2a study wherein a patient reached an undetectable level of HCV-RNA and robust activity was exhibited against viral load [53,54].

Since miRNAs are pivotal in cancer, they have been associated with every hallmark of cancer. Due to the challenges involved in using nucleotide analogs to target miRNAs, the development of small-molecule drugs targeting specific miRNAs was proposed to be a promising approach. The interaction of small molecules with miRNA was termed as "SMIR" by Melo and Calin et al. [55,56], commonly referred as small molecule inhibitors of specific miRNAs. This opened doors for a very specific, targeted cancer therapy. The SMIR-concept is attractive, as it takes shorter time in drug development and also reduces the time for approval and production, thus reducing the overall cost of drug discovery. However this approach is also challenging and risky. RNA transcripts, in the past, were not explored fully as drug targets, due to their electronegative charge and flexible structure. Additionally, X-Ray crystallography as well as Nuclear Magnetic Resonance structures for miRNAs were not well developed. The lower availability of miRNA-Dicer or RISC complex structures made drug discovery difficult. If these barriers are overcome, the SMIR approach will result in having effective drug delivery to patients.

More recently, there is advancement in the development of efficient LNAs, targeting various cancers. miR-221/222 antisense LNA oligonucleotides reduce tumor growth by increasing intra-tumor p27Kip1 protein expression in prostate carcinoma [57]. Other researchers showed the possibility to extend the results to human studies, prompting investigators to develop a novel 13-mer locked nucleic acid (LNA)-chemically modified miR-221 inhibitor, a fully phosphorothioate (PS)-modified backbone, the LNAi-miR-221 [58]. LNA modifications conferred prolonged effects and higher stability. This novel approach was also prompted by recent successful treatment of chronic hepatitis C patients with miR-122 LNA inhibitor [59]. Maria Eugenia Gallo Cantafio et al., demonstrated short half-life, optimal tissue biovailability and minimal urine excretion of LNA-i-miR-221 in mice and monkeys. LNA-i-miR-221 was still detectable in mice vital organs and in xenografted tumors, upto 3 weeks, along with p27 target upregulation. No toxicity in the pilot study with monkey model was observed [60]. In summary, these data suggest a prominent role of miR-221/222 in the development and progression of solid and hematological tumors. The recent findings on the use of selective inhibitors, as LNA-i-miR-221, both *in vitro* and *in vivo* are opening a new avenue for the design of miRNA-based therapies to be tested in clinical trials.

RNA as a target

RNA is an attractive target for drug development as it folds into three dimensional structures and is involved in various cellular processes [61,62]. Two main targeting strategies are (1) Oligonucleotides and (2) Small molecules. Due to ease of base-pairing, as per Watson-Crick rules, RNAs can be targeted by oligonucleotides. They are however high molecular weight compounds that cause difficulties in delivery into the cells. Modifications of oligonucleotides have helped traverse the cell membrane and reach the diseased tissues. Oligonucleotides can be synthesized to block translation, inhibit toxic RNA-protein interactions and avoid cryptic splice sites associated with disease [63]. Studies have shown that RNA can be used as a therapeutic target via small molecule interactions [64-66]. Small molecules have been designed, to target RNA repeats in genetic diseases [67-73] and miRNA precursors involved in cancer and other diseases [74-76].

Development of Oligonucleotides

Antisense oligonucleotides (ASOs) were first developed by Zamecnik et al., to target Rous sarcoma virus [77,78]. Complementary DNAs were used to bind to the virus and reduce the production of its RNA. Studies showed that DNA oligonucleotides form DNA-RNA hybrids in cells, which then recruit RNase H and result in cleavage of the RNA strand [79]. Modified oligonucleotides were made to improve stability in the presence of endonucleases, while retaining the RNase-dependent activity [80]. Site-specific modifications in the backbone and sugar moiety were created to increase the potential of these molecules to treat human diseases [81-83].

The most common accepted mechanism of action of ASOs, involves formation of mRNA-ASO duplex, through Watson-Crick pairing, leading to RNase-dependent cleavage of target mRNA [84-86]. Other mechanisms involve blockage of mRNA transport, modulation of splicing, translational arrest and formation of a triple helical structure through ASO binding to double stranded DNA resulting in inhibition of transcription. Chemical modifications of ASO drugs will increase *in vivo* half life, improve distribution to diseased tissue, increase potency and reduce toxic effects. Better understanding of importance of therapeutic target, dose optimization and scheduling, increasing trials with tumors that are sensitive to inhibition of relevant target and their use as combination therapy will increase the likelihood of success.

Small molecules to target RNA

Targeting RNA with small molecules is a challenging task, due to binding problems, non-specific interactions, and structural redundancy. A major advantage is that RNA folds into different structures and is composed of base pairs, and non-canonically paired regions, such as hairpins, loops and bulges. Base-paired regions are common to all RNAs, whereas the latter are unique to a single RNA or a smaller subset. A lead identification strategy, named Inforna [74,75] was proposed, that compares secondary structural elements in a target RNA to known RNA motif-small molecular interactions that are highly selective. Inforna has reported the design of small molecule leads that target RNAs involved in microsatellite disorders and miRNAs linked to cancer. Other methods in the design of small molecules to target RNA are structure-dependent. This has proven useful to generate molecules mimicking the binding of proteins to RNA. Another method is docking of small molecules into RNA, using NMR spectroscopy and molecular dynamics simulations [87,88].

CONCLUSION

There is an increased interest in RNA-drug discovery, owing to the roles played by RNA in healthy and diseased tissues. Precise medicines can be developed, that target RNA

and affect cellular function. Oligonucleotides as well as small molecules are of therapeutic potential. There is need, not only for making effective RNA inhibiting molecules, but to validate them. Rational computational methods have been validated, that enable functional studies on lead compounds, by predicting 3D structures of miRNAs. Undoubtedly miRNAs are being considered as potential molecules for targeting in cancer.

A more precise way to silence specific miRNAs, would be to find a compound that binds to the primary, precursor or mature sequence of a miRNA in a specific manner. For instance, miR-21 is overexpressed in breast, ovary, cervix, colon, lung, liver, brain, esophagus, prostate, pancreas and thyroid cancers. A SMIR that is specific to miR-21 is most likely to treat patients with these cancers via expression of OncomiR-21, as it negatively the tumor suppressive targets PTEN, PDCD4 and RECK. However we should also expect off-target effects, in both tissue of interest and throughout the body. Therefore newly discovered SMIRs have to undergo thorough *in vitro* and *in vivo* testing, before being released for patient use.

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Cite this article

Pamarthy D, Chowdhury D, Bhadra U, Bhadra MP (2016) Small Molecule Modulators of Micro RNA and Their Role in Cancer Therapy. JSM Biotechnol Bioeng 3(5): 1070.