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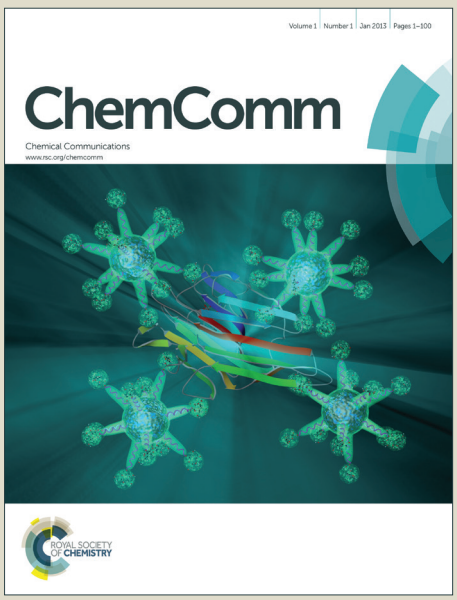

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ARTICLE TYPE

Nonconventional Chemical Inhibitors of microRNA: therapeutic scope

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MicroRNAs (miRNA) are a class of genomically encoded small RNA molecules (~ 22nts in length) which regulate gene expression post transcriptionally. The term microRNA or miRNA was coined in 2001 [1], and research in past decade has shed light on their widespread occurrence, evolutionary conservation and tissue specific functions. It is estimated that they modulate gene expression of approximately 60 % of the mammalian genes by regulating levels of target mRNAs to which they can bind on the basis of sequence complementarities [2]. miRNAs are produced in a well co-ordinated series of steps from being transcribed in the nucleus to exerting their function in the cytoplasm. miRNAs are now implicated in diverse biological phenomena ranging from development[3] to stress response[4] which makes miRNA as one of the central regulatory molecules which modulate information flow along the central dogma of gene expression. More importantly, like any regulatory molecule, deregulation of miRNAs are causally associated with several diseases (mainly cancer) and are directly involved in a variety of pathophysiologies owing to their aberrant expression [5]. Thus, modulation of miRNA levels is of prime therapeutic importance. Conventional methods of miRNA knockdown using chemically modified antisense-oligonucleotides have been explored extensively but face the challenges of modes of delivery, biostability and biodistribution. This calls for development of more alternate and non-conventional methods to target miRNA. Small molecules targeting RNA chemical and structural space provide one such timely opportunity. In this article we first provide brief overview of miRNA biogenesis and their disease associations. We then summarize the major developments of conventional oligonucleotide based approaches to miRNA knockdown and their status. We then focus on the more non-conventional methods like oligonucleotide enzymes and small molecules and provide an outlook on the future of such methods.

Biogenesis of microRNA – a series of catalytic chemical reactions

The cell can be imagined essentially as an assembly of compartmentalized reaction vessels in which molecular reactions take place in limited volumes. These compartments are further able to communicate with each other and thus are able to co-ordinate their respective reaction rates to maintain a homeostatic environment. miRNAs as mentioned, are small endogenous non-coding RNA thus retaining all the obvious chemical properties of RNA in general. They vary from 19-25 nucleotides in length from organism to organism and even from tissue to tissue[2,6]. However, they are produced initially as long primary transcripts in the nucleus of the cell. These transcripts then undergo a series of processing steps by the end of which they are trimmed down to mature duplex miRNA in the cytoplasm of the cell.

MicroRNAs are produced as long primary transcripts most of which are under the transcriptional control of RNA polymerase II [2] in the nuclear compartment of the cell. The long primary transcripts are called primary miRNA (pri-miRNA) and can be several kilobases in length. The pri-miRNA consists of stem-loop structures, which are found along its length and contain the precursors to the mature miRNA. These stem loop structures typically consist of a ~33 bp double stranded stem region, a variable terminal loop and flanking single stranded segments. The pri-miRNAs are then processed into an approximately 70 nucleotide precursor by the microprocessor complex (of which Drosha and DGCR8 form the core components)[7]. They do so by concerted action of DGCR8 binding to the single stranded and double stranded junction and Drosha cleaving approximately 11 bp upstream along the stem region [7]. This processed form is called the precursor miRNA or pre-miRNA which then becomes a substrate for further downstream processes.

After the formation of pre-miRNA, it is exported to the cytoplasmic compartment of the cell with the help of another protein, Exportin-5. A high resolution X- ray structure of Exportin-5 with pre-miRNA reveals that it binds to the end of the stem region and covers it along its length[8]. This binding is also sequence independent and thus is a universal exporter of pre-miRNA into the cytoplasm where it would be further processed by another protein of RNase III family – Dicer.

The pre-miRNA upon export are then taken up by Dicer which recognizes the end of the pre-miRNA and then cleaves at the base of the stem-loop junction to produce the mature miRNA duplex which consists of complementary strands with 2nt 3' overhangs[9]. One of the sequences is called the guide strand (which binds to target mRNA) and the other is called the passenger strand (which

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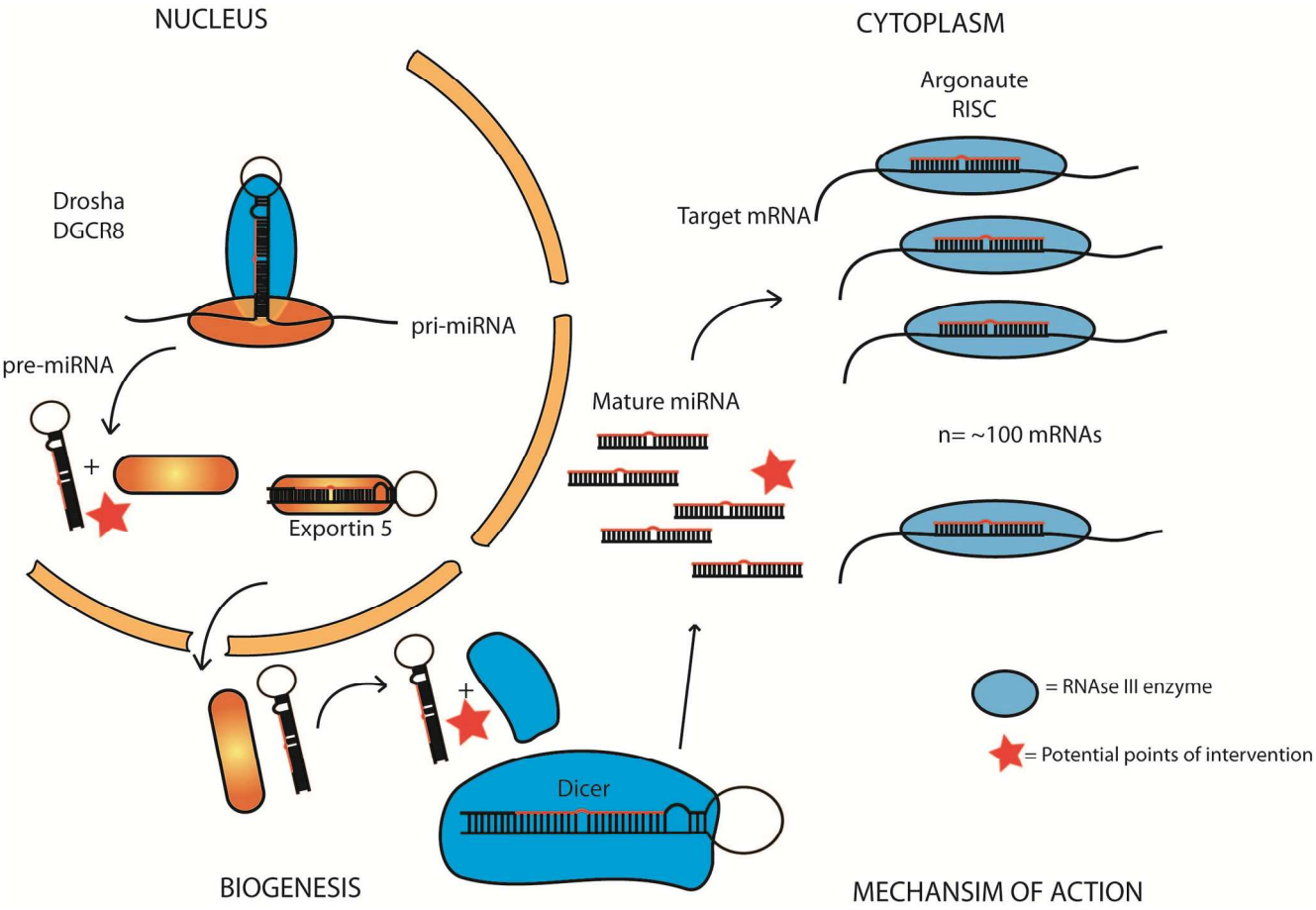

Fig.1 microRNA are genomically encoded and transcribed as long primary transcripts in the nucleus. These primary transcripts (pri-miRNA) are processed by the nuclear RNase complex Drosha-DGCR8 to form the pre-miRNA. The pre-miRNA is then exported (by Exportin 5) to the cytoplasm where the cytosolic RNase Dicer, processes the pre-miRNA to the mature duplex miRNA which then in concerted action with Argonaute regulates target mRNA abundance. As depicted in the illustration, the miRNA processing pathway offers atleast three stages of therapeutic intervention. ASOs (antisense oligonucleotides) have typically targeted the cytosolic mature miRNA and prevent their functional silencing of target mRNAs. However the presence of secondary structures in the pri and pre forms of miRNA offer an unconventional targeting space by use of small molecules.

is generally degraded). These generic terms have been recently revised to 5p and 3p where these represent the strand being loaded from the 5' arm or 3' arm of the pre-miRNA stem-loop. However for the sake of simplicity we restrict our usage to 'guide' and 'passenger'. The proteins Drosha and Dicer are a class of RNaseIII domain containing proteins which are highly conserved [10]. As shown in Fig 1. They operate to process the long precursor forms of miRNA to its mature form. However it is important to note that these processing events are structure dependent and sequence independent (discussed in detail in later sections).

These miRNA duplexes are now loaded into a protein complex called RNA induced silencing complex (RISC). The RISC complex consists of the core Argonaute (AGO) proteins and several accessory proteins. Within the RISC complex, a preferential loading of the guide strand of miRNA takes place on to the AGO proteins thus forming the miRISC[11].

The functional miRISC then targets mRNA (typically in the 3'UTR) on the basis of a partial sequence complimentarity (called the seed sequence) between the loaded guide miRNA and its target mRNA. Upon binding, inhibition of protein production by repression of translational initiation of the target gene takes place [12]. This is typically followed by deadenylation of the mRNA and reduction in mRNA levels[13]. Any given miRNA is predicted to regulate hundreds of target mRNA, while a single mRNA may have several miRNA binding sites[2]. Thus miRNA-mRNA interactions form a complex regulatory network which finally influences cellular function.

A more biological and descriptive explanation can be found in several reviewing articles[2, 6]. The take home lesson from a

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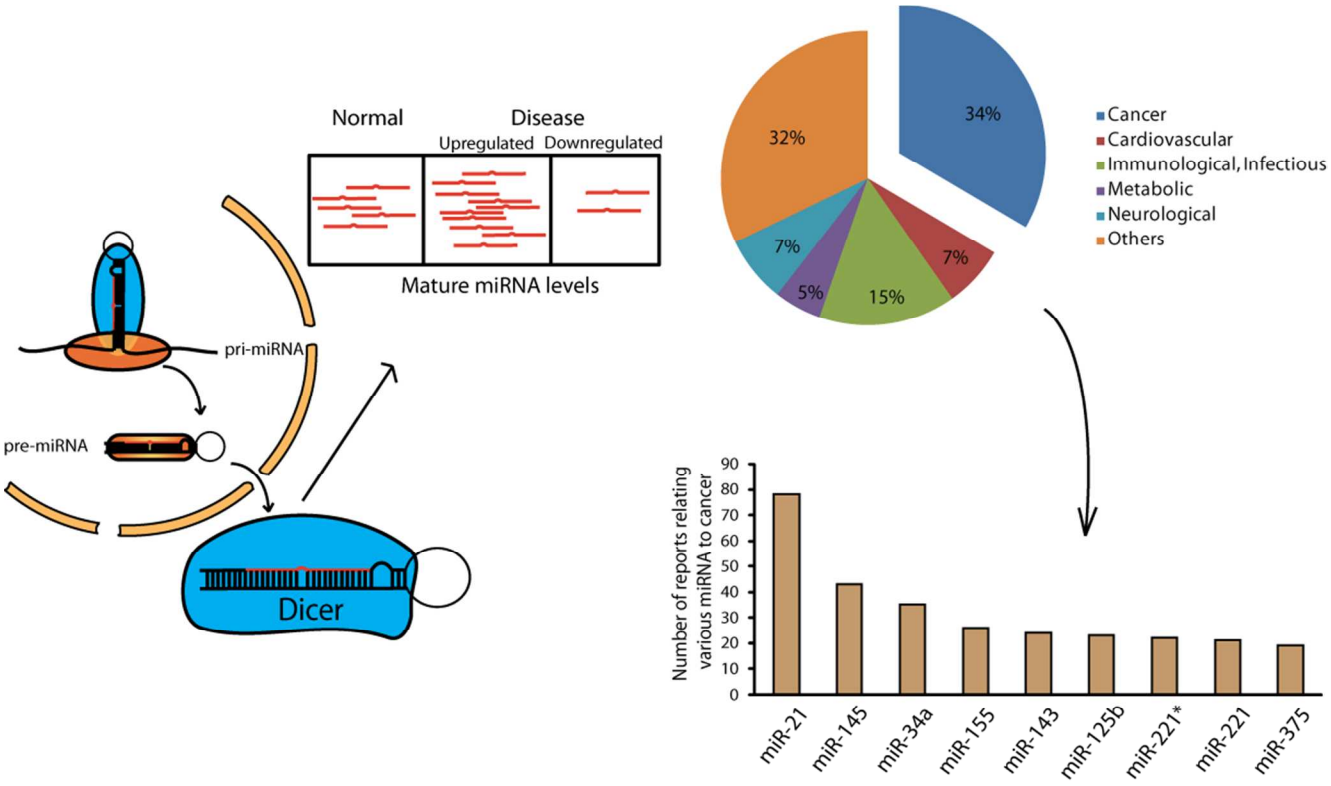

Fig.2 miRNAs contribute to disease onset and severity by alteration of their abundance. The left panel of the illustration shows the scenarios where in pathophysiological states, miRNAs may be repressed or over expressed compared to the normal scenario as indicated in each box. The right panel is a representation of the associated miRNA with various diseases which have been documented in literature. As seen in the pie chart, cancer as a disease group has most number of deregulated miRNA. In the lower right panel - among different cancers miR-21 is reported to be most frequently associated– The y axis indicates the number of different reports which have identified any given miRNA to be associated with cancer (data taken from reference 15a-d).

chemist's perspective is that miRNAs are produced in a series of chemical reactions involving proteins and RNA. These reactions have certain kinetic rates associated with them which allow one to design a variety of inhibitory modules to disrupt their function. Secondly precursor miRNAs are enriched in prototypic secondary structures (discussed in detail in later sections) which then serve as targets for small molecules which could potentially inhibit miRNA maturation and hence function.

miRNA burden in disease etiology

Like any multi-component machine, the cell consists of parts which can get damaged or which malfunction during the course of its operation leading to its reduced or aberrant performance. Generally, diseased states are a result of such abnormal performance. miRNAs being important regulatory molecules, when present in higher or lower levels (abundances or expression levels) also contribute to the pathophysiology of disease. This fact can be reconciled with the fact that a single miRNA has the capability of regulating hundreds of target mRNA, hence it is conceivable that imbalance in the levels of one miRNA can destabilize the entire cellular machinery.

Although, the causal association of miRNA with human disease conditions have been explored and reviewed in detail elsewhere[5, 14], we here give a brief introduction to the spectrum of diseases where miRNA malfunction is implicated. The availability of miRNA-disease databases [15] which contain manually curated information, has afforded easy ways to mine disease associations with miRNA malfunction. The advantage inherent to such databases is the regular updates, which reduces tedious literature mining. According to recent estimates, miRNA's are involved majorly in cancer, cardiovascular, infection and immune disease conditions (Fig2) amongst other conditions. For conceptual purposes, we restrict our description to cancer, which forms the major chunk of disease spectrum (approx 35%).

The case of Cancer

Cancer is a genetic disease thought to arise from mutational accumulation [16]. Mounting evidence suggests that in addition to mutations, regulatory paradigms also change in the onset of cancer. The first association of miRNAs with cancer came from miRNA profiling studies that revealed significant differences in expression levels of miRNAs between normal and tumor tissue type. miRNAs that contribute to oncogenesis may function as an oncogene or a tumor suppressor. This could be understood in terms of the target repertoire of each miRNA being implicated. If the miRNA under question is upregulated and regulates a set of genes which are bonafide tumor suppressors, it can be termed as an oncogenic miRNA (or oncomirs). Conversely, if an miRNA is downregulated (and it regulates several oncogenic target genes) it is termed as a tumor suppressor miRNA. Several miRNAs have been identified either as bonafide tumor suppressors or oncogenes. Tumor suppressor miRNAs (e.g miR-15, mir-16) are generally downregulated in most cancers, while oncogenic miRNAs – oncomiRs (such as miR-21[17])- are upregulated and contribute to the onset and maintenance of the tumor phenotype. A simple meta-analysis [15d] of all documented human cancers and the differential miRNA expression levels reveals that more than 40% of miRNAs associated with cancer are upregulated. It is imperative to understand that the overexpression or amplification of oncogenic miRNA could occur by virtue of abnormal promoter activity or increased efficiency of miRNA biogenesis machinery. As mentioned before, due to the highly concerted action of a single miRNA, in either case it is conceivable that correction of this expression imbalance would result in a complete phenotypic reversal to normalcy. With this view in mind, miRNAs form important therapeutic targets and thus, several strategies have been devised to either silence an oncomiR or replenish tumor suppressor miRNAs [18].

Conventional methods of miRNA inhibition

As mentioned in the previous section, after the discovery that certain miRNAs are causally upregulated in several cancers, intuitive strategies to use antisense oligonucleotides (ASO) against miRNA were adopted. The very first demonstration for the functional inhibition of miRNA was achieved by using simple antisense DNA oligonucleotides[19]. However, unmodified DNA oligonucleotides were not effective enough to inhibit miRNA activity in subsequent attempts in other organisms. This is due to the fact that biological fluids contain highly active nucleases that degrade the oligonucleotide even before it reaches its destination. Moreover, one must consider the inherent off-targeting dangers of unmodified ASOs to miRNAs of very similar sequences (miRNA families). Considering these two parameters, several chemical modifications to ASOs have been made to improve their biostability and bioavailability. Early on, various studies demonstrated that modifications to the backbone (PS) and 2'OH (in the nucleobases of the ASO) showed greater biostability and nuclease resistance and were also used in mice models[20]. These first generation ASOs were further modified to increase uptake and bioavailability with cholesterol conjugation (termed 'antagomirs') and extended to whole animal models for inhibition of miRNA function [21]. Some of the prototype ASOs used in the past is summarized briefly in supplementary table S1.

Locked Nucleic Acid (LNA) – a success story.

Although the utilization of first generation ASOs for determination of miRNA function in cellulo have been extended to pre-clinical studies in animal models, their use as therapeutic modalities have been limited[22]. These strategies have been superseded by the use of LNA (Locked nucleic acids) which are modified ribonucleotide analogues having a methylene bridge connecting the 2' oxygen and 4' carbon (of the ribose moiety) thus 'locking' the sugar in a C'3-endo conformation[23]. This modification enhances the hybridization affinity manifold over other chemical moieties, by increasing the thermal stability by a factor of upto 10˚C (by substitution of single LNA monomer)[23] and hence reducing the off-targeting. The first utility of LNA based ASOs against miRNA was the detection (rather than inhibition) of miRNA in-vitro [24] and in-situ [25]. It was then followed by a few pioneering loss of function studies of miRNA using LNA ASOs in cell lines and mice models [26] where its biostability and increased efficacy was gauged. In a first experiment of its kind, Elmen and colleagues used an unconjugated LNA modified ASO against miR-122 in Africa green monkeys [27] where the authors demonstrated a systemic delivery and dose and tissue dependent reduction in miRNA levels without any toxicities. This study furthered the promise of LNA- based ASOs against disease associated miRNA. miR-122 is a vertebrate (liver) restricted miRNA implicated in fatty acid metabolism [28] and hepatitis C virus (HCV) replication during infection [29]. The therapeutic efficacy of LNA-mediated miRNA silencing was demonstrated in a landmark study when a LNA modified ASO (SPC3649- later dubbed as Miravsen) was used in chimpanzee models of chronic HCV infection[30]. The administration of the LNA based ASO led to a complete suppression of the viral titre and had long lasting effects without resistance or side-effects. This was followed up in a successful phase II clinical trial in patients with chronic HCV infection[31]. Through a series of experiments, Miravsen has been demonstrated to interfere with the biogenesis of miR-122 as well titrate out the mature forms and may be attributable to the high efficacy of miRNA silencing observed [32]. Functional silencing of whole miRNA families have been achieved with the use of 'tiny LNAs' which are continuous LNA monomers (8 mers) which target the 'seed' sequence of mature miRNA[33]. A more comprehensive and systematic review about diverse chemically modified ASOs can be found elsewhere[22,34].

Nucleic Acid Enzyme based miRNA silencing

Although conventional ASO based targeting strategies of miRNA have had reasonable success, these methods are limited by the need of prolonged dosage. It is believed that most of the ASOs targeting miRNAs form stable heteroduplexes and inhibit the cytoplasmic interaction between miRNA and its target and there is a subsequent reduction in the mature miRNA levels via

degradation [21, 28, 35]. However extensive investigation [36] suggests that high affinity ASOs (like LNA, LNA/O-Me etc.) do not reduce miRNA levels post-sequestering. This creates a 'demand and supply' scenario where the active working dosage of ASOs must be matched to the dynamic concentrations of newly transcribed miRNA. Catalytic oligonucleotide enzymes such as DNAzymes[37] and synthetic ribozymes cleave the RNA substrate in a sequence dependent manner. They bind the target RNA through Watson Crick base pairing interactions, cleave the bound RNA, release the products and are recycled so that this process is repeated multiple times, thus overcoming the shortcoming of perpetuated dosage. As a proof of principle, we used this approach to antagonize miRNA function (antagomirzymes)[38]. In order to increase the binding affinity and specificity, we subsequently incorporated LNA modifications in the flanking arms (LNAzymes)[39] and utilized it in an *in vivo* model[40] to knockdown miRNA.

Restoration of miRNA levels

As mentioned before, in pathological conditions, a miRNA may be down-regulated. While, efforts towards inhibition of over expressed miRNA have been a major focus over the past years, a few successful strategies have been employed to restore levels of depleted miRNA in various conditions. For the sake of brevity, we restrict our discussion to seminal examples (one can find detailed discussion of therapeutic substitution for loss of miRNA elsewhere [34a, 34b]). Conventionally, restoration of miRNA levels is achieved using oligonucleotide based 'miRNA mimics' or by means of miRNA expression plasmids. One of the first studies using the former strategy reported successful restoration of tumor suppressor miR-34a leading to reduced cellular proliferation in a xenograft model of colon cancer[41]. Following this report, other studies[42] have shown successful use of miRNA mimics, and the first clinical trial[43] using a miR-34 mimic (MRX34) was reported recently. It is important to remember that miRNA mimic based strategies are still in principle oligonucleotide based and hence heavily depend on the parallel development of efficient delivery systems which may become a rate limiting step for many candidates to enter clinical trials. Another potential concern is the dosage regime of such approaches. To overcome continuous dosing, an alternate strategy is to use miRNA expression vectors which were demonstrated to be viable in a murine model of hepatocellular carcinoma[18a]. The authors systemically delivered a AAV (adeno-associated virus) based vector which expressed miR-26a which caused inhibition of cancer cell proliferation. Although no study so far has reported the clinical usage of such a strategy for miRNA restoration, AAV vector based gene therapy holds great promise.

Paradigm shift – the use of small molecule inhibitors of miRNA function

The development of antisense nucleic-acid based therapeutic strategies has been often plagued by the recalcitrant complications of targeted delivery with optimal bioavailability [44]. Although several advances have been made in the field of gene delivery, the cost of synthesis [45] and storage impede its large scale success as highly successful drug candidates. Another complication is the poor pharmacokinetics and pharmacodynamics [46]. Of course, a few siRNA and ASO based modalities [31, 44] have reached human trials, it remains that nucleic acid based therapeutic strategies of knocking down miRNA have a long way to go. Conversely, small molecules have been at the forefront of discovery and approval of new therapeutic modalities [47] owing to their favourable physico-chemical and pharmacological properties. Historically, drug targets have rarely extended beyond the domain of proteins and the 'druggability of RNA' has been doubtful due to its inherent intrinsic flexibility, highly charged surface and poor understanding of drug/small molecule-RNA binding complexity. However since the druggability of the proteome is restricted [48] it is foreseeable that RNA may form an alternate drug target (for any given disease). As more comprehensive studies of RNA structure and dynamics emerge [49], it is becoming clearer that RNA can be targeted by small molecules. As discussed by the authors in this excellent review [50] RNA consists of suitable secondary structures (Fig 3a) which can act as binding pockets for small molecules.

As explained earlier, the miRNA biogenesis pathway can be visualized as a series of chemical reactions beginning from the expression of the pri-miRNA transcript in the nucleus to its processing as mature duplex miRNA in the cytoplasm. During the course of its biogenesis, the miRNA molecules are maintained in structural entities (namely stem-loop structures and bulges along the stem) for proper recognition by the microprocessor and Dicer enzymes [51].Thus in general, the inherent nature of miRNA to form secondary structures paves the way for the testing of small molecule ligands which could modify their activity by altering the flexibility of the terminal loop[51b] or by physically perturbing the action of the RNaseIII enzymes. While conventional ASO based methods typically function to stoichiometrically restrict activity of the mature miRNA species, targeting precursor forms offer a unique advantage of restricting the production of mature miRNA. With this perspective, targeting miRNA function with small molecules offers an alternate strategy to explore [52]

Typically drug discovery strategies involve either a screening of a chemical library or rational drug design, both of which involve inclusion of structural information about the intended biological target. However, in the case of miRNA, it must be noted that due to the paucity of structural information, targeting miRNA with small molecules has often been done using a screening procedure, although the availability of accurate 3D modelling may enable efforts to complement the screening strategies (discussed later).

One of the earliest reports of using small molecules for the possible inhibition of miRNA function was the design and development of helix-threading peptides[53] which can bind to pre-miRNA. Although a functional outcome for their inhibitory capacity was not demonstrated, these peptides formed the beginnings of the foray of small molecules in miRNA knockdown and inhibition. The first use of small molecules for inhibition of miRNA activity was reported in an in vitro fluorescence- based assay developed for screening compounds which could inhibit pre-miRNA let-7 processing by the Dicer enzyme [54]. The major disadvantage of this approach is the use of the pre-miRNA as the reporter with a fluorophore and quencher

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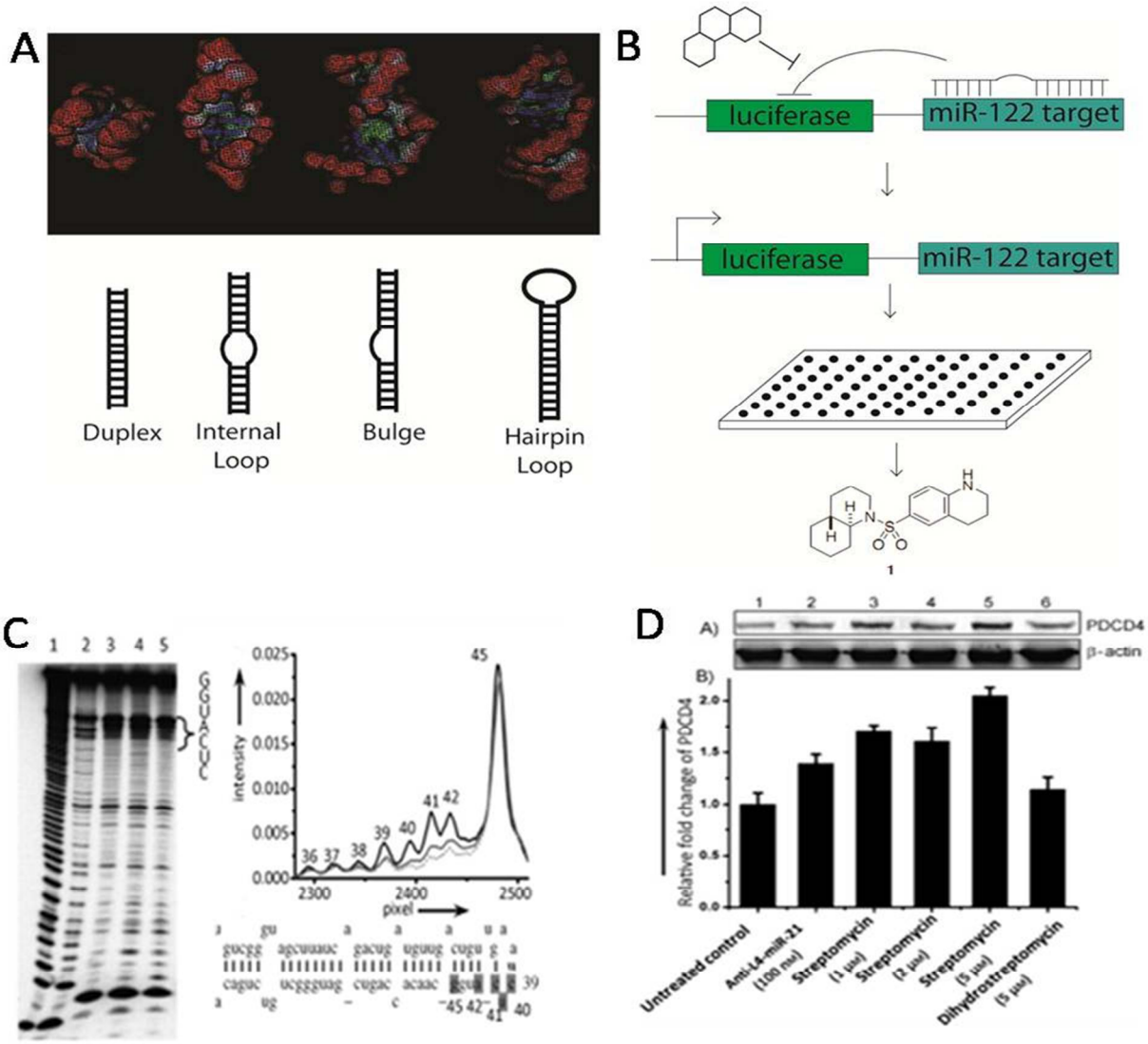

Fig.3 A) Schematic illustration of prototypical RNA secondary structures which are enriched for in the miRNA biogenesis pathway especially in the pre-miRNA (adapted from ref 50). B) High-throughput screening strategy using a luciferase based cellular screen (adapted from ref 65). A vector containing a validated target of miR-122 placed downstream of a luciferase reporter is stably integrated into a test cell line where miR-122 levels are high. The cells are then treated with a library of compounds in a high-throughput format using microplates. Small molecule candidates are then identified based on relative luciferase levels (an increase). C) Identification and characterization of Streptomycin as an inhibitor of miR-21 function (adapted from ref 68) Streptomycin binds to a secondary structure (bulge – indicated by shaded nucleotides in grey) near the terminal loop and inhibits the processing by Dicer, thus reducing mature miRNA levels. D) Functional inhibition of miRNA function revealed through western blot analysis of a validated target (PDCD4) of miR-21. The efficacy of Streptomycin mediated inhibition is comparable to that of LNA based ASO mediated inhibition of miR-21 function.

attached to the ends of the pre-miRNA, which may interfere with the Dicing activity itself [9] and hence confounding the results of the assay. Moreover it was not clear if the compounds tested in-vitro would inhibit specific miRNA function in vivo. The advantage of using minimal in vitro platforms of screening is the scalability of the assay in a high throughput manner. To overcome the drawbacks mentioned above, the same group improved the method based on BRCA (branched rolling circle amplification)[55], thus omitting the need for artificial fluorescent labelling in the pre-miRNA. As an alternative strategy, we recently developed in vitro assay [56] using a molecular beacon which is independent of the pre-miRNA and is complementary to the mature miRNA produced upon Dicer cleavage. Using this beacon based screen, we identified potential inhibitors of miR27a

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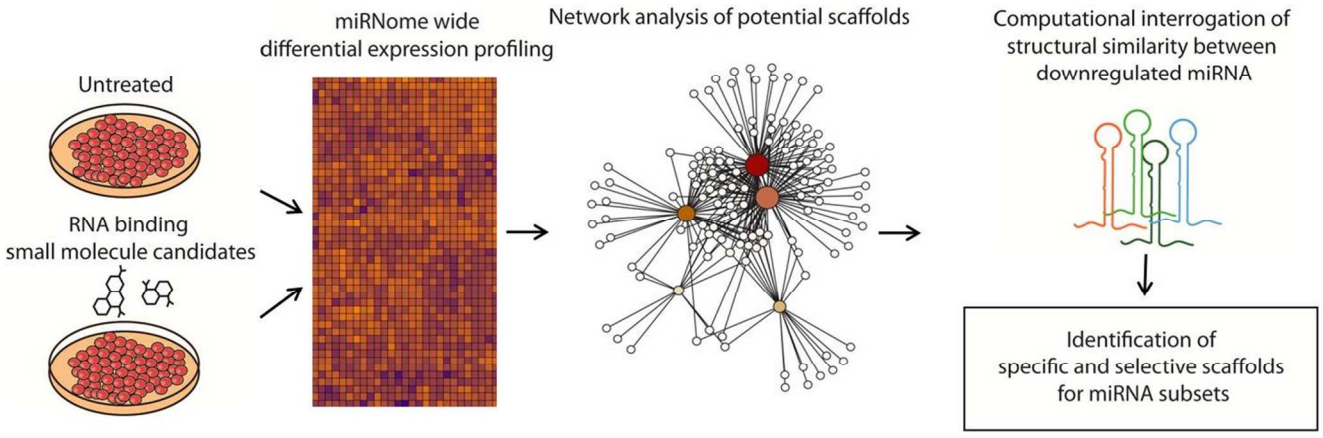

Fig.4 A schematic illustration of a systems approach to small molecule inhibitor discovery. Cell lines are treated with small molecule candidates and high through put miRNA profiling using multiplex qPCR or next generation sequencing will reveal miRnome wide perturbations which can be then deconvoluted using network analysis to discover specific subsets of responsive miRNA to each candidate drug. The structural basis of this perturbation can be estimated by computational interrogation of secondary structure similarity between members of a given subset – a high similarity score would indicate that the perturbation (in this case inhibition/down regulation) may operate via a mechanism similar to that of Streptomycin inhibiting pre-miRNA dicing.

and validated these candidates with downstream biological assays.

A generic modifier of the miRNA and siRNA pathways was also discovered [57] using a cell based screen which led to the identification of compounds which could affect miRNA/siRNA output albeit non-specifically. This compound was later characterized to bind to and affect protein components of the miRNA biogenesis pathway [58]. The first specific small molecule inhibitor was identified in a study [59] where the authors screened a library of over 1000 compounds to test inhibition of activity of miR-21 (oncomir) using a luciferase based assay. After screening at a treatment concentration of 10 uM, the azobenzene derivatives discovered here were elucidated to be non generic and were able to target miRNA-21 transcription (but not miRNA maturation) specifically when compared to other control miRNAs used in the assay. However its miRnome wide specificity remains untested.

Following this, another study characterized small molecule modifiers of miRNA-122 [60] which is known to be causally associated with Hepatitis (where its levels are high) and hepatocellular carcinoma (where its levels are reduced). The authors again used a high throughput luciferase screening of about 1300 compounds from the NCI (National Cancer Institute) developmental therapeutics program. They discovered transcriptional regulators (compounds which could both activate and inhibit miRNA-122 expression) of miRNA-122 which did not affect the general miRNA biogenesis pathway. Independently, another screen of about 530 compounds led to the identification of poly-L-lysine (PLL) and trypaflavine (TPF) as generic inhibitors of miRNA biogenesis pathway [61]. Further investigation showed that PLL was able to inhibit pre-miRNA processing by binding to Dicer and TPF could inhibit mature miRNA/siRNA loading onto the AGO2 of the RISC complex. Another example generic modification of the RISC activity was demonstrated subsequently using an in vitro fluorescence polarization assay enabling the identification of aurintricarboxylic acid, suramin and oxidopamine as potent RISC loading inhibitors[62].

Although all the above examples discuss the general potential of small molecules for miRNA knockdown, most (with the exception of the helix threading peptides and the candidates from the in vitro screens) do not interact directly with the miRNA. In search for the molecules with specificity directly to miRNA, Chirayil et al [63] screened a combinatorial library of N-substituted oligoglycines (7680), commonly called peptoids employing a microarray format against the precursor miR-21. Using this approach functional groups that contribute towards binding affinity and specificity with the miR-21 precursor were identified, leading to synthesis of compounds having better binding potential. These compounds were determined to have binding affinities in range exhibited by Drosha to its substrates. However, the functional inhibition of miRNA-21 using these candidate peptoids is yet to be reported. The same group in a recent study [64] identified a peptoid ligand (out of 14,024 N-substituted oligoglycines) which can bind to the apical loop of pri-miRNA 21 and inhibit Drosha processing.

As described, the development of high-throughput cell based screening assays (Fig 3b) [65] and microarray format (as above) will enable identification of novel miRNA activity inhibitors from the several available chemical libraries. However from a therapeutic and clinical standpoint, such a process is,

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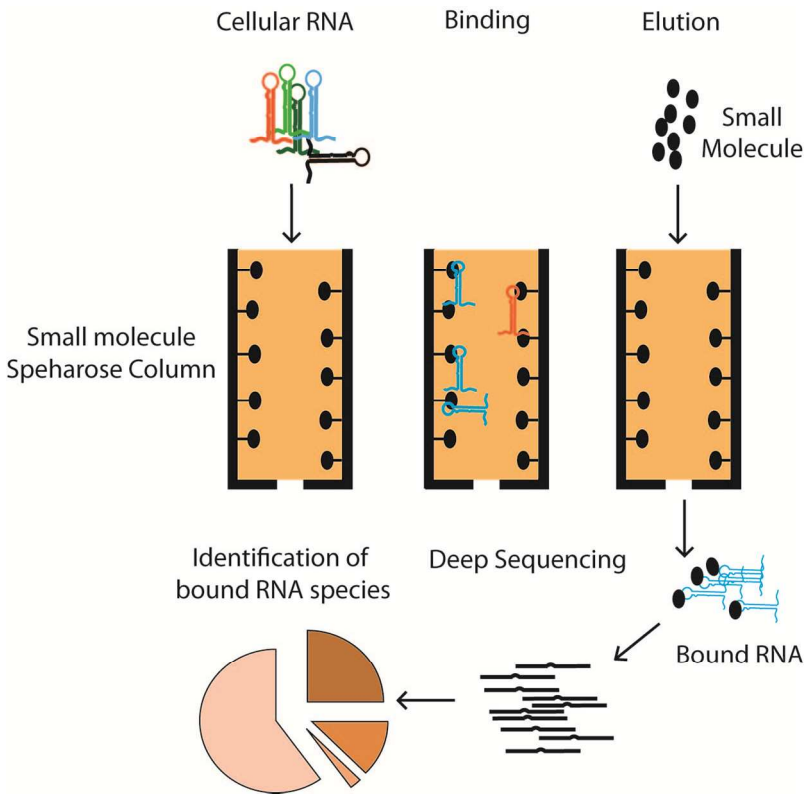

Fig.5 A complementary strategy to identify direct targets and off-targets of small molecule candidates. In conditions where the small molecule can be coupled to a sepharose or agarose matrix, cellular RNA (total or size fractionated) can be added to the column. Non-specific or unbound RNA can be washed using stringent conditions, and bound RNA can be competitively eluted using the same small molecule. The bound RNA can be then subjected to next generation sequencing to identify all possible direct targets and off-targets. This method should be broadly applicable to screen for off-targets of RNA binding drugs which are currently in use. .

costly[66].To circumvent this, a more recent approach in the pharmaceutical industry exploits the polypharmcological potential of approved drugs for drug repurposing or drug repositioning[67]. We hypothesized that one can theoretically screen the array of FDA approved RNA binding drugs for miRNA inhibitory activity. We screened 15 FDA-approved aminoglycoside antibiotics for their potential to inhibit miR-21 function. Aminoglycosides are well known RNA secondary structure binders. Using a low throughput luciferase assay, we identified that Streptomycin (a well know tuberculosis drug) is a potent inhibitor of miR-21 function [68](independently another group identified pre-miRNA binding aminglycosides using an in-vitro binding assay without testing further in vivo effects[69]. Further molecular characterization using a combination of biochemical (Fig 3c) and computational methods revealed that it binds to a bulge close to the terminal loop and structurally hinders pre-miRNA (Fig 3d) processing by Dicer in a dose dependent manner as show by q-RT-PCR. Other related oncomirs (8 out of 9) which were tested were relatively unaffected by streptomycin giving clues to its empirical specificity. Nevertheless our results indicated that streptomycin in general can be used as a scaffold for designing and development of more specific small molecule ligands for miR-21 knockdown. The study also raised the possibility of each aminoglycoside antibiotic having a specific miRNA target(s). Independently, Vo and colleagues [70] have reported the synthesis of bimodal molecules consisting of neomycin and nucleobases which target oncomirs - miR 372 and miR-373 and inhibit their Dicer mediated maturation and thus showed anti-proliferative activity.

On the contrary when such small molecules are tested, with a view of inhibiting miRNA function, the question of specificity always takes prime importance. We thus formulated an alternate workflow to deconvolute this contradiction which does not involve the tedious luciferase based screening for each miRNA of interest. We speculated that global profiling of miRNA using high throughput RT-PCR profiling post drug treatment would a provide list of miRNA which are differentially affected. By computationally interrogating structural similarity, we would be able to filter down to drug specific miRNA subsets and further scrutinize their selectivity (Fig 4). To directly identify binding

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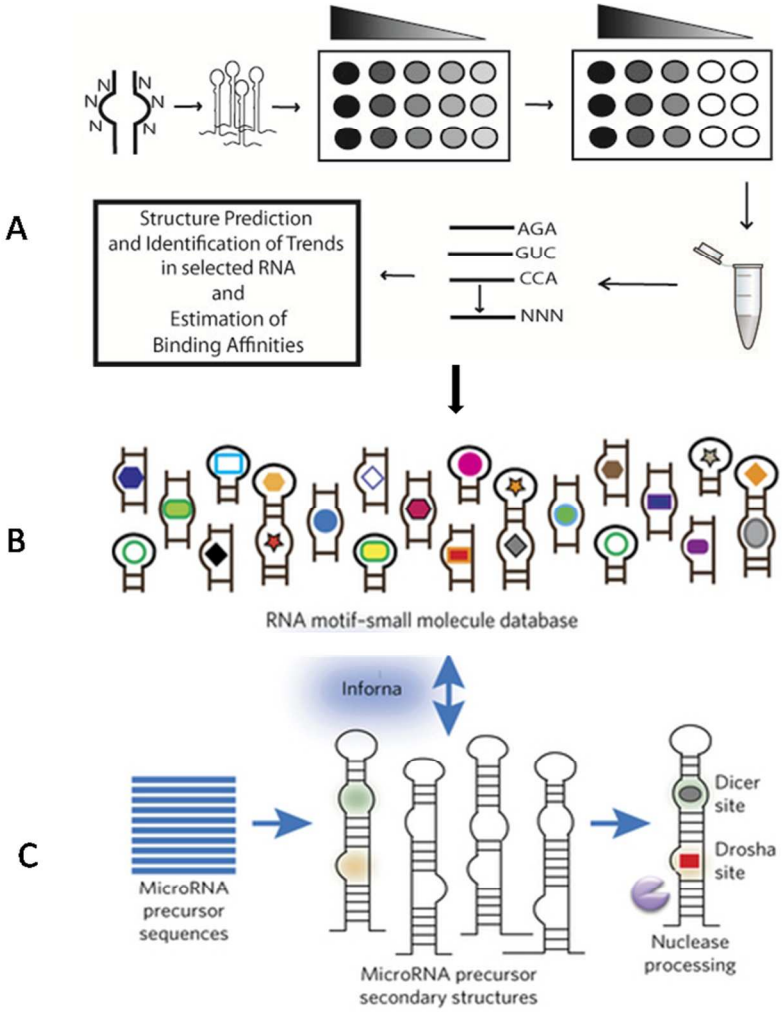

Fig.6 A schematic illustration of the Inforna[71] approach used to identify lead small molecules for miRNA inhibition. A) Two dimensional combinatorial screening (2DCS) enables simultaneous identification of RNA-structural motifs which can bind to an array of small molecules. Shown in order – is the utilization of a radiolabelled RNA-motif (can be any NxN c0.3ombination as depicted – here it is a 3x3) library. The chemicals are immobilized in a microarray format in a gradient of concentrations (gradient circles). Each row represents decreasing concentrations of a unique chemical/small molecule. The RNA library is then allowed to react and hybridize on the microarray, and the unbound RNA is washed away. The bound fractions (empty circles on right side) are then excised, RNA is extracted and sequenced to identify features which allow binding to each small molecule. This is followed by a secondary structure and binding affinity calculation. B) All the binding pairs of RNA-motif and small molecules are then recorded into a database. C) Estimation of structural motifs from sequence information of precursor miRNA is done and statistically evaluated for the best small molecule binder to any given miRNA of interest, provided the binding region overlaps cut sites of the miRNA biogenesis RNaseIII enzymes Drosha and Dicer.

miRNA, small molecule based pull downs or affinity purification (Fig 5) coupled with next generation sequencing may be used. This strategy is built on prior important work which used small molecule coupled beads to bind RNA and identify them on microarrays[72]. Current work in the laboratory is focused on deriving such potential scaffolds using the above framework and combining these scaffolds with moieties which impart high specificity and selectivity (Manuscript in preparation). These scaffolds can then be used to synthesize bimodal molecules as reported recently. In parallel, the availability of 3D modelling of pre-miRNA structures[73] and advances in molecular docking platforms like DOCK[74] will accelerate preliminary virtual screening of several molecules computationally in a high throughput manner (vHTS – virtual high throughput screening - discussed in [52c]). A recent study [75] employed this approach and identified a potent inhibitor (AC1MMYR2 from a library of 1990 compounds) of miR-21 maturation.

Rational 'bottom-up' approach

As an alternate approach altogether, Disney and colleagues devised a novel strategy to identify RNA and ligand binding partners simultaneously, called 2-DCS [76] (two dimensional combinatorial screening). The method utilizes a small molecule microarray (agarose) against which a library of RNA secondary structures (loops, bulges etc) is titrated (Fig 6a). This screening enables the simultaneous probing and identification of specific RNA-ligand interactions. Given that the library space encompasses all possible combinations of sequence-structure, screening and identification against multiple ligands would generate an enormous amount of interactions and their affinities thereof. For the sake of analysis and specific identification, the authors then developed a statistical method[77] to derive features of the RNA sequence which contribute to the high affinity binding for any given ligand. Thus, sequence-structure features which can bind to any given ligand with high affinity can be gauged from 2-DCS of vast libraries of RNA vs. Ligands which is stored in a database (Fig6b). For identification of miRNA targeting molecules, the authors intuitively developed Inforna[71] – a pipeline which combines these two methods. Very briefly, using the computed sequence-features and cross referencing it in the context of pre-miRNA (Fig 6b and 6c), the authors were able to identify a benzamidole based lead molecule which could inhibit the Dicer mediated maturation of miR-96. The specificity of the molecule was confirmed by conducting a miRnome wide RT-PCR which surprisingly revealed it had greater discriminatory power over a sequence based LNA ASO against miR-96. Again using 2-DCS, the same group identified a guanidylated neomycin B (G Neo B) inhibitor of miR-10b[78]. In this case, the G Neo B bound to the Drosha processing site inhibited the maturation into precursor and mature forms.

Small molecule based restoration of miRNA

As indicated above, efforts of small molecule based modulation of miRNA levels have been inclined to inhibit candidate miRNA which are over-expressed. While restoration of mRNA levels using oligonucleotide based approaches are straightforward, using small molecules for the same purpose is non-trivial. It can be envisaged that a rational design of small molecules to upregulate specific miRNA at present is not possible, and the few studies which have identified small molecule candidates for specific miRNA have been a result of high throughput screens[60] or global upregulators of miRNA expression[79].

Outlook – Opportunities and Challenges

Nucleic acids as drug targets offer potential solutions to the limited druggability of the proteome [48, 50]. Although, a few drugs (e.g. Cisplatin, Doxorubicin and Acylovir amongst others) are currently being used in clinical scenarios for the treatment of cancer, targeting DNA poses several restrictions due to its relative conformational uniformity. RNA – which is more than a mere information bearer – functions by folding into a variety of simple to complex shapes to create structural modules influencing almost all sequence dependent functions like splicing, translation and turnover of RNA [49]. It can be thus surmised that RNA offers more targetable chemical space making them excellent choices as drug targets [50, 80].

It is evident that the 'miRNA burden in disease etiology' [5, 34d, 81] will only increase as more sensitive detection and profiling technologies emerge. As mentioned earlier, strategies for targeting miRNA are guided not only by basic gene function discovery, but also by the need for therapeutic intervention in many pathophysiological conditions and diseases. Oligonucleotide based targeting of miRNA has been at the forefront of such an endeavour with varying degrees of success, limited by the constraints of delivery and good pharmacokinetics and pharmacodynamics. Despite these impediments LNA based ASOs have been tested clinically, with promising results. These shortcomings however, do not hinder the use of ASOs as tools for functional characterization of miRNA in non-clinical setups. Efforts focussed on circumventing problems posed by such constraints, have led to the exploration of small molecules as an alternative approach to target miRNA.

A comparative note on off-target effects

Conventional wisdom suggests that ASO based strategies should have lesser off target effects as compared to other approaches due to the inherent and direct sequence complimentarity as a pre-requisite. ASO moieties with a mean length equal to that of the mature miRNA and chemical modifications (like LNA) which improve binding affinity (hence specificity) afford more on-targeting capabilities. However despite such a fundamental advantage, side effects due to off targeting may arise from imperfect pairing between the ASO and partially complementary sequences in the transcriptomic space akin to siRNA off-targeting[82]. Another potential source of off-targeting and undesired effects involves the non-specific responses of cells and animal models to the delivery systems[83] used for oligonucleotide based therapeutics. On the other hand small molecules modulate miRNA levels either by direct or indirect binding (transcriptional[60] or RISC protein machinery modulation)[62]. Direct binding typically will depend on the availability of cognate secondary structures as binding pockets for the small molecule moiety. Such secondary structures like loops and bulges are abundant in the miRnome and transcriptome thus potentially hindering their use as miRNA-specific moieties prompting more thorough investigations of identified lead small molecules for miRNA modulation. Although, till date no small molecule candidate has been promoted to clinical trials, a direct comparison of a recently identified lead molecule against miR-96 and a LNA modified ASO against the same has shown promising results of similar specificities[71].

Future Prospects

In the context of the human genome alone, there are approximately 1900 miRNAs [84] which have been identified. However, in terms of functional non-protein coding transcriptional output, miRNAs form only the tip of the iceberg. The advent of next generation sequencing has heralded a tremendous increase in cataloguing of the transcribed genome (> 85%) in the form of thousands of non-coding RNAs [85]. Long non-coding RNAs (lncRNAs) which form a major proportion of these,

serve as functional regulatory RNA molecules influencing gene expression in almost identical ways as proteins do [86]. More importantly, the mechanisms by which lncRNAs appear to exert their function seem to centre on a bipartite manner involving both sequence and higher order secondary and tertiary structures [86-87]. Further, the evolutionary conservation of lncRNAs across different species seems to be guided by principles of structure rather than plain sequence [88]. Their functional importance is highlighted further with their increasing ascription to various diseases [89]. Although at a nascent stage, non-coding RNA functional discovery has caused a re-examination of our views of RNA biology. Collectively, it is clear that non-coding RNA serve as a rich sink of secondary structures within the cellular milieu, prompting two immediate conjectures – Firstly, functional perturbation of lncRNA function may be explored by the use of small molecules. Second and more important, is the realization that any small molecule drug targeting miRNA can have a potential cross talk with other non-coding RNAs of unforeseen functions [90]. In parallel, it is possible that side effects of many RNA binding drugs may also funnel through this mechanism (currently being explored in the lab). One straightforward method to identify off-target binding sites would be to use a pull down based strategy as explained in the previous sections (Fig 6).

In this article, we have tried to highlight the present state of the field and various strategies which have been tried so far. Within a short span of time, rapid progress has been made, and small molecules hold a great translational potential. However, the field is still at its infancy, and more combinatorial and multidisciplinary approaches, including animal studies and evaluation of potential side effects are called for.

Notes and references

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1 G. Ruvkun, *Science*, 2001, **294**, 797.
2 D. P. Bartel, *Cell*, 2009, **136**, 215.
3 E. Wienholds and R. H. Plasterk, *FEBS Lett*, 2005, **579**, 5911.
4 A. K. Leung and P. A. Sharp, *Mol Cell*, 2010, **40**, 205.
5 J. T. Mendell and E. N. Olson, *Cell*, 2012, **148**, 1172.
6 J. Krol, I. Loedige and W. Filipowicz, *Nat Rev Genet*, 2010, **11**, 597.
7 J. Han, Y. Lee, K. H. Yeom, J. W. Nam, I. Heo, J. K. Rhee, S. Y. Sohn, Y. Cho, B. T. Zhang and V. N. Kim, *Cell*, 2006, **125**, 887.
8 C. Okada, E. Yamashita, S. J. Lee, S. Shibata, J. Katahira, A. Nakagawa, Y. Yoneda and T. Tsukihara, *Science*, 2009, **326**, 1275.
9 J. E. Park, I. Heo, Y. Tian, D. K. Simanshu, H. Chang, D. Jee, D. J. Patel and V. N. Kim, *Nature*, 2011, **475**, 201.
10 V. N. Kim, J. Han and M. C. Siomi, *Nat Rev Mol Cell Biol*, 2009, **10**, 126.
11 Y. Kim and V. N. Kim, *Mol Cell*, 2012, **46**, 384.
12 H. A. Meijer, Y. W. Kong, W. T. Lu, A. Wilczynska, R. V. Spriggs, S. W. Robinson, J. D. Godfrey, A. E. Willis and M. Bushell, *Science*, 2013, **340**, 82.
13 S. Djuranovic, A. Nahvi and R. Green, *Science*, 2012, **336**, 237.
14 M. Lu, Q. Zhang, M. Deng, J. Miao, Y. Guo, W. Gao and Q. Cui, *PLoS One*, 2008, **3**, e3420.
15(a) B. Xie, Q. Ding, H. Han and D. Wu, *Bioinformatics*, 2013, **29**, 638; (b) Q. Jiang, Y. Wang, Y. Hao, L. Juan, M. Teng, X. Zhang, M. Li, G. Wang and Y. Liu, *Nucleic Acids Res*, 2009, **37**, D98; (c) Y. Li, C. Qiu, J. Tu, B. Geng, J. Yang, T. Jiang and Q. Cui, *Nucleic Acids Res*, 2014, **42**, D1070; (d) A. Ruepp, A. Kowarsch, D. Schmidl, F. Buggenthin, B. Brauner, I. Dunger, G. Fobo, G. Frishman, C. Montrone and F. J. Theis, *Genome Biol*, 2010, **11**, R6.
16 B. Vogelstein and K. W. Kinzler, *Nat Med*, 2004, **10**, 789.
17 P. P. Medina, M. Nolde and F. J. Slack, *Nature*, 2010, **467**, 86.
18(a) J. Kota, R. R. Chivukula, K. A. O'Donnell, E. A. Wentzel, C. L. Montgomery, H. W. Hwang, T. C. Chang, P. Vivekanandan, M. Torbenson, K. R. Clark, J. R. Mendell and J. T. Mendell, *Cell*, 2009, **137**, 1005; (b) R. Garzon, G. Marcucci and C. M. Croce, *Nat Rev Drug Discov*, 2010, **9**, 775.
19 A. Boutla, C. Delidakis and M. Tabler, *Nucleic Acids Res*, 2003, **31**, 4973.
20(a) G. Hutvagner, M. J. Simard, C. C. Mello and P. D. Zamore, *PLoS Biol*, 2004, **2**, E98; (b) G. Meister, M. Landthaler, Y. Dorsett and T. Tuschl, *RNA*, 2004, **10**, 544.
21 J. Krutzfeldt, N. Rajewsky, R. Braich, K. G. Rajeev, T. Tuschl, M. Manoharan and M. Stoffel, *Nature*, 2005, **438**, 685.
22 J. Stenvang, A. Petri, M. Lindow, S. Obad and S. Kauppinen, *Silence*, 2012, **3**, 1.
23 H. Kaur, B. R. Babu and S. Maiti, *Chemical Reviews*, 2007, **107**, 4672.
24 A. Valoczi, C. Hornyik, N. Varga, J. Burgyan, S. Kauppinen and Z. Havelda, *Nucleic Acids Res*, 2004, **32**, e175.
25 W. P. Kloosterman, E. Wienholds, E. de Bruijn, S. Kauppinen and R. H. Plasterk, *Nat Methods*, 2006, **3**, 27.
26(a) I. Naguibneva, M. Ameyar-Zazoua, N. Nonne, A. Polesskaya, S. Ait-Si-Ali, R. Groisman, M. Souidi, L. L. Pritchard and A. Harel-Bellan, *Biomed Pharmacother*, 2006, **60**, 633; (b) U. A. Orom, S. Kauppinen and A. H. Lund, *Gene*, 2006, **372**, 137; (c) J. Elmen, M. Lindow, A. Silahtaroglu, M. Bak, M. Christensen, A. Lind-Thomsen, M. Hedtjarn, J. B. Hansen, H. F. Hansen, E. M. Straarup, K. McCullagh, P. Kearney and S. Kauppinen, *Nucleic Acids Res*, 2008, **36**, 1153.
27 J. Elmen, M. Lindow, S. Schutz, M. Lawrence, A. Petri, S. Obad, M. Lindholm, M. Hedtjarn, H. F. Hansen, U. Berger, S. Gullans, P. Kearney, P. Sarnow, E. M. Straarup and S. Kauppinen, *Nature*, 2008, **452**, 896.
28 C. Esau, S. Davis, S. F. Murray, X. X. Yu, S. K. Pandey, M. Pear, L. Watts, S. L. Booten, M. Graham, R. McKay, A. Subramaniam, S. Propp, B. A. Lollo, S. Freier, C. F. Bennett, S. Bhanot and B. P. Monia, *Cell Metab*, 2006, **3**, 87.
29 C. L. Jopling, M. Yi, A. M. Lancaster, S. M. Lemon and P. Sarnow, *Science*, 2005, **309**, 1577.

30 R. E. Lanford, E. S. Hildebrandt-Eriksen, A. Petri, R. Persson, M. Lindow, M. E. Munk, S. Kauppinen and H. Orum, *Science*, 2010, **327**, 198.

31 H. L. Janssen, H. W. Reesink, E. J. Lawitz, S. Zeuzem, M. Rodriguez-Torres, K. Patel, A. J. van der Meer, A. K. Patick, A. Chen, Y. Zhou, R. Persson, B. D. King, S. Kauppinen, A. A. Levin and M. R. Hodges, *N Engl J Med*, 2013, **368**, 1685.

32 L. F. Gebert, M. A. Rebhan, S. E. Crivelli, R. Denzler, M. Stoffel and J. Hall, *Nucleic Acids Res*, 2014, **42**, 609.

33 S. Obad, C. O. dos Santos, A. Petri, M. Heidenblad, O. Broom, C. Ruse, C. Fu, M. Lindow, J. Stenvang, E. M. Straarup, H. F. Hansen, T. Koch, D. Pappin, G. J. Hannon and S. Kauppinen, *Nat Genet*, 2011, **43**, 371.

34(a) M. Y. Shah and G. A. Calin, *Wiley Interdiscip Rev RNA*, 2014; (b) H. Ling, M. Fabbri and G. A. Calin, *Nat Rev Drug Discov*, 2013, **12**, 847; (c) E. van Rooij and E. N. Olson, *Nat Rev Drug Discov*, 2012, **11**, 860; (d) T. Thum, *EMBO Mol Med*, 2012, **4**, 3.

35 S. L. Ameres, M. D. Horwich, J. H. Hung, J. Xu, M. Ghildiyal, Z. Weng and P. D. Zamore, *Science*, 2010, **328**, 1534.

36 A. G. Torres, M. M. Fabani, E. Vigorito and M. J. Gait, *RNA*, 2011, **17**, 933.

37 S. W. Santoro and G. F. Joyce, *Proc Natl Acad Sci U S A*, 1997, **94**, 4262.

38 V. M. Jadhav, V. Scaria and S. Maiti, *Angew Chem Int Ed Engl*, 2009, **48**, 2557.

39 H. Kaur, V. Scaria and S. Maiti, *Biochemistry*, 2010, **49**, 9449.

40 H. Suryawanshi, M. K. Lalwani, S. Ramasamy, R. Rana, V. Scaria, S. Sivasubbu and S. Maiti, *Chembiochem : a European journal of chemical biology*, 2012, **13**, 584.

41 H. Tazawa, N. Tsuchiya, M. Izumiya and H. Nakagama, *Proc Natl Acad Sci U S A*, 2007, **104**, 15472.

42(a) P. Trang, J. F. Wiggins, C. L. Daige, C. Cho, M. Omotola, D. Brown, J. B. Weidhaas, A. G. Bader and F. J. Slack, *Mol Ther*, 2011, **19**, 1116; (b) B. C. Bernardo, X. M. Gao, C. E. Winbanks, E. J. Boey, Y. K. Tham, H. Kiriazis, P. Gregorevic, S. Obad, S. Kauppinen, X. J. Du, R. C. Lin and J. R. McMullen, *Proc Natl Acad Sci U S A*, 2012, **109**, 17615.

43 A. Bouchie, *Nat Biotechnol*, 2013, **31**, 577.

44 D. Castanotto and J. J. Rossi, *Nature*, 2009, **457**, 426.

45 E. R. Rayburn and R. Zhang, *Drug Discov Today*, 2008, **13**, 513.

46 C. L. Esposito, S. Catuogno, V. de Franciscis and L. Cerchia, *Discov Med*, 2011, **11**, 487.

47 D. C. Swinney and J. Anthony, *Nat Rev Drug Discov*, 2011, **10**, 507.

48 J. P. Overington, B. Al-Lazikani and A. L. Hopkins, *Nat Rev Drug Discov*, 2006, **5**, 993.

49 J. A. Cruz and E. Westhof, *Cell*, 2009, **136**, 604.

50 J. R. Thomas and P. J. Hergenrother, *Chemical Reviews*, 2008, **108**, 1171.

51(a) J. Krol, K. Sobczak, U. Wilczynska, M. Drath, A. Jasinska, D. Kaczynska and W. J. Krzyzosiak, *J Biol Chem*, 2004, **279**, 42230; (b) X. Zhang and Y. Zeng, *Nucleic Acids Res*, 2010, **38**, 7689; (c) J. Starega-Roslan, E. Koscianska, P. Kozlowski and W. J. Krzyzosiak, *Cell Mol Life Sci*, 2011, **68**, 2859.

52(a) A. Deiters, *AAPS J*, 2010, **12**, 51; (b) Y. Li, C. He and P. Jin, *Chem Biol*, 2010, **17**, 584; (c) S. Zhang, L. Chen, E. J. Jung and G. A. Calin, *Clin Pharmacol Ther*, 2010, **87**, 754.

53(a) B. D. Gooch and P. A. Beal, *J Am Chem Soc*, 2004, **126**, 10603; (b) M. Krishnamurthy, K. Simon, A. M. Orendt and P. A. Beal, *Angew Chem Int Ed Engl*, 2007, **46**, 7044.

54 B. P. Davies and C. Arenz, *Angew Chem Int Ed Engl*, 2006, **45**, 5550.

55 S. Neubacher, C. M. Dojahn and C. Arenz, *Chembiochem : a European journal of chemical biology*, 2011, **12**, 2302.

56 D. Bose, G. G. Jayaraj, S. Kumar and S. Maiti, *ACS Chem Biol*, 2013, **8**, 930.

57 G. Shan, Y. Li, J. Zhang, W. Li, K. E. Szulwach, R. Duan, M. A. Faghihi, A. M. Khalil, L. Lu, Z. Paroo, A. W. Chan, Z. Shi, Q. Liu, C. Wahlestedt, C. He and P. Jin, *Nat Biotechnol*, 2008, **26**, 933.

58 S. Melo, A. Villanueva, C. Moutinho, V. Davalos, R. Spizzo, C. Ivan, S. Rossi, F. Setien, O. Casanovas, L. Simo-Riudalbas, J. Carmona, J. Carrere, A. Vidal, A. Aytes, S. Puertas, S. Ropero, R. Kalluri, C. M. Croce, G. A. Calin and M. Esteller, *Proc Natl Acad Sci U S A*, 2011, **108**, 4394.

59 K. Gumireddy, D. D. Young, X. Xiong, J. B. Hogenesch, Q. Huang and A. Deiters, *Angew Chem Int Ed Engl*, 2008, **47**, 7482.

60 D. D. Young, C. M. Connelly, C. Grohmann and A. Deiters, *J Am Chem Soc*, 2010, **132**, 7976.

61 K. Watashi, M. L. Yeung, M. F. Starost, R. S. Hosmane and K. T. Jeang, *J Biol Chem*, 2010, **285**, 24707.

62 G. S. Tan, C. H. Chiu, B. G. Garchow, D. Metzler, S. L. Diamond and M. Kiriakidou, *ACS Chem Biol*, 2012, **7**, 403.

63 S. Chirayil, R. Chirayil and K. J. Luebke, *Nucleic Acids Res*, 2009, **37**, 5486.

64 J. P. Diaz, R. Chirayil, S. Chirayil, M. Tom, K. J. Head and K. J. Luebke, *RNA*, 2014, **20**, 528.

65 C. M. Connelly and A. Deiters, *Methods Mol Biol*, 2014, **1095**, 147.

66 S. M. Paul, D. S. Mytelka, C. T. Dunwiddie, C. C. Persinger, B. H. Munos, S. R. Lindborg and A. L. Schacht, *Nat Rev Drug Discov*, 2010, **9**, 203.

67 T. T. Ashburn and K. B. Thor, *Nat Rev Drug Discov*, 2004, **3**, 673.

68 D. Bose, G. Jayaraj, H. Suryawanshi, P. Agarwala, S. K. Pore, R. Banerjee and S. Maiti, *Angewandte Chemie-International Edition*, 2012, **51**, 1019.

69 M. Maiti, K. Nauwelaerts and P. Herdewijn, *Bioorg Med Chem Lett*, 2012, **22**, 1709.

70 D. D. Vo, C. Staedel, L. Zehnacker, R. Benhida, F. Darfeuille and M. Duca, *ACS Chem Biol*, 2014, **9**, 711.

71 S. P. Velagapudi, S. M. Gallo and M. D. Disney, *Nat Chem Biol*, 2014, **10**, 291.

72 F. S. Liang, W. A. Greenberg, J. A. Hammond, J. Hoffmann, S. R. Head and C. H. Wong, *Proc Natl Acad Sci U S A*, 2006, **103**, 12311.

73 M. Parisien and F. Major, *Nature*, 2008, **452**, 51.

74 P. T. Lang, S. R. Brozell, S. Mukherjee, E. F. Pettersen, E. C. Meng, V. Thomas, R. C. Rizzo, D. A. Case, T. L. James and I. D. Kuntz, *RNA*, 2009, **15**, 1219.

75 Z. Shi, J. Zhang, X. Qian, L. Han, K. Zhang, L. Chen, J. Liu, Y. Ren, M. Yang, A. Zhang, P. Pu and C. Kang, *Cancer Res*, 2013, **73**, 5519.

76 M. D. Disney, L. P. Labuda, D. J. Paul, S. G. Poplawski, A. Pushechnikov, T. Tran, S. P. Velagapudi, M. Wu and J. L. Childs-Disney, *J Am Chem Soc*, 2008, **130**, 11185.

77 S. P. Velagapudi, S. J. Seedhouse and M. D. Disney, *Angew Chem Int Ed Engl*, 2010, **49**, 3816.

78 S. P. Velagapudi and M. D. Disney, *Chem Commun (Camb)*, 2014, **50**, 3027.

79 S. Nahar, D. Bose, S. Kumar Panja, S. Saha and S. Maiti, *Chem Commun (Camb)*, 2014, **50**, 4639.

80 T. Hermann and E. Westhof, *Curr Opin Biotechnol*, 1998, **9**, 66.

81(a) G. A. Calin and C. M. Croce, *Nat Rev Cancer*, 2006, **6**, 857; (b) H. S. Soifer, J. J. Rossi and P. Saetrom, *Mol Ther*, 2007, **15**, 2070; (c) C. M. Croce, *Nat Rev Genet*, 2009, **10**, 704; (d) D. Sayed and M. Abdellatif, *Physiol Rev*, 2011, **91**, 827; (e) M. R. Schneider, *Br J Dermatol*, 2012, **166**, 22.

82 A. L. Jackson and P. S. Linsley, *Nat Rev Drug Discov*, 2010, **9**, 57.

83 Y. Fedorov, A. King, E. Anderson, J. Karpilow, D. Ilsley, W. Marshall and A. Khvorova, *Nat Methods*, 2005, **2**, 241.

84 A. Kozomara and S. Griffiths-Jones, *Nucleic Acids Res*, 2014, **42**, D68.

85(a) S. Djebali, C. A. Davis, A. Merkel, A. Dobin, T. Lassmann, A. Mortazavi, A. Tanzer, J. Lagarde, W. Lin, F. Schlesinger, C. Xue, G. K. Marinov, J. Khatun, B. A. Williams, C. Zaleski, J. Rozowsky, M. Roder, F. Kokocinski, R. F. Abdelhamid, T. Alioto, I. Antoshechkin, M. T. Baer, N. S. Bar, P. Batut, K. Bell, I. Bell, S. Chakrabortty, X. Chen, J. Chrast, J. Curado, T. Derrien, J. Drenkow, E. Dumais, J. Dumais, R. Duttagupta, E. Falconnet, M. Fastuca, K. Fejes-Toth, P. Ferreira, S. Foissac, M. J. Fullwood, H. Gao, D. Gonzalez, A. Gordon, H. Gunawardena, C. Howald, S. Jha, R. Johnson, P. Kapranov, B. King, C. Kingswood, O. J. Luo, E. Park, K. Persaud, J. B. Preall, P. Ribeca, B. Risk, D. Robyr, M. Sammeth, L. Schaffer, L. H. See, A. Shahab, J. Skancke, A. M. Suzuki, H. Takahashi, H. Tilgner, D. Trout, N. Walters, H. Wang, J. Wrobel, Y. Yu, X. Ruan, Y. Hayashizaki, J. Harrow, M. Gerstein, T. Hubbard, A. Reymond, S. E. Antonarakis, G. Hannon, M. C. Giddings, Y. Ruan, B. Wold, P. Carninci, R. Guigo and T. R. Gingeras, *Nature*, 2012, **489**, 101; (b) M. J. Hangauer, I. W. Vaughn and M. T. McManus, *PLoS Genet*, 2013, **9**, e1003569.

86 K. C. Wang and H. Y. Chang, *Mol Cell*, 2011, **43**, 904.

87 Y. Wan, M. Kertesz, R. C. Spitale, E. Segal and H. Y. Chang, *Nat Rev Genet*, 2011, **12**, 641.

88 S. Diederichs, *Trends Genet*, 2014, **30**, 121.

89 O. Wapinski and H. Y. Chang, *Trends Cell Biol*, 2011, **21**, 354.

90 T. R. Cech and J. A. Steitz, *Cell*, 2014, **157**, 77.