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PERSPECTIVE

Development and utilization of non-coding RNA–small molecule interactions

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RNA plays a crucial role in cellular biology as a carrier of genetic information. However, beyond this passive role, RNA has been shown to regulate various cellular processes in a form that is not translated into protein. Non-coding RNA (ncRNA) has been shown to be important in gene regulation, and its aberrant activity has been associated with several disease states. As such, ncRNAs represent a novel target for small molecule regulation and recently, significant advances have been made towards elucidating small molecule regulators of ncRNAs. Herein, we provide an overview of miRNA, siRNA, RNA aptamers, riboswitches, and ribozymes, within the context of recent findings regarding the exogenous regulation of these ncRNAs by small molecules. The development of these small molecule tools has far-reaching applications in the advancement of molecular therapeutics.

Introduction

RNA was once thought to act solely as an intermediate in the transfer of genetic information; however, over the last thirty years, scientific advancements have demonstrated its important role in gene regulation, and its propensity for catalytic activity.[1–5] Within the human genome, less than 2% of all nucleotides make up the open reading frames that encode proteins. As such, the remaining 98% was at one time believed to be evolutionary relics that lacked function.[6–8] However, the more recent discovery that non-coding RNAs (ncRNAs) have important and complex roles in regulating cellular function has enhanced our understanding of fundamental molecular and evolutionary biology.[9,10]

NcRNAs have been shown to affect chromatin structure, control transcription factor activity, dictate the posttranslational outcome of coding RNAs, and facilitate RNA-mediated catalysis.[11–18] More recently, aberrant ncRNA activity has been associated with the abnormal regulation of cellular processes in disease states.[19–21] This growing understanding of the vital roles that ncRNAs play in normal cellular function has made them important therapeutic targets. While most "druggable" targets are proteins, RNA possesses several features which suggest it is a viable candidate for drug intervention, specifically its ability to adopt complex secondary structures capable of specific ligand binding.[22] Small molecules thus provide an attractive means to regulate ncRNA activity as they possess favorable pharmacokinetic/pharmacodynamic (PK/PD) properties and are amenable to cellular delivery.[22,23]

Small molecule interaction with ncRNA can potentially disrupt the native nucleic acid conformation, altering biological activity. Previously discovered drugs that target RNA (*e.g.* aminoglycosides) are complex antibiotics which affect ribosomal RNA; however, new RNA targets are now being investigated and evolved.[24,25] Additionally, due to the prevalence of ncRNAs in gene regulation, pathways involving their biogenesis and processing are also viable candidates for novel small molecule therapies. While current approaches are in the early stages of development, one major challenge of targeting RNA with small molecules is the achievement of target specificity, either to the ncRNA–small molecule interaction itself, or to the global ncRNA pathway.

Herein we focus on several of the rapidly expanding classes of ncRNAs, namely miRNA, siRNA, RNA aptamers, riboswitches and ribozymes, and their potential for novel therapeutic applications; however, many other types of ncRNAs also provide interesting candidates in the context of small molecule regulation. Recently, several reviews have also investigated this interesting field,[22,23,26–31] and consequently we have attempted to focus on more recent discoveries.

Small molecule effectors of the miRNA/siRNA pathway

The RNA interference (RNAi) pathway is perhaps the most exciting target for small molecule regulation. RNAi pathways are highly conserved and tightly regulated networks consisting of both proteins and RNA that modulate gene expression. These pathways have also been shown to be prevalent in a range of organisms, including humans.[4] RNAi involves small single-stranded ncRNAs that are typically 19–24 nucleotides in length and derived from either longer primary transcripts or exogenously introduced double-stranded RNA (dsRNA).[15,32,33] These ncRNAs anneal to their target mRNA in a sequence specific fashion to silence gene expression *via* translational suppression or transcript degradation. The RNAi pathway utilizes exogenous

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small interfering RNAs (siRNA) or noncoding microRNAs (miRNAs) from the host genome as templates for directed gene silencing. These networks are considered to be one of the major regulatory mechanisms in eukaryotic cells and are thought to be responsible for regulating up to 30% of all genes.[34,35] miRNAs have been discovered to be involved in an array of biological processes, including development, apoptosis, cell proliferation, and chromosomal maintenance.[26,29] Additionally, several reports identify misregulation of miRNA as a key component in a variety of human diseases such as cancer, immune disorders, diabetes, neurodegeneration, and cardiovascular disease.[12,13,19] Due to its prevalence and regulatory nature, RNAi has recently become an ideal candidate for the rapidly advancing field of small molecule targeting and has been the subject of several previous reviews.[23,26,27]

Understanding the RNAi components

While all of the major participants of the RNAi pathway have been characterized, their mechanisms of regulation are still being elucidated (Fig. 1).[33] Endogenous miRNAs are initially subject to transcriptional regulation and are then transcribed as primary transcripts (pri-miRNA), ranging from 100–1000 nucleotides in length.[36] The pri-miRNAs are then processed in the nucleus by the enzyme Drosha, which facilitates cleavage of the pri-miRNA to yield precursor miRNA (pre-miRNA), before being exported by an exportin-5/RanGTP complex into the cytoplasm.[37,38] The miRNA and siRNA pathways now converge and pre-miRNAs or shRNAs/dsRNAs are further processed by Dicer in conjunction with TAR RNA-binding protein (TRBP) to yield mature miRNA/siRNA.[39] The appropriate guide strand is then unwound by a helicase and loaded into the RNA-induced silencing complex (RISC). RISC is a large multi-protein complex which includes

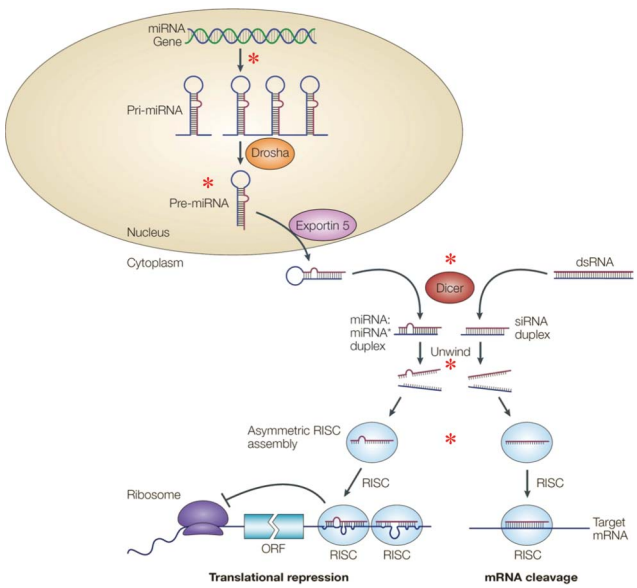


Fig. 1 RNAi and miRNA pathway. The biogenesis and processing of these ncRNAs result in gene silencing *via* either translational repression or mRNA cleavage, and misregulation can significantly influence cellular homeostasis leading to a disease state. As such, the pathway is a viable target for small molecule therapeutic intervention. Red asterisks indicate pathway components for which small molecule effectors have been discovered. *Adapted with permission from He et al., 2004.*[33]

Dicer and other proteins, including Argonaute (AGO), from which the complex derives its cleaving activity.[40] Based on the RNA sequence loaded into RISC, complementary mRNA is targeted for either degradation or translational repression, resulting in decreased expression of the cognate gene. Recently, miRNAs have alternatively been demonstrated to increase the expression of targeted genes.[41,42] Importantly, any point in this complex cascade is a potential target for small molecule effectors, which can dramatically alter the silencing potential of the entire pathway, and produce significant downstream effects.

Discovery of small molecules targeting the RNAi pathway

Traditionally, modified antisense oligonucleotides (*e.g.* phosophorothioate DNA, locked nucleic acids, antagomirs) that are complementary to a specific miRNA/siRNA have been used to regulate the RNAi pathway.[43–45] While the target specificity of antisense technology is robust, drawbacks to antisense approaches (*e.g.* difficult cellular delivery, poor PK/PD profiles, and high cost) are currently prohibitive,[23] and small molecules are now being explored to regulate RNAi. Additionally, it may be advantageous to target a specific protein involved in the RNAi pathway that is more accessible by small molecules. There are two major strategies for the discovery of small molecule modulators: 1) *in vitro* component-based assays which isolate and target a specific component of the pathway; and 2) *in vivo* assays which probe the entire pathway simultaneously, but require deconvolution of the small molecule target (Fig. 2).

1) Component-based assays for small molecule effectors. The ability to rapidly screen a large collection of small molecules *in vitro* is advantageous, as one can directly target a specific component of the RNAi pathway in a high throughput fashion. However, initial activity may not translate to *in vivo* efficacy. To date, only two *in vitro* assays to screen small molecule effectors of the RNAi pathway have been described.

Arenz *et al.* developed the first assay to identify small molecule regulators of Dicer.[46,47] Their fluorescence-based assay employed a hairpin miRNA precursor probe (pre-let-7; a well studied pre-miRNA) containing a 5′ fluorescein label and a 3′ dabcyl quencher (Fig. 2A). Due to the proximity of the fluorophore and quencher, only Dicer processing of the pre-miRNA enables fluorescence. Incubation with either recombinant or cell lysate Dicer *in vitro* afforded a 3–10 fold increase in fluorescence. The reporter system was then investigated with the known inhibitors kanamycin (an RNA binder) and dodecapeptides derived from the Dicer sequence (competitive inhibitors), to afford 10–85% inhibition of Dicer. Interestingly, although initially published in 2006, no small molecule screens employing this assay have been reported. This may suggest that the selection of an appropriate small molecule library to disrupt Dicer function is somewhat challenging or that the assay may not be robust enough to function in a high-throughput setting. Additionally, this is currently the only reported *in vitro* assay which targets a protein component of the RNAi pathway. Future work will likely involve targeting other prominent components of the pathway with greater efficacy, as the prevalence of several key proteins within this pathway represents the most direct approach for pathway targeting.

A second *in vitro* assay developed by Luebke and co-workers targeted miRNA directly to elucidate specific ligands for

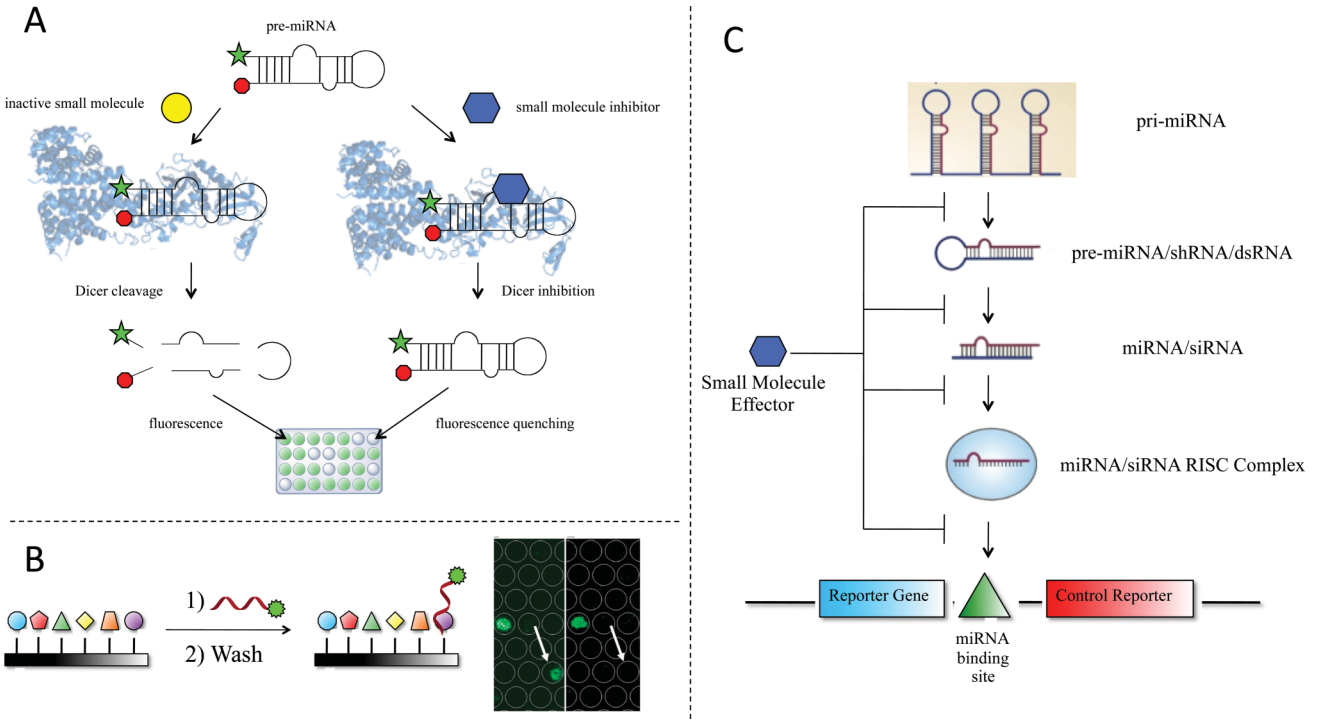


Fig. 2 Assays developed to elucidate small molecule effectors of miRNA and siRNA. A) Schematic of an *in vitro* assay for inhibition of Dicer processing. A molecular beacon pre-miRNA containing a fluorophore and a quencher are incubated in the presence of Dicer. Proper Dicer processing results in cleavage, and a fluorescent signal, while small molecule inhibition results in no pre-miRNA processing, maintaining the fluorescence quenching. B) An *in vitro* assay for pre-miRNA binders. Peptoid libraries were immobilized in a spatially addressable fashion on a surface and incubated with a fluorescently labeled pre-miRNA. After washing, peptoids capable of binding the pre-miRNA retain the fluorescent probe. C) Schematic for *in vivo* small molecule assays. A transfected reporter plasmid (GFP or luciferase) is directly affected by another introduced siRNA/miRNA or by endogenous miRNA. The effect of the small molecule on gene expression is assessed followed by target identification. This assay can be employed for both RNAi activators and inhibitors.

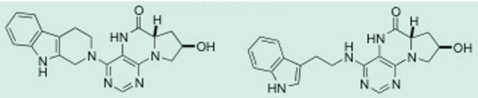
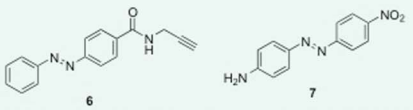
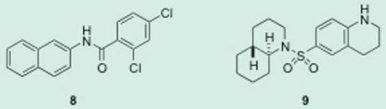
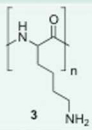
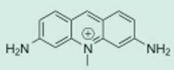
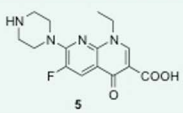
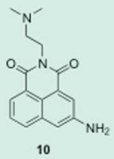
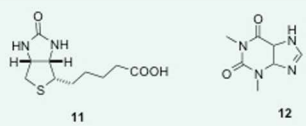
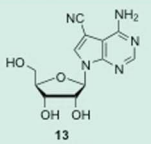
miRNA-21 (miR-21), since ligand binding may ultimately affect miRNA processing and inhibit its downstream effect.[48] To rapidly identify ligands, microarrays of spatially isolated peptoids were synthesized using 21 monomers, theoretically representing 9261 compounds. Microarrays were then incubated with a fluorescently labelled pri-miRNA or pre-miRNA and were imaged for fluorescence (Fig. 2B). Two peptoids showed promise as specific miR-21 binders with one possessing a 1.9 μM dissociation constant and a 20-fold discrimination against a closely related hairpin RNA. No cell-based assays have yet been reported to determine the effects of this peptoid on the RNAi pathway, or if its association/hairpin stabilization will affect the processing and activity of miRNA. Additionally, the assay requires immobilization of potential ligands, which may ultimately limit its utility in the identification of other small molecule effectors. However, this assay suggests the potential for miRNA target specificity, which will be useful in the development of drugs that target specific ncRNAs (rather than the entire pathway), minimizing off-target effects.

2) System-based assays for small molecule effectors. A more common approach to the identification of small molecule regulators of the RNAi pathway has been to use cell-based assays to screen all components of the pathway simultaneously. This approach takes into account all potential mechanisms of regulation, but is subject to an increase in false-positive hits due to off-target effects, as well as requiring the identification of the small molecule mechanism of action (Fig. 2C). Such cell-based assays utilize a reporter gene that is regulated by the siRNA/miRNA pathway, and changes in expression levels are correlated to small molecule interaction.

Chiu and co-workers reported the first small molecule/RNAi screen in 2005 in which HeLa cells were co-transfected with a GFP-targeting siRNA and a plasmid encoding both GFP and RFP.[49] A small library of dihydropteridinone ATP analogs was individually screened, and the GFP/RFP ratio was monitored in the absence of either small molecule or siRNA. Two compounds, **1** and **2**, were identified as potential inhibitors of the pathway because they afforded a dose dependent increase in GFP fluorescence (Table 1). These compounds were also able to affect siRNA silencing of an endogenous CDK9 gene. Based on the time dependent nature of small molecule inhibition, a biotinylated siRNA pull-down and a FRET assay determined that **1** and **2** were involved in an early step in the RNAi pathway, most likely helicase unwinding of siRNA. Due to this effect in cell culture, future studies of the efficacy of these compounds in an animal model would be especially interesting.

Another example of a system based screen towards small molecule regulators of the RNAi pathway was recently demonstrated by the Jeang laboratory[50] in which the homology to the siRNA pathway was exploited to discover modulators of the miRNA pathway. HEK293T cells were transfected with plasmids encoding firefly and *Renilla* luciferase as well as a shRNA

Table 1 Small molecule effectors of ncRNAs

Effector(s)	Function	Target	Reference
 1 2	Inhibitor	RNA unwinding; most likely RNA helicase	Chiu et al., 2005
 6 7	Inhibitor	miRNA-21; most likely a transcription factor	Gumireddy et al., 2008
 8 9	Inhibitor	miRNA-122; most likely a transcription factor	Young et al., 2010
 3	Inhibitor	Dicer mediated processing	Watashi et al., 2010
 4	Inhibitor	TRBP/AGO2; disrupts protein-protein association	Watashi et al., 2010
 5	Activator	TRBP; facilitates interaction with RNA	Shan et al., 2008 Zhang et al., 2008
 10	Activator	miRNA-122; most likely a transcription factor	Young et al., 2010
 11 12	Binder	aptamer regulation of RNA constructs	Jenison et al., 1994 Wilson et al., 1998
 13	Inhibitor	ribozyme activitiy	Yen et al., 2004

plasmid that targets firefly luciferase. Screening of 530 compounds identified two compounds, polylysine **3** and trypflavine **4**, which reproducibly suppressed the luciferase ratio (firefly/*Renilla*) at ~1 μM concentrations, while having little effect on a negative control GFP assay (Table 1). To probe the mechanism of action, a FLAG-AGO2 cell line was used to enable the pull down of argonaute–RNA complexes within RISC. Ratios of total siRNA *versus* RISC-associated siRNA afforded insight into possible modes of small molecule action, as a decreased ratio indicated poor siRNA loading into RISC, while a higher ratio indicated an effect further upstream in the pathway. Interestingly, **3** and **4** had opposite ratios, suggesting differing mechanisms of action. Using several Dicer processing and association assays, **3** was found to be an inhibitor of Dicer–RNA complexes, resulting in reduced processing of the shRNA/pre-miRNA, while **4** was found to directly block siRNA/miRNA loading into RISC by disrupting either the TRBP/AGO2 or RHA/AGO2 associations. The effects of these compounds were then examined with endogenous miRNAs, facilitating a detectable knockdown on all of the six tested miRNAs. Excitingly, these compounds effectively demonstrated that miRNA is indeed a target for small molecule therapeutics. Over-expression of miR-93 has been linked with tumorgenesis, and implantation of cells over-expressing miR-93 and miR-130 in a mouse model led to tumor formation (Fig. 3B). Interestingly, treatment with either **3** or **4** significantly reduced the ability of the cells to form tumors.

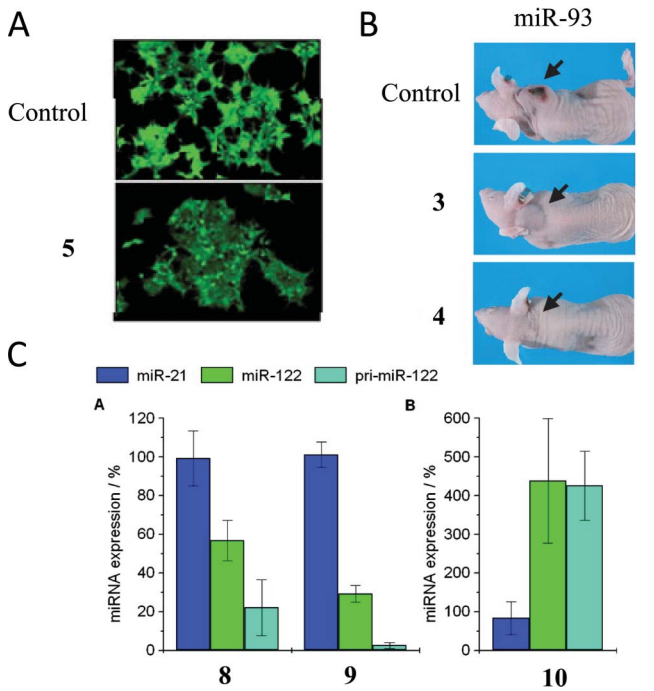


Fig. 3 Elucidation of small molecule effectors of miRNA/siRNA. A) Compound **5** was found to be an activator of the RNAi pathway *via* facilitating the TRBP interaction with RNA. As seen in the figure the degree of EGFP silencing is higher in the compound treated cells *versus* a control. *Adapted with permission from Shan et al.*, 2008. B) Utilization of small molecules in a nude mouse model. 3T3-miR93 cells were pretreated without or with either **3** or **4** and transplanted subcutaneously into mice. Treated cells did not form tumors in the mice, potentially due to the inhibition of miR-93 by the small molecules. *Adapted with permission from Watashi et al., 2010.* C) RT-PCR assay of miR-122 inhibitors **8** and **9**, as well as miR-122 activator **10**. Treatment with compounds relative to a DMSO control lead to alterations in both miR-122 and pre-miR-122 levels, but had no effect on miR-21 levels, demonstrating a degree of target specificity. *Adapted with permission from Young et al., 2010.*[54]

While the previous studies elucidated small molecule inhibitors, both the Jin and Xi laboratories simultaneously discovered a small molecule RNAi activator, and interestingly, both screens converged on the same chemical compound.[51,52] The Jin laboratory assayed HEK293T cells stably expressing both GFP and a short hairpin RNA (shRNA) precursor targeting the GFP gene. The authors note that the stable introduction of these components reduced experimental variability associated with transient transfection utilized in other assays. Additionally, effort was taken to select a proper clone that did not exhibit complete knockdown, enabling the screening of both inhibitors and activators. Using this assay, 2000 FDA approved compounds were screened. Enoxacin (**5**), a fluoroquinolone, was identified as an enhancer of the RNAi pathway (Table 1). In the absence of **5**, decreased fluorescence was detected relative to the shRNA control (Fig. 3A). Other quinolones that were screened had little to no effect relative to **5**, suggesting that the molecule does not follow the typical fluoroquinolone mechanism to reduce steady-state mRNA levels. Analysis of miRNA levels in the presence and absence of **5** indicated that it promotes processing of the miRNA/siRNA effectors. Processing of pre-*let*-7 and pre-miR-30a in the presence of **5** was only enhanced when incubated with both Dicer and TRBP (but not Dicer alone), and TRBP knockdown suggested that enoxacin activity is TRBP dependent. Enoxacin was then examined in a GFP transgenic mouse model and found by RT-PCR to reduce siRNA knockdown from 20% to 60% with no knockdown in the absence of siRNA construct. Thus, although **5** acts as a small molecule effector of RNAi, the generality of the target may be prohibitive as it can lead to a global increase of all miRNAs, offsetting the delicate balance of their expression. While **5** holds clinical promise in regulating RNAi, examination of off-target effects and further compound optimization are required to assess its therapeutic potential.

The Deiters lab focused specifically on the miRNA pathway in an attempt to elucidate both small molecule inhibitors and activators.[53,54] Employing a luciferase based reporter system, the miRNA binding site of specific miRNAs was cloned into the 3′ untranslated region of the luciferase gene (as opposed to the previously described studies which employed siRNAs that recognize a specific region within the gene). In the absence of the small molecule, mature miRNAs were produced which silenced the luciferase reporter. However, small molecules that inhibited the pathway decreased the concentration of mature miRNA, preventing their binding and resulting in increased luciferase signal. Initial studies targeted miR-21 (due to its misregulation in various cancers) and generated HeLa stable cell lines expressing a miR-21 luciferase reporter construct.[53] HeLa cells were employed due to their high levels of endogenous miR-21, which eliminates the need for exogenous introduction. Approximately 1000 compounds were screened for increases in luciferase expression, and after a small SAR study, the molecules **6** and **7** were discovered, with **6** leading to a 485% increase in luciferase expression relative to a DMSO treated control (Table 1). Further investigation indicated that this molecule is somewhat specific to regulation of miR-21, as constructs that contained the recognition sequence of other miRNAs did not lead to increased expression in the presence of **6**. Additionally, RT-PCR analyses showed that levels of both mature miR-21 and pri-miR-21 were decreased. While the direct target of small molecule intervention was not determined, these results suggest it is active on a pri-miRNA transcriptional level, which is most likely where the specificity of **6** is derived. Thus, this molecule has therapeutic potential in specific regulation of miR-21, but no further investigations into its use have been reported.

More recently, a similar screen has been conducted against miR-122, which has been shown to be necessary for hepatitis C virus (HCV) replication.[54] It is also significantly downregulated in hepatocellular carcinomas (HCC). The assay was modified with a dual Firefly/*Renilla* luciferase reporter transiently transfected into Huh7 cells (which have high levels of miRNA-122 expression). The NCI Diversity set of 1364 compounds was screened, and compounds **8** and **9** were found to significantly increase luciferase expression. RT-PCR studies confirmed the decreased levels of both mature miRNA-122 and pri-miRNA-122, again suggesting transcriptional regulation (Fig. 3C). Even more exciting, compound **10** was discovered in the same screen that further decreased luciferase expression, suggesting it is capable of activating the miRNA pathway; this was also confirmed by increased mature miR-122 and pri-miR-122 levels by RT-PCR (Fig. 3C). The inhibitory compounds **8** and **9** were then utilized to demonstrate a decrease in HCV levels relative to DMSO controls in HCV infected Huh7 cells (Table 1). Additionally, **10** was employed in HepG2 HCC

cells to induce apoptosis *via* increasing the levels of miR-122, further demonstrating the therapeutic relevance of small molecule effectors of the miRNA pathway. While an advancement to the field, the therapeutic targets of these molecules still have yet to be elucidated, and their efficacy in animal models has not been determined. However, the specificity gained by targeting transcriptional pathways *versus* actual components of the RNAi pathway is a valuable strategy.

All of these results contribute to the importance of small molecule effectors of RNAi. However, further studies are required for compounds that specifically affect the pathway and do not result in global modification of all miRNA processing, potentially resulting in more deleterious than beneficial effects. Further work in this exciting field will be able to address these concerns and identify viable targets of the RNAi pathway.

Small molecule effectors of RNA aptamers, ribozymes, and riboswitches

RNA aptamers

Nucleic acid aptamers provide another exciting example of the interaction of ncRNAs and small molecules. RNA aptamers are short oligonucleotides typically 60–80 nucleotides in length that recognize and bind to their target with extremely high affinity and specificity.[55,56] RNA aptamers are illustrative of the ability of RNA to specifically recognize small molecules, a feature which can be exploited to achieve a higher level of control over biological systems. Aptamers occur naturally, or can be rapidly engineered from a library of random sequences using an *in vitro* selection strategy known as systematic evolution of ligands by exponential enrichment (SELEX)[57,58] to recognize small molecules (for a comprehensive review of aptamer design and applications, see ref. 59). Many different aptamers have been evolved for small molecules such as ATP (K_d of 0.7 μM),[60] biotin, (**11**; K_d of 6 μM, Table 1)[61] and theophylline (**12**; K_d of 0.29 μM, Table 1).[62] Impressively, these aptamers are capable of distinguishing with high stringency between their targets and structurally similar small molecules. For example, the theophylline aptamer binds caffeine, which is nearly structurally identical to theophylline except for an additional methyl group, with a K_d of ~3500 μM, or with approximately 10 000 times less specificity than **12**. In these cases, instead of screening a small molecule library for a specific RNA effector, an RNA library is screened against a specific small molecule.

The use of ncRNA aptamer–small molecule interactions has had various applications in biological systems. An illustrative example was reported by the Yokobayashi lab, employing aptamers in combination with RNAi to regulate gene expression.[63] A vector was designed with a shRNA targeting GFP fused to the theophylline aptamer at its hairpin loop. When the aptamer domain was incubated with **12** the putative Dicer cleavage sites were blocked, inhibiting Dicer cleavage and suppressing RNAi *in vitro*. Similarly, co-expression of the shRNA-aptamer with GFP in HEK293T cells led to theophylline-mediated inhibition of RNAi, where greater fluorescence was observed as the small molecule concentration increased. This was the first report of dose-dependent aptamer-mediated post-transcriptional inhibition at a key point in the RNAi pathway and provides a general platform for targeting Dicer activity that is adaptable to other small molecules. More recently, the previously described aptamer system was used to target endogenous expression of albumin in HepG2 cells.[64] The addition of **12** again inhibited RNAi, restoring up to 80% of wild type albumin concentration. Quantitative real-time PCR showed decreased transcript levels in cells expressing the shRNA-aptamer or a shRNA control; however, in the presence of theophylline, cells expressing shRNA-aptamer had recovered levels of transcript. In a similar approach, Henn *et al.* abrogated Dicer activity with a guanosine-rich shRNA substrate that formed a G-quadruplex; the resulting quadruplex blocked Dicer and prevented shRNA processing.[65] However, the small molecules bisquinoline and porphyrazin, known to target the G-rich regions, were bound by the shRNA. This prevented quadruplex formation and successfully knocked down expression of a luciferase reporter gene in HEK293T cells. These methods demonstrate the ability to utilize evolved small molecule–ncRNA interactions to perturb gene expression. However, one substantial drawback to this approach in the context of therapeutic intervention is the required cellular delivery of both an aptamer construct and its small molecule effector.

Additional investigations into aptamer-mediated control of gene expression have led to the design of riboregulators that both down- and up-regulate translation. Bayer and Smolke coupled aptamer and antisense technology in *Saccharomyces cerevisiae* to engineer allosteric, ligand-responsive switches termed antiswitches.[66] An antiswitch is comprised of an antisense domain that is initially sequestered in a stem-loop; in this "off" state, the antiswitch cannot bind mRNA (Fig. 4). However, the change in conformation that occurs when an adjacent aptamer domain binds to its small molecule effector activates the antiswitch. Once "on," the freed antisense domain hybridizes to its target transcript and sterically inhibits ribosomal translation.

A theophylline-inducible antiswitch that targets GFP mRNA, s1, was designed so that co-expression *in vivo* with GFP resulted in a 30% decrease in fluorescence. In the presence of >0.8 mM **12**, the antiswitch was activated and fluorescence levels decreased to that of background. Conversely, expression in the presence of caffeine resulted in GFP expression levels comparable to the inactive antiswitch. Quantitative RT-PCR evaluation of cells expressing both the s1 antiswitch and GFP showed a constant amount of GFP transcript both in the absence and in the presence of **12**, confirming that the antiswitch affects gene expression by inhibiting translation.

Bayer and Smolke also varied the aptamer domain to create antiswitches that respond to other small molecules, showing that a new antiswitch can be readily constructed from a known aptamer sequence, or with new aptamers evolved *in vitro*. Of these, a tetracycline-inducible antiswitch, s9, was utilized in combination with s1 to obtain small molecule-mediated control over the expression of two genes simultaneously. In yeast, co-expression of s9 (which targets a variant of yellow fluorescent protein (YFP)), and s1 in the absence of their small molecule effectors led to minimal translational suppression of target mRNAs. However, with the addition of **12**, GFP expression was reduced to background levels while YFP expression was unaffected. Conversely, addition of tetracycline to activate s9 suppressed only YFP fluorescence. Addition of both small molecules turned on both s1 and s9, inhibiting translation of their respective mRNA targets. The antiswitch

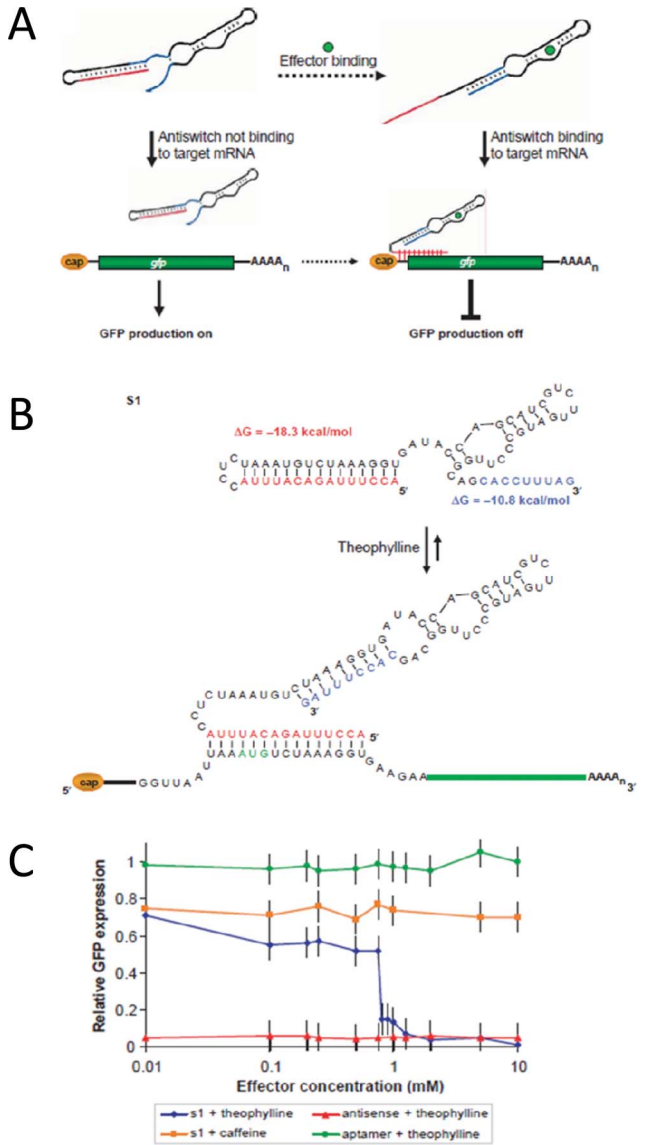


Fig. 4 Design of the s1 theophylline antiswitch and its use in controlling GFP expression. A) In the absence of the **12**, the s1 antiswitch cannot bind to its mRNA target and GFP is expressed. Binding of **12** alters the conformation of the aptamer domain (blue), enabling the antisense domain (red) to bind to the GFP transcript and inhibit translation. B) Sequence and predicted structure of the s1 antiswitch before and after binding to the GFP transcript. C) GFP expression levels decrease in the presence of s1 at concentrations between 0.8 and 1 mM of **12**(blue). In contrast, caffeine shows little effect on s1 activity (orange). The theophylline aptamer control has no effect on GFP expression when incubated with **12** (green), and the antisense control maintains activity regardless of **12** addition (red). *Adapted with permission from Bayer et al.*, 2005.[66]

platform thus serves as an effective and modular riboregulating platform to obtain small molecule-mediated control over gene expression.

Aptamers provide an exciting means for obtaining small molecule control over biological processes. Aptamers alone continue to evolve as important biological mediators and future work will provide additional insight into their therapeutic relevance. Importantly, aptamers can be coupled with other ncRNAs to provide regulation of gene expression and subsequent therapeutic targets.

Riboswitches

Similar small molecule–ncRNA interactions form the basis of a riboswitch. Riboswitches are naturally occurring ncRNAs that regulate gene expression in response to metabolites or secondary messengers, thereby serving as molecular sensors.[67] They consist of an aptamer domain in the 5′ UTR of the transcript and a downstream expression platform that modulates expression by either sequestering the ribosome binding site (RBS) of the mRNA to inhibit translation, or forming a stem loop in the growing transcript to inhibit transcription by RNA polymerase.[68] Regulation of gene expression is thus governed by conformational changes in the expression platform that occur in response to effector binding within the aptamer domain. (For detailed structural and mechanistic reviews of natural and synthetic riboswitches, see refs. 69, 70.)

Riboswitches were first identified in the Breaker lab in 2002.[71] It was discovered that mRNA from the *btuB* gene in *E. coli* contained an aptamer domain that bound coenzyme B12 to autoregulate *btuB* expression. Such modulation in response to effector concentration demonstrated that riboswitches function as metabolic sensors. In the years since, more than a dozen classes of riboswitches have been identified in bacteria, plants, and fungi, and synthetic riboswitches have been engineered in *E. coli* and *Bacillus subtilis*.[72–75] Additionally, riboswitches have recently been discovered with ribozyme domains that enable *cis*-cleavage of cognate mRNA. In 2004, Suess *et al.* reported the first rationally designed riboswitch that was used to control gene expression in *B. subtilis*.[74] The switch was designed with a theophylline aptamer domain upstream of a helix slipping module whose native conformation inhibited access to the RBS. Introduction of **12** induced a shift by one nucleotide in the secondary structure of the module to free the RBS and enable translation in a dose-dependent manner.

Harvey *et al.* demonstrated a riboswitch approach *in vitro* and *in vivo* by engineering up to three aptamer domains (both **11** and **12**) into the 5′ and 3′ untranslated region (UTR) of the mRNA of a CAT reporter gene.[76] Translation *in vitro* in the presence of **12** was almost completely inhibited when CAT mRNA contained three copies of the theophylline aptamer. In the absence of **12**, only a slight reduction in translation was noted, presumably due to a new adoptive folding conformation facilitated by the presence of the aptamer regions. No effect was noted in the presence of caffeine. *In vitro* translation of the biotin aptamer constructs in the presence of **11** was inhibited only when the aptamers were placed in the 5′ UTR. Using a eukaryotic translational system supplemented with **11**, they found that formation of the 80S ribosome initiation complex and binding of the 40S ribosomal subunit to the wild type mRNA template occurred normally. However, formation of the initiation complex and binding to the target mRNA were inhibited when the transcript contained 5′ biotin aptamers. Similar results were also observed *in vivo* in xenopus embryos microinjected with both **11** and either wild type mRNA or mRNA harboring biotin aptamer sequences. CAT activity was analyzed in oocyte extracts, and radiolabeled mRNA was imaged to ensure that the transcript was not degraded, which

would result in a false-positive decrease in expression. Co-injection of **11** and CAT mRNA containing three 5′ UTR biotin aptamer sequences inhibited expression, whereas co-injection with biotin and CAT mRNA (without aptamers) showed no knock-down. These experiments successfully demonstrated that tuned small molecule–aptamer interactions are readily capable of disrupting gene expression at the translational level.

More recently, the development of high-throughput screening methodologies has identified a number of riboswitches that modulate gene expression.[77] In prokaryotes, these riboswitches provide attractive targets for small molecule strategies that may constitute the next generation of antibiotics to treat increasingly antibiotic-resistant bacteria. Previous work in the Breaker group has shown that the antimicrobial compounds pyrithiamine and *S*-(2-aminoethyl)-L-cysteine are small molecule effectors that modulate *B. subtilis* thiamine pyrophosphate (*TPP*) and lysine (*lysC*) riboswitches, respectively.[78,79] Additionally, the antibiotic roseoflavin from *Streptomyces davawensis* was identified as an effector of the flavin mononucleotide (FMN) riboswitch.[80] In the future, additional screens will identify riboswitches that are candidates for small molecule antibiotics, while alternatively, small molecules drugs can be synthesized to specifically target known riboswitches. In this manner, exploitation of small molecule–riboswitch interactions holds great therapeutic promise.

Allosteric ribozymes

Ribozymes, or RNA enzymes, are a unique class of naturally occurring ncRNAs that are capable of catalyzing chemical reactions.[81] The first ribozymes were discovered independently in the laboratories of Sidney Altman and Thomas Cech.[82,83] Prior to their work, only protein enzymes were thought to have catalytic properties. However, in the thirty years since these seminal findings, several ribozymes have been identified that catalyze self-cleavage or cleavage of a complementary RNA substrate. Catalysis occurs when the ribozyme adopts a specific tertiary structure that enables its 2′-OH to cleave an adjacent intra- or intermolecular phosphodiester bond.[84,85] A variety of ribozyme classes exist and have been thoroughly reviewed.[73] Self-cleaving ribozymes embedded in the 5′ UTR of mature mRNA (such as the *glm*S ribozyme that functions as a riboswitch; see *Aptamers* and *Riboswitches*) or in the 3′ UTR (as in the C-type lectin type II (CLEC2) genes) have been shown to regulate gene expression in prokaryotes and eukaryotes, respectively. This activity represents a target for small molecule regulation.

Through *in vitro* evolution, ribozymes have been engineered with aptamer domains to mediate their activity.[86] These so-called allosteric ribozymes cleave their target only in the presence of a small molecule effector. Of these, the hammerhead ribozyme has been widely utilized to allosterically control catalysis.[87] This was first demonstrated in the Breaker laboratory, who designed a hammerhead ribozyme with a theophylline aptamer domain with RNA cleavage occurring only in the presence of **12**.[88] Several additional applications have been reported for this ribozyme, including photochemical control over its catalysis *in vitro* by photocaged theophylline to obtain spatio-temporal control over activity.[89]

Allosteric hammerhead ribozymes have been utilized to regulate gene expression in bacteria and yeast and more recently, in mammalian cells.[88,90,91] Previous work by the Breaker and Mulligan labs identified theophylline-responsive hammerhead ribozymes that cleaved their substrates with high efficiency *in vitro*, but possessed poor activity when coupled with mammalian transcripts *in vivo*. As such, Auslander *et al.* recently optimized a theophylline hammerhead ribozyme for use in a mammalian culture and demonstrated its increased efficiency in HEK293T and HeLa cells.[92] The authors removed two AUG sites within the ribozyme that were thought to function as alternative start codons, and employed *in vivo* screens to identify ribozyme sequences with greater differences in activity when bound *versus* unbound to **12**. The resulting optimized hammerhead ribozymes showed greater activity in mammalian cells and facilitated better small molecule-mediated control over gene expression.

Alternatively, Kumar *et al.* reported the use of allosteric hammerhead ribozymes in conditionally mediating RNAi in HEK293T cells.[91] They designed a transcript containing a double-stranded pri-miRNA hairpin coupled to the theophylline hammerhead ribozyme, with an inhibitory strand that forms a stable stem with the 5′ end of the pri-miRNA. In the absence of **12**, Drosha cannot process the pri-miRNA, which is sequestered by the inhibitory strand. However, in the presence of **12**, the ribozyme cleaves itself, freeing the pri-miRNA from the inhibitory strand and providing a substrate for Drosha. Once processed, the miRNA would activate the RNAi mechanism to ablate target gene expression. With this strategy they obtained knock-down of GFP expression and detected ribozyme-cleaved pri-miRNA and mature miRNA only in cells exposed to **12**. This methodology is the first application of allosteric ribozymes to the activation of RNAi and subsequent control over gene expression. The combination of small molecules and ncRNA in this case is especially useful, as RNAi activity can be modulated in a temporal fashion *via* introduction of the small molecule effector.

Employing a different approach, the Mulligan lab employed high-throughput cell-based screens with small molecule libraries to identify small molecules capable of disrupting the activity of a standard hammerhead ribozyme (without an aptamer based recognition element) embedded upstream in a luciferase transcript.[93,94] From the screen, toyocamycin (**13**, 1.5 μM) was found to be capable of inhibiting ribozyme activity, as evidenced by increased expression of the luciferase reporter in HEK293T cells (Fig. 5A). Furthermore, when virally delivered to mice, the ribozyme self-cleaved its downstream mRNA until the introduction of **13** inhibited cleavage and restored luciferase expression. Thus, the identification of **13** as an inhibitor of a hammerhead ribozyme provides an exciting alternative to theophylline-based ribozymes and demonstrates novel control over gene expression in both cell culture and an animal model (Fig. 5B) using small molecule–ncRNA interactions without the need for an engineered aptamer component. Moreover, photochemical regulation of this system has also been achieved *via* installation of a photolabile protecting group onto **13** to afford spatial and temporal control over ribozyme cleavage.[95]

Ribozymes play an important role in the regulation of gene function and thus serve as promising therapeutic agents. Allosteric ribozymes may be particularly useful, as they can be evolved to respond to specific small molecules (*e.g.* metabolites). Further investigation into the therapeutic use of ribozymes will provide necessary insight into their potential role as gene therapy agents

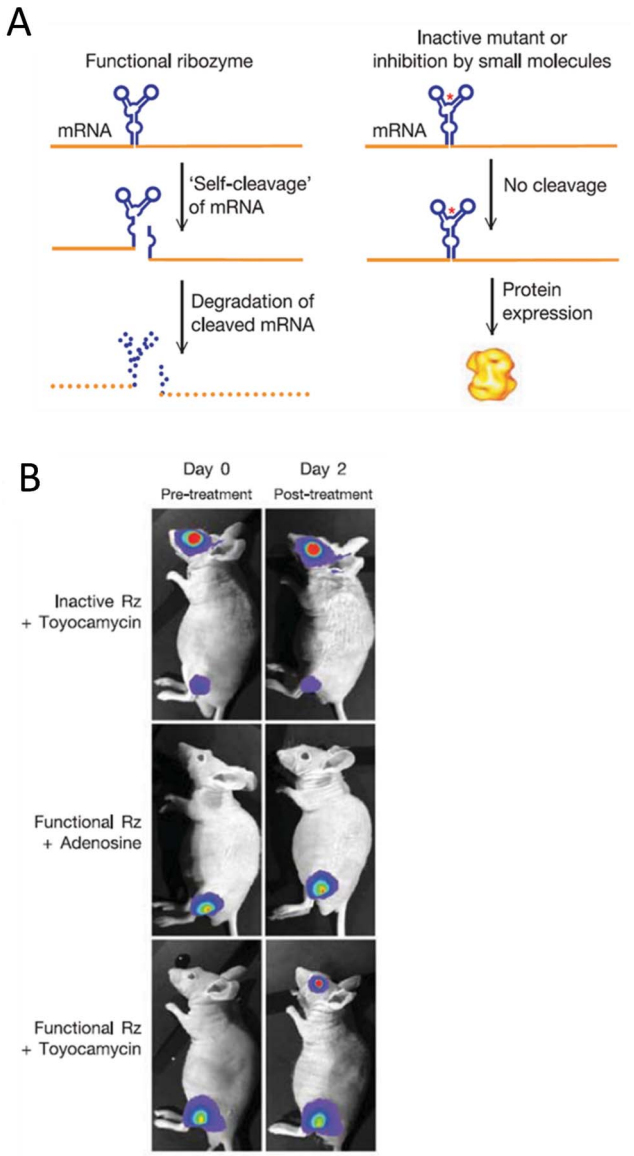


Fig. 5 Small molecule-mediated control of gene expression using a hammerhead ribozyme. A) In the absence of the small molecule inhibitor, the functional ribozyme self-cleaves its mRNA to inhibit translation. Incubation with **13** inactivates the ribozyme and enables protein expression. B) AAV-mediated viral sub-retinal delivery of the functional ribozyme-luciferase construct (Rz) shows reporter expression only upon administration of **13**, as compared to administration of adenosine. The inactive Rz control had no effect on luciferase expression. All animals were also injected with inactive Rz in the hind limb. *Adapted with permission from Yen et al., 2004.*[93]

and will offer progress toward challenges, such as intracellular delivery, that are currently preventing their widespread application.

Conclusions

In summary, we hope to have demonstrated the relevance and excitement surrounding several small molecule–ncRNA interactions. These important interactions are advancing our understanding of the role of ncRNA in cellular processes and enabling small molecule-mediated control of gene expression and silencing. Moreover, though this field is still in its infancy, it holds a great deal of promise towards the development of small molecule-mediated ncRNA therapeutics. Due to the prevalence of ncRNAs in a variety of disease states, it is likely that if several key issues (including delivery, stability, and specificity) can be overcome, small molecule–ncRNA interactions will play a significant role in the diagnosis and treatment of a variety of diseases.

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