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Minireview

Emergence of Chemical Biology Approaches to the RNAi/miRNA Pathway

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RNA interference (RNAi) is a well-conserved mechanism that uses small noncoding RNAs to silence gene expression posttranscriptionally. Gene regulation by RNAi is now recognized as one of the major regulatory pathways in eukaryotic cells. Although the main components of the RNAi/miRNA pathway have been identified, the molecular mechanisms regulating the activity of the RNAi/miRNA pathway have only begun to emerge within the last couple of years. Recently, high-throughput reporter assays to monitor the activity of the RNAi/miRNA pathway have been developed and used for proof-of-concept pilot screens. Both inhibitors and activators of the RNAi/miRNA pathway have been found. Although still in its infancy, a chemical biology approach using high-throughput chemical screens should open up a new avenue for dissecting the RNAi/miRNA pathway, as well as developing novel RNAi- or miRNA-based therapeutic interventions.

Introduction

Increasingly complex networks of small RNAs act through RNA interference (RNAi) pathways to regulate gene expression in simple multicellular organisms to humans. RNAi is a well-conserved mechanism in which small regulatory RNAs silence gene expression posttranscriptionally (Bartel, 2004; Hannon, 2002). Gene regulation by RNAi has been recognized as one of the major regulatory mechanisms in eukaryotic cells (Plasterk, 2006). Small regulatory RNAs, including microRNAs (miRNAs), endogenous small interfering RNAs (esiRNAs), Piwi-interacting RNAs (piRNAs), and promoter-associated sRNAs (PASRs, or transcription initiation RNAs [tiRNAs]), are 18–30 nucleotides in length and can shape diverse cellular pathways, from chromosome architecture, development, and growth control to apoptosis and stem cell maintenance (Table 1) (Czech et al., 2008; Ghildiyal and Zamore, 2009; Kawamura et al., 2008; Kim et al., 2009; Okamura et al., 2008; Okamura and Lai, 2008; Plasterk, 2006; Taft et al., 2009). Mounting evidence suggests that the misregulation of miRNAs could contribute to the pathogenesis of a wide range of human diseases. Moreover, the RNAi mechanism has been adopted by researchers and is of broad utility in gene-function analysis, drug-target discovery and validation, and therapeutic development (Dykxhoorn and Lieberman, 2005, 2006). Given the pivotal roles of miRNAs in diverse biological pathways and human disease as well as the broad application of RNAi, understanding the mechanism of the RNAi/miRNA pathway is essential.

Although the major components within the RNAi/miRNA pathway have been identified, the molecular mechanisms regulating the activity of the RNAi/miRNA pathway have only begun to emerge within the last couple of years. Chemical biology, in particular the use of diverse chemicals to interrogate molecular processes, provides a novel means of rapidly and effectively dissecting biological mechanisms and gene networks in ways not feasible with mutation-based genetic approaches (Hergenrother, 2006; Lipinski and Hopkins, 2004; Schreiber, 2005). Unlike the traditional genetic approach, wherein nucleotide changes (mutations) are fixed, chemical biology offers a more dynamic way to monitor and study the activity of specific pathways. Thus, identification of the small molecules modulating the biological activity of the RNAi pathway will allow us to explore miRNA/siRNA biogenesis from a unique angle that could open up a whole new approach for dissecting the RNAi/miRNA pathway, as well as developing novel therapeutic interventions. Although a chemical biology approach to the RNAi/miRNA pathway is still in its infancy, with assays just in place and only a handful of screens being run, we highlight, in this article, recent discoveries of small-molecule modulators of the RNAi/miRNA pathway, which illustrate how a chemical biology approach could be used to dissect the RNAi/miRNA pathway.

The RNAi/miRNA Pathway and Its Regulation

Over the last several years, research has uncovered key protein components involved in the RNAi/miRNA pathway (Figure 1). In mammals, the majority of endogenous miRNA genes are transcribed initially as primary transcripts (pri-miRNAs) that range from hundreds to thousands of nucleotides (nt) in length and contain one or more extended hairpin structures (Du and Zamore, 2005). The nuclear RNase III enzyme Drosha, working with DGCR8, cleaves both strands near the base of the primary stem-loop and yields the precursor miRNA (pre-miRNA), an ~65 nt stem-loop that harbors the miRNA in the 5′ or 3′ half of the stem (Lee et al., 2003). The cleavage by Drosha defines one end of the mature miRNA and generates a 5′ phosphate and an ~2 nt 3′ overhang (Du and Zamore, 2005). However, recent studies have also identified short hairpin introns, called "mirtrons," that provide an alternative source for microRNA biogenesis (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). Mirtrons use the splicing machinery to bypass Drosha cleavage in initial maturation to produce pre-miRNAs. After being exported to the cytoplasm by exportin-5/RanGTP, pre-miRNAs are further cleaved by the RNase III Dicer along with a dsRNA-binding protein, TAR RNA-binding protein (TRBP), to define the other end of the mature miRNA and produce the double-stranded

Table 1. Types of Endogenous Small Regulatory RNAs

	Definition
MicroRNA (miRNA)	18- to 25-nucleotide noncoding RNAs that can regulate translation of target mRNA molecules in a sequence-specific manner.
Endogenous small interfering RNAs (esiRNAs)	Endogenous siRNA derived from bidirectional transcripts or the transcript of an inverted repeat.
Piwi-interacting RNAs (piRNAs)	26- to 31-nucleotide noncoding RNAs associated with Piwi-subfamily proteins. Most piRNAs are mapped to the repetitive regions of the genome and thought to control the activity of transposons.
Promoter-associated sRNAs (PASRs, or transcription initiation RNAs [tiRNAs])	Small RNAs mapped to the 5′-end or promoter region of the protein-coding genes that are proposed to have a role in divergent transcription.

miRNA/miRNA* duplexes (Du and Zamore, 2005). Dicer-TRBP complex is also required for the processing of short hairpin RNA (shRNA) into small interference RNA (siRNA) of ~21 bp (Zamore and Haley, 2005). After cleavage by Dicer and unwinding, one strand of the miRNA/miRNA* or siRNA duplexes (the antisense, or guide strand) is then preferentially incorporated into the RNA-induced silencing complex (RISC), while the other strand (the sense, or passenger strand) is degraded (Zamore and Haley, 2005). The RISC is a large and heterogeneous multiprotein complex. The core components of the RISC include Dicer, TRBP, and Argonaute 2 protein (AGO2); of these, AGO2, which was identified as the sole protein required for mRNA-cleaving RISC activity (Slicer activity), is the key RISC component (Figure 1) (Liu et al., 2004; Rand et al., 2004; Rivas et al., 2005).

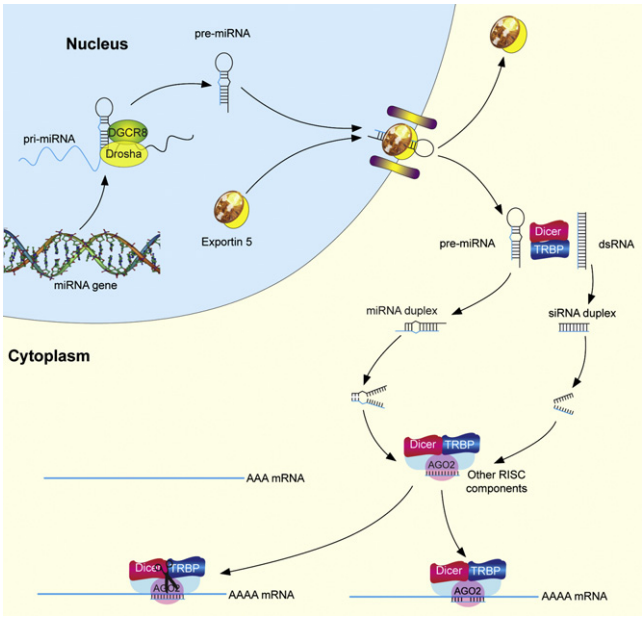


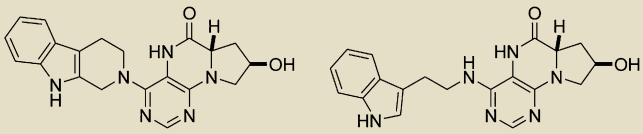
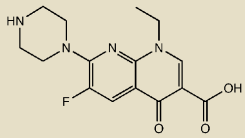
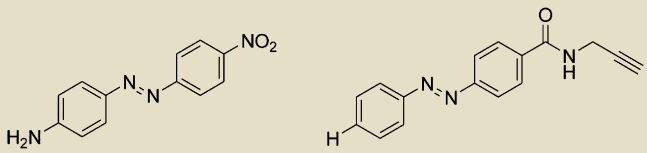
Figure 1. The RNAi/miRNA Pathway

Genes encoding microRNAs are initially transcribed by RNA polymerase II or III to generate the primary miRNA transcripts (pri-miRNA) within the nucleus. The stem-loop structure of the pri-miRNA is recognized and cleaved on both strands by a Microprocessor complex, which consists of the nuclear RNase III enzyme Drosha and an RNA-binding protein, DGCR8, to yield a precursor miRNA (pre-miRNA) 60-70 nucleotides in length. The pre-miRNA is then exported from the nucleus through a nuclear pore by exportin-5 in a Ran-GTP-dependent manner and processed in the cytoplasm by the RNase III Dicer-TRBP. Sliced RNA strands are further unwound. One strand of the miRNA/miRNA* or siRNA duplex (the antisense, or guide strand) is then preferentially incorporated into the RNA-induced silencing complex (RISC) and will guide the RISC to a target mRNA in a sequence-specific manner. Once directed to a target mRNA, the RISC can mediate translational regulation by inhibiting the initiation or the elongation step or through destabilization of the target mRNA. The illustrated number of base pairs does not reflect the actual length of either miRNA or siRNA.

The assembly and function of RISC complex can be divided into at least two catalytically controlled steps (Rana, 2007). The first kinetic checkpoint involves the loading of small RNAs onto RISCs. Dicer along with TRBP can process both shRNAs and pre-miRNAs into an ~21-nt RNA helix, on which RISC is assembled (Zamore and Haley, 2005). The passenger strand is cleaved by AGO2 and destroyed, and the guide strand is incorporated into the RISC to become active RISC (Leuschner et al., 2006; Matranga et al., 2005; Rand et al., 2005). The process of RISC loading is found to be influenced by the thermodynamics of the RNA duplex (Khvorova et al., 2003; Reynolds et al., 2004; Schwarz et al., 2003). The second step involves target recognition and cleavage by AGO2. RISC finds its mRNA target, cleaves the mRNA, and is recycled for potential additional cleavage. The kinetics of this step can be modulated by the target mRNA structure, structural reorganization of RISC, and product release (Rana, 2007). The protein components of RISC can also modulate the activity of RISC, particularly at these two checkpoints.

As key regulators in cellular functions, miRNAs themselves are shown to be tightly controlled at multiple levels. First, at the transcriptional level, transcription factors can directly regulate the expression of specific miRNAs, while specific miRNAs can then target another transcription factor and regulate its expression posttranscriptionally, resulting in a transcription factor—to miRNA—to another transcription factor (sometimes even of the miRNA itself) paradigm for gene regulation (Gangaraju and Lin, 2009). This represents a novel evolutionarily conserved strategy to maintain the balance between the miRNAs and their transcriptional regulatory programs. Second, miRNA processing, pri- to pre-miRNAs and pre- to mature miRNAs, can be regulated as well. Lin28, a developmentally regulated RNA-binding protein, was found to selectively block the processing of pri-let-7 miRNAs in embryonic cells by competing for binding to conserved nucleotides in the loop region of the let-7 precursor (pre-let-7) and inducing uridylation of pre-let-7 (Newman et al., 2008; Viswanathan et al., 2008). A noncanonical poly (A) polymerase, TUTase4 (TUT4), was identified as the uridylyl transferase involved in this process (Hagan et al., 2009; Heo et al., 2008, 2009). Furthermore, the KH-type splicing regulatory protein (KSRP, also known as KHSRP), a previously identified mediator of mRNA decay, was found to bind with high affinity to the terminal loop of the selective miRNA precursors and promotes their maturation (Trabucchi et al., 2009). These findings suggest that, besides the general components involved, other proteins could be involved in the efficient processing of specific miRNAs. Finally, posttranslational modifications can also regulate the activities of the core RISC factors. It was found that TRBP could be phosphorylated in response to Erk pathway activity (Paroo et al., 2009). Phosphorylation of TRBP enhances

Table 2. Small-Molecule Modulators of the RNAi/miRNA Pathway Identified via Chemical Screens

Small Molecule	Chemical Structure	Function and Target	Reference
ATP analogs: ATPA-18 (left) and ATPA-21 (right)		RNAi inhibitor, targets an early unwinding step in the RNAi pathway, possibly an ATP-dependent RNA helicase.	Chiu et al., 2005
Enoxacin		RNAi activator, targets TRBP and facilitates the interaction between TRBP and RNA	Shan et al., 2008
miR-21 Inhibitor		Specific inhibitor of miR-21, targets the transcription of miR-21	Gumireddy et al., 2008

its stability as well as that of its partner, Dicer, which increases the steady level of miRNA processing complex (Paroo et al., 2009). Furthermore, the hydroxylase C-P4H (I) was also shown to catalyze hydroxylation of Ago2 (Qi et al., 2008). Proline hydroxylation stabilizes the Ago2 protein, facilitating small RNA-guided mRNA cleavage (Qi et al., 2008). In addition, Ago2 could be phosphorylated via the p38 MAPK pathway (Zeng et al., 2008). Both the phosphorylation and hydroxylation enhance Ago2 localization to the P-body, though the physiological significance of P-body localization is unclear. These findings illustrate the different regulatory mechanisms of miRNA biogenesis and function, which could serve as potential targets for modulating the activity of the RNAi/miRNA pathway with small molecules.

Small Molecules Modulating the Activity of the RNAi/miRNA Pathway

The key step in any small molecule-based screen is the development of a reliable and robust assay, and both in vitro and in vivo assays have now been created. An in vitro fluorescence assay has been developed to measure the activity of Dicer (Davies and Arenz, 2006). In this assay, based on the pre-miRNA sequence of let-7, an RNA hairpin with a fluorescence emitter at the 5′ terminus and a fluorescence quencher at the 3′ terminus was prepared. Due to the close proximity of the fluorophore and the quencher, no fluorescence is detected in the case of an intact hairpin. Thus, the cleavage activity of Dicer can be measured by the increase of fluorescence signal. However, the application of this assay to small molecule screens has yet to be evaluated.

The in vivo assay was developed initially by transient cotransfection of EGFP-expressing plasmid and siRNA against EGFP (Chiu et al., 2005). In this assay, plasmids harboring enhanced green- and red-fluorescent proteins (EGFP and RFP, respectively) are cotransfected into HeLa cells with EGFP siRNA that targets EGFP mRNA for degradation. The ratio of EGFP/RFP fluorescence in the presence of siRNA is calculated and normalized to the EGFP/RFP ratio of mock-treated cells. Given the multiple ATP-dependent steps of the loading of the siRNA duplex onto active RISC, a small chemical library of substituted dihydropteridinones as ATP analogs that contain both rigid and flexible scaffolds was used for this screen; two nontoxic compounds (ATPA-18 and ATPA-21) that specifically inhibit ATP-dependent events during RNAi were found (Table 2) (Chiu et al., 2005). A series of in vitro and in vivo analyses demonstrate that these compounds specifically affect an early unwinding step in the RNAi pathway, suggesting that its target is an ATP-dependent RNA helicase, although the precise molecular target remains a mystery (Chiu et al., 2005). Interestingly, these compounds had no effect on the endogenous miRNA pathway. These results establish the timing of siRNA unwinding and suggest that siRNA helicase activity is required for RNAi.

In our own studies, we found that transient transfections with the DNA plasmid and the siRNA duplex have variable efficacy, making them unsuitable for future high-throughput screening. To address this problem, we developed a RNAi reporter system in which both an EGFP protein and an shRNA against EGFP are stably expressed (Figure 2) (Shan et al., 2008). In addition, selecting the proper cell clone for the chemical screen is also critical. Although the use of a clone with a strong knockdown of EGFP would have enabled us to identify inhibitors of the RNAi pathway robustly (EGFP expression would increase if the RNAi pathway were inhibited), it would nevertheless offer little chance of finding any small molecules that could enhance RNAi. We therefore chose to use a specific clone with a modest EGFP knockdown, thereby enabling us to identify both inhibitors and enhancers (Figure 2). Using this assay, we performed a proof-of-principal pilot screen with a collection of 2000 FDA-approved compounds and natural products and showed that the small molecule enoxacin enhances RNA interference induced by either shRNAs or siRNA duplexes and significantly reduces the siRNA dosage required to achieve gene knockdown in mammalian cells (Table 2) (Shan et al., 2008). Enoxacin was identified as an RNAi enhancer in an independent chemical screen from another group as well (Zhang et al., 2008). Enoxacin belongs to a family of synthetic antibacterial compounds based on a fluoroquinolone skeleton (Bhanot et al., 2001). A study of structure-activity

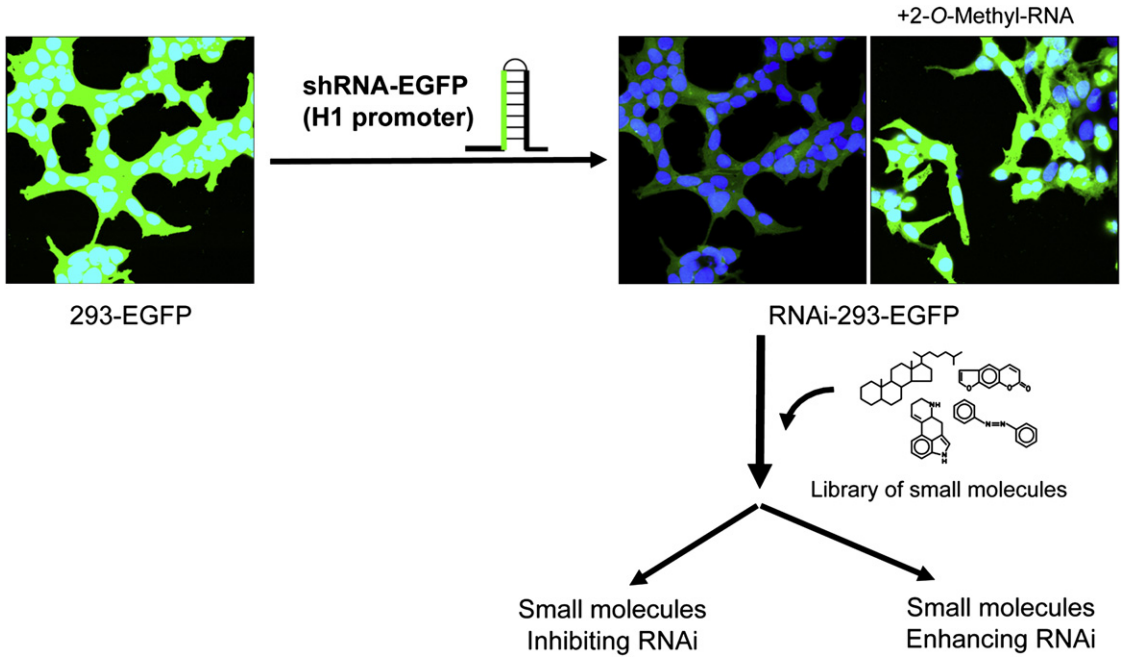


Figure 2. Development of a Reporter System to Monitor the Activity of the RNAi Pathway
Human 293 cells stably expressing EGFP (293-EGFP) were infected with lentivirus producing shRNA against EGFP (shRNA-EGFP); the resulting RNAi-293-EGFP cells with reduced GFP expression were isolated. The RNAi-293-EGFP cells transfected with 2-O-methyl RNA against the GFP siRNA are shown on the right with the recovery of GFP expression. The RNAi-293-EGFP cells could be used for a chemical screen to identify both inhibitors and enhancers of the RNAi pathway.

relationships has suggested that the RNAi-enhancing activity of enoxacin does not depend on general fluoroquinolone activity but rather on the unique chemical structure of enoxacin (Shan et al., 2008). Besides enhancing RNAi, enoxacin can also promote the biogenesis of endogenous miRNAs. Using a series of in vitro and in vivo analyses, we found that the enoxacin-mediated RNAi-enhancing activity is TRBP-dependent, and enoxacin could facilitate the interaction between TRBP and RNAs (Shan et al., 2008). Furthermore, we found that enoxacin had no effect on an in vitro RISC-cleavage assay, which argues against the potential involvement of enoxacin in the step of mRNA-target recognition and cleavage. Rather, these results together suggest that enoxacin targets the step of RISC loading by enhancing the interaction between TRBP and RNAs. Although the previous studies demonstrated the role of TRBP in the processing and loading of miRNAs/siRNAs onto the RISC, our findings reveal that TRBP plays important roles in modulating the activity or efficacy of siRNAs, and one could potentially increase RISC-loading efficiency and enhance RNAi by targeting TRBP-RNA interactions (Chendrimada et al., 2005; Förstemann et al., 2005; Jiang et al., 2005). Our findings also underscore the power of chemical biology, through which we could monitor the dynamic modulation of the RNAi pathway. It would have been quite a challenge to uncover this modulation using traditional genetic or biochemical approaches.

Besides unbiased chemical screens using different reporter systems, small molecules that modulate the activity of the RNAi/miRNA pathway could also be developed based on our understanding of RNAi/miRNA pathway regulation. As discussed above, both Ago2 and TRBP can be phosphorylated. Thus, inhibitors of specific kinases that phosphorylate these proteins could potentially be used to modulate the activity of the RNAi/miRNA pathway as well (Paroo et al., 2009; Zeng et al., 2008). However, those inhibitors are unlikely to specifically target the RNAi/miRNA pathway, since the identified kinases are well known to be involved in the regulation of many other biological pathways.

Small Molecules Modulating the Activity of Specific miRNAs

A similar screening strategy can also be developed for individual miRNAs. There is a luciferase-based screen system for miR-21, which has been directly linked to several human malignancies and may make a promising new target for the development of cancer therapeutics (Krichevsky and Gabriely, 2009). In this assay, the luciferase-miRNA complementary sequence plasmid serves as a sensor to detect the presence of specific mature miRNA. More importantly, this assay monitors the endogenous miR-21 in transfected cells, which is not biased toward or against any specific components of the miRNA pathway. Through a primary screen of > 1200 compounds, a compound with the diazobenzene core structure was discovered to inhibit the suppression mediated by miR-21 (Gumireddy et al., 2008). A further screen of ~100 structurally modified molecules related to the diazobenzene core structure led to the identification of a highly active compound (Table 2, miR-21 inhibitor-right) (Gumireddy et al., 2008). Interestingly, this compound is not a general inhibitor of the miRNA pathway but displays specificity for miR-21. Additional molecular analyses suggest that this compound is a selective inhibitor by targeting the transcription of the miR-21 gene, but not the downstream processes of the common miRNA pathway. However, the exact cellular target of this miR-21 inhibitor is still unknown.

Future Perspectives on Chemical Biology Approaches to the RNAi/miRNA Pathway

Through proof-of-concept pilot screens of limited numbers of small molecules, small-molecule modulators targeting specific steps in the RNAi/miRNA pathway have been identified. Therefore, it would be interesting to use the existing reporter systems to expand the screening to more comprehensive large-scale compound libraries containing hundreds of thousands of compounds with diverse scaffolds. Once small molecules capable of modulating the activity of the RNAi/miRNA pathway are identified, a multidisciplinary approach that integrates chemical synthesis, proteomics, biochemistry, and genetics should be adopted to understand how the identified molecules modulate the RNAi/miRNA pathway. Since a wide variety of chemical structures would enable us to probe many classes of potential targets, besides identifying small molecules targeting known components within the RNAi/miRNA pathway, large-scale chemical screens might reveal novel components of the RNAi/miRNA pathway, a feat not easily achieved by way of traditional forward and reverse genetic screens. Although this chemical biology or chemical genetic approach is indeed very powerful, there are some limitations that should be considered as well. Despite significant progress in proteomics recently, it is still a technical challenge to identify proteins specifically affected by novel small molecules. Small molecules, even well-characterized ones, might have multiple targets that could give rise to the observed modulation. After the identification of candidate protein targets, additional studies using other molecules targeting the same protein(s) and genetic experiments are necessary.

Although all the miRNAs in the genome use the same pathway to process pri-miRNAs to produce the functional mature miRNAs, we are now aware that miRNAs can be differentially processed in a given cell, and a given miRNA can be processed differently in different cell types (Thomson et al., 2006; Viswanathan et al., 2008). The mechanism underlying the regulation of miRNA processing has yet to be determined. Uncovering this mechanism is particularly important for those miRNAs that have been linked to human diseases and could serve as new therapeutic targets. Therefore, identifying small molecules that can modulate the activity of specific miRNAs is key. Although this may pose a significant challenge to chemical biology, its feasibility has been demonstrated by the identification of the miR-21 inhibitor (Gumireddy et al., 2008). Besides potentially targeting the protein(s) involved in the regulation of the processing of selective miRNAs, several small molecules have been reported to bind directly to RNAs, including miRNAs (Gooch and Beal, 2004; Henn et al., 2008; Thomas and Hergenrother, 2008; Tor, 2003). Through screening of a library of 7680 N-substituted oligoglycines (peptoids), specific ligands for the RNA hairpin precursor of miR-21 were identified; however, whether these ligands affect miRNA processing remains to be determined (Chirayil et al., 2009). Given the differences in RNA secondary structure among different miRNA precursors, it is likely that small molecules targeting specific pri-, pre-, or mature miRNA will be found.

Since miRNAs play major roles in nearly every cellular process, the identification and characterization of small-molecule modulators of the RNAi/miRNA pathway will yield fresh insights into fundamental mechanisms behind human disease. By studying the mode of action of these small-molecule modulators and identifying their protein targets, we could learn more about the dynamic regulation of the miRNA pathway and uncover additional factors that modulate the activity of the miRNA pathway. These small molecules could also be used to modulate the kinetics of specific step(s) in the miRNA pathway or specific miRNAs both experimentally and for therapeutic purposes. Moreover, these RNAi modulators, particularly RNAi enhancers, could potentially facilitate the development of RNA interference as a tool for biomedical research and therapeutic interventions. Although this field is still in its infancy, given the progress that has already been made and the widespread use of small molecule probes targeted to specific proteins, small-molecule RNAi modulators are likely to show their true potential in the very near future.

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Note Added in Proof

Most recently, two compounds that could suppress RNAi activity in cultured cells were identified and characterized; one could impair Dicer activity, while the other blocks small RNA loading into an Argonaute 2 complex (Watashi, K., Yeung, M.L., Starost, M.F., Hosmane, R.S., and Jeang, K.T. (2010). Identification of small molecules that suppress microRNA function and reverse tumorigenesis. J. Biol. Chem., in press. Published online June 7, 2010. 10.1074/jbc.M109.062976.). In addition, the first small molecule inhibitors and activators of the liver-specific microRNA miR-122 were also reported (Young, D.D., Connelly, C.M., Grohmann, C., and Deiters, A. Small molecule modifiers of microRNA miR-122 function for the treatment of the hepatitis C virus infection and hepatocellular carcinoma. J. Am. Chem. Soc. *132*, 7976–7981).