Bioconjugate Chemistry

Communication

pubs.acs.org/bc

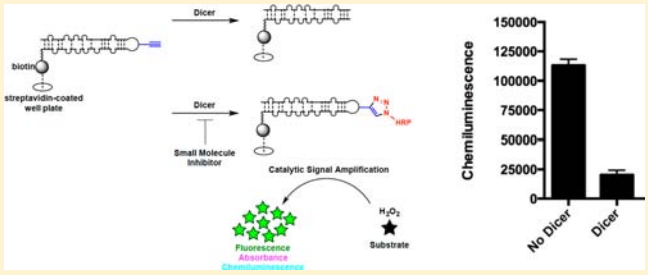
High-Throughput Platform Assay Technology for the Discovery of pre-microRNA-Selective Small Molecule Probes

Daniel A. Lorenz,[†] James M. Song,[†] and Amanda L. Garner*[,†,‡]

[†]Program in Chemical Biology and [‡]Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109, United States

Supporting Information

ABSTRACT: MicroRNAs (miRNA) play critical roles in human development and disease. As such, the targeting of miRNAs is considered attractive as a novel therapeutic strategy. A major bottleneck toward this goal, however, has been the identification of small molecule probes that are specific for select RNAs and methods that will facilitate such discovery efforts. Using pre-microRNAs as proof-of-concept, herein we report a conceptually new and innovative approach for assaying RNA−small molecule interactions. Through this platform assay technology, which we term catalytic enzyme-linked click chemistry assay or cat-ELCCA, we have designed a method that can be implemented in high throughput, is virtually free of false readouts, and is general for all nucleic acids. Through cat-ELCCA, we envision the discovery of selective small molecule ligands for disease-relevant miRNAs to promote the field of RNA-targeted drug discovery and further our understanding of the role of miRNAs in cellular biology.



INTRODUCTION

miRNAs comprise a large family of evolutionarily conserved ∼21−23 nucleotide noncoding RNAs that have emerged as key post-transcriptional regulators of gene expression. To date, there are ∼1000 predicted human miRNAs believed to control the activity of >60% of all protein-coding genes.[1] Although the exact mechanism of miRNA-mediated control is not known, these micromanagers function by binding to the 3′ untranslated region of specific target mRNAs and inhibit protein synthesis by either repressing translation or promoting mRNA degradation.[2] Increasing evidence supports a role for miRNAs in nearly all aspects of human biology, including the development of diseases such as cancer, obesity, diabetes, viral infections, and autoimmune, neurodegenerative, and cardiovascular diseases.[2] Although antisense oligonucleotide inhibitors of aberrantly expressed miRNAs are in preclinical and clinical trials, the targeting of miRNAs using small molecule probes remains an unmet need.[2]

Despite the great diversity of structure−function and relevance to human health, RNA continues to be an underexploited arena for drug discovery.[3,4] Major challenges include its highly electronegative surface that is prone to promiscuous and nonspecific ligand binding by positively charged molecules (e.g., aminoglycoside antibiotics) and its structural flexibility. To date, the only clinically approved drugs that target RNA are antibiotics (aminoglycosides, macrolides, tetracyclines, and oxazolidinones), which bind to rRNA. Interestingly, these compounds were discovered via phenotypic assays; the de novo design of chemically diverse modulators that are specific for select RNAs remains a difficult task. Recent advances in the field indicate that small molecules can be "rationally" designed to sequence-selectively target RNA; however, these focused studies have been biased toward previously known RNA-binding motifs such as aminosugars and benzimidazoles and indoles found in the Hoechst dyes and the well-known nuclear stain, DAPI, respectively.[3−12]

Small molecule modulators of miRNA function have been reported. Early studies used a luciferase-based cellular reporter assay of miRNA activity; however, lack of mechanistic information due to this pathway-based approach hindered medicinal chemistry followup of these compounds.[13,14] More recently, small molecule and peptoid microarays have been employed for the identification of primary miRNA (pri-miRNA) and precursor miRNA (pre-miRNA) ligands.[5−8] A number of issues exist with this focused approach, the most significant of which is the requirement for compound libraries to be immobilized onto a glass slide. Not only does this greatly limit the diversity of ligands that can be assayed (only three classes of compounds have been examined to date: aminoglycosides,[7,8,15,16] benzimidazoles,[7,9] and peptoids;[5,6] all previously known chemical space for RNA binding), but library members must also contain chemical handles for covalent conjugation to the microarray slide. A related shortcoming of ligand immobilization is the distortion of RNA binding affinities with different results obtained on the plate and in solution, likely due to linker effects.[6,10,15,16] Additionally, the selectivity

Received: November 24, 2014
Revised: December 10, 2014
Published: December 15, 2014

   dx.doi.org/10.1021/bc500544v | *Bioconjugate Chem.* 2015, 26, 19−23

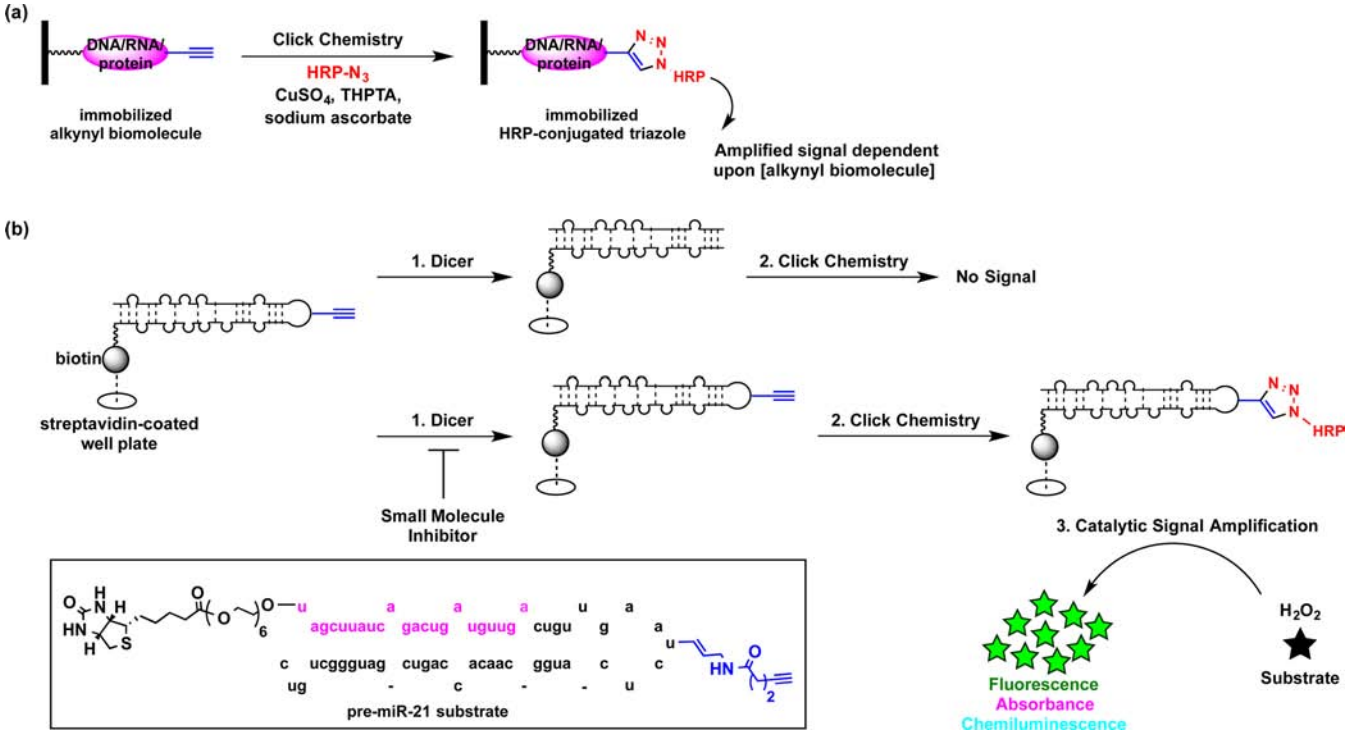


Figure 1. cat-ELCCA. (a) General assay scheme. (b) cat-ELCCA for Dicer-mediated pre-miRNA maturation. The structure of the pre-miR-21 cat-ELCCA substrate is shown (the highlighted nucleotides indicate the mature miR-21 sequence).

of these compounds has only been assessed against a limited panel of pre-miRNAs and/or other RNA molecules due to the lack of a high-throughput screening (HTS) method for assessing RNA-ligand binding. In fact, a recent review of the field highlighted the present lack of general, high-throughput methods for the identification of RNA–ligand interactions as a great limitation in promoting the future of RNA-targeted drug discovery.[3] Thus, while these studies provide proof-of-concept that the activity of miRNAs can be manipulated using small molecules, new methods are required for the facile discovery of structurally unique miRNA-selective ligands for target validation in human disease and future drug discovery and development efforts.

Current assays for RNA–ligand binding rely on single-turnover methods such as fluorescence resonance energy transfer (FRET)[17−19] and fluorescent ligand displacement.[10] While these homogeneous assay formats are typically straightforward to implement, they suffer from a number of drawbacks with respect to screening, the most significant of which is compound interference, which can stem from inner filter effects, fluorescence quenching, or autofluorescence.[20−22] Since RNA is a challenging biological target, a paradigm shift in assay technology will be necessary to enable the rapid discovery of small molecule modulators to further illuminate its therapeutic relevance. Herein, we describe the development of high-throughput platform assay technology for the discovery of small molecule probes of Dicer-catalyzed pre-miRNA maturation. Through our efforts, we have designed a method that is high-throughput, is virtually free of false readouts, and will be general for all nucleic acids.

RESULTS AND DISCUSSION

Inspired by ELISA and cat-ELISA,[23] a conceptually new assay system termed **cat**alytic **E**nzyme-**L**inked **C**lick **C**hemistry **A**ssay, or **cat-ELCCA**, was designed that takes advantage of catalytic signal amplification obtained with enzyme-linked detection antibodies and is necessary for ultrasensitive detection (Figure 1a).[24] The key step is the use of copper(I)-catalyzed alkyne–azide cycloaddition chemistry to "click" an azido-horseradish peroxidase (HRP) to an alkynyl biomolecule for enzyme-mediated signal amplification. In addition to catalyzed signal turnover, a significant advantage of cat-ELCCA with respect to ligand screening is the fact that this assay approach is not subject to compound interference like traditional fluorescence assays.[25] More specifically, because screening and signal measurement are conducted in separate steps, fluorescent compounds or quenchers will not interfere with the assay readout since all compounds are removed prior to detection. Thus, cat-ELCCA will provide reproducible and reliable screening results. An additional advantage is the capacity for multimode detection, as pro-fluorescent, -absorbant, and -chemiluminescent substrates are available for HRP. As such, cat-ELCCA is amenable for use in any laboratory and the readout mode can be tailored to available equipment.

cat-ELCCA overcomes all limitations of current assays used for RNA-ligand detection. First, because it does not rely on small molecule immobilization, access to all chemical space as potential ligands is possible. Along similar lines, it is able to detect both direct and allosteric inhibitors of Dicer cleavage, and thus is not limited to only one RNA structural motif.[7−11] cat-ELCCA is more sensitive due to catalytic signal amplification via HRP linkage using click chemistry so weaker binders can be discovered and will not simply be washed away. The assay is also not subject to compound interference by fluorescent molecules and quenchers because screening compounds are removed prior to readout, thus significantly decreasing the number of false hits identified. Moreover, all reagents required for cat-ELCCA are either commercial or can

dx.doi.org/10.1021/bc500544v | Bioconjugate Chem. 2015, 26, 19−23

be readily accessed; thus, our approach is user-friendly and we envision effortless dissemination of this technology to interested researchers in the field. It is also important to note the advantage of cat-ELCCA over ELISA, which is that it obviates the need for antibodies for detection. This is not only cheaper, but also necessary for targets for which antibodies do not exist like RNA.

As proof-of-concept for this assay approach, our laboratory has applied cat-ELCCA toward the development of innovative platform assay technology for the discovery of pre-microRNA-selective ligands using Dicer-mediated maturation as a readout (Figure 1b).[26] Dicer recognizes a pre-miRNA via its imperfectly base-paired double-stranded RNA duplex, 3′ 2-nt overhang and terminal loop motif, and acts as a "molecular ruler" where it counts from the 3′ end to digest the precursor into a mature miRNA 21−23 nucleotides in length.[27,28] Based on this, our assay design is as follows: chemically synthesized, 5′-biotinylated pre-miRNA tagged with an alkyne in the hairpin loop (5 pmol) is immobilized in the wells of a 384-well streptavidin microtiter plate and treated with recombinant Dicer. In the absence of a small molecule inhibitor, no signal will be observed due to Dicer-catalyzed cleavage of the tagged apical loop; however, in the presence of an inhibitor, signal will be emitted by covalent conjugation of HRP via the click reaction. As a model click reaction using our pre-miRNA substrate and a rhodamine azide was successful, we were encouraged for our assay design strategy (see Figure S1 of the Supporting Information).

To test the applicability of cat-ELCCA for Dicer processing of pre-miRNAs, a modified pre-miR-21 substrate (Figure 1b) was immobilized and treated with recombinant Dicer (6 h, 37 °C). Following the click reaction with azido-HRP, the wells were treated with SuperSignal West Pico and chemiluminescence signal was measured. As Figure 2a shows, our preliminary

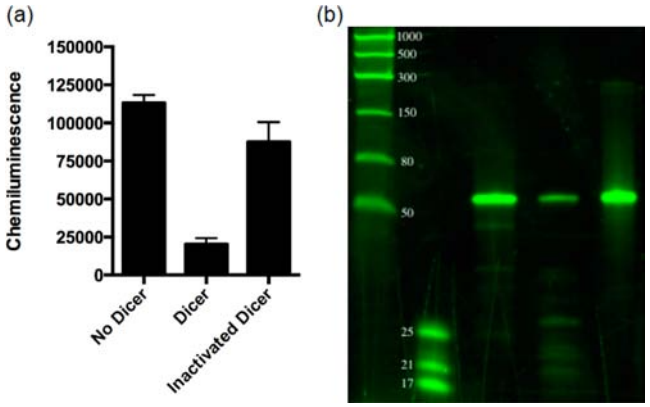


Figure 2. Proof-of-concept. (a) Raw chemiluminescence signal from cat-ELCCA using an immobilized pre-miR-21 substrate. (b) Fluorescence gel. Lane 1 = Low MW RNA ladder; Lane 2 = miRNA ladder; Lane 3 = pre-miR-21 + denatured Dicer; Lane 4 = pre-miR-21 + Dicer; Lane 5 = pre-miR-21.

experiments were successful and a ∼6-fold decrease in chemiluminescence signal was observed in the presence of Dicer, whereas restoration of signal was observed with heat and EDTA-denatured enzyme. Importantly, these results correlated to data obtained in solution detected via fluorescence gel (Figure 2b). Similar results were obtained when using a pro-fluorescent substrate (QuantaRed) (Figure 4b). Thus, the presence of alkyne and biotin tags do not interfere with Dicer-catalyzed cleavage. Moreover, we observed no difference in kinetics as compared to previous reports with the enzyme.[17−19,29] Additionally, each of these experiments was performed in the presence of 5% DMSO, indicating the applicability of our assay for small molecule HTS; all subsequent experiments were performed using these conditions.

Based on these successful results, we next characterized cat-ELCCA for Dicer-catalyzed pre-miRNA processing. As Figure 3

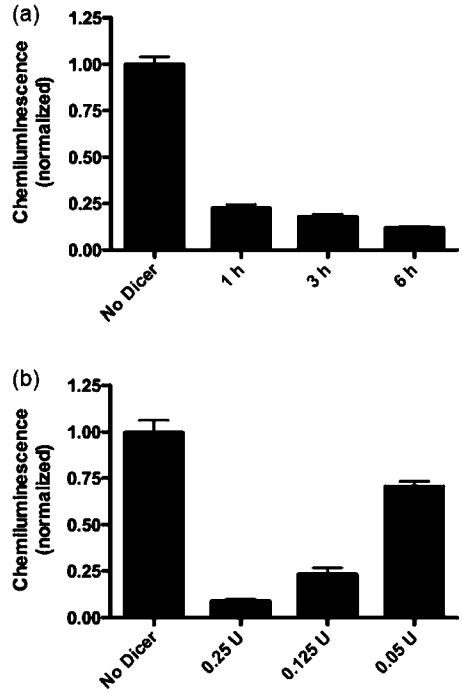


Figure 3. Assay characterization. (a) Time-dependence. (b) Dependence on Dicer.

shows, the enzymatic reaction was time-dependent and complete by 6 h, and dependent upon the units of Dicer used. In attempts to examine the dependence of signal generation on the concentration of pre-miRNA, we consistently saw similar signal regardless of immobilization amount (1−5 pmol). Further investigation revealed that of the 5-pmol capacity of the 384-well streptavidin-coated plate, we are actually immobilizing ∼1−2 pmol of RNA per well (see Figure S2 of the Supporting Information). Although the immobilization protocol could be improved, these findings demonstrate the superior sensitivity and dynamic range of cat-ELCCA as we are able detect such a minute amount of labeled RNA and still monitor its enzymatic degradation with high accuracy.

Because our goal is to use this assay for screening, we assessed the suitability of cat-ELCCA for HTS: the signal-to-noise (S/N) ratio was >100; the signal-to-background (S/B) was 11.4 and the Z′ factor was 0.60 (Figure 4a). Most important of these statistical parameters is the Z′ factor, which is reflective of both dynamic range and data variation associated with the signal measurement, and Z′ values between 0.5 and 1.0 are regarded as excellent assays.[30] To determine the effect of common false hits (i.e., fluorophores and fluorescence quenchers) in HTS, we intentionally doped the Dicer reaction with 2′,7′-dichlorofluorescein (DCF) or guanosine diphosphate (GDP), each at 10 μM, a typical screening concentration for HTS. As Figure 4b shows, neither compound interfered with

dx.doi.org/10.1021/bc500544v | *Bioconjugate Chem.* 2015, 26, 19−23

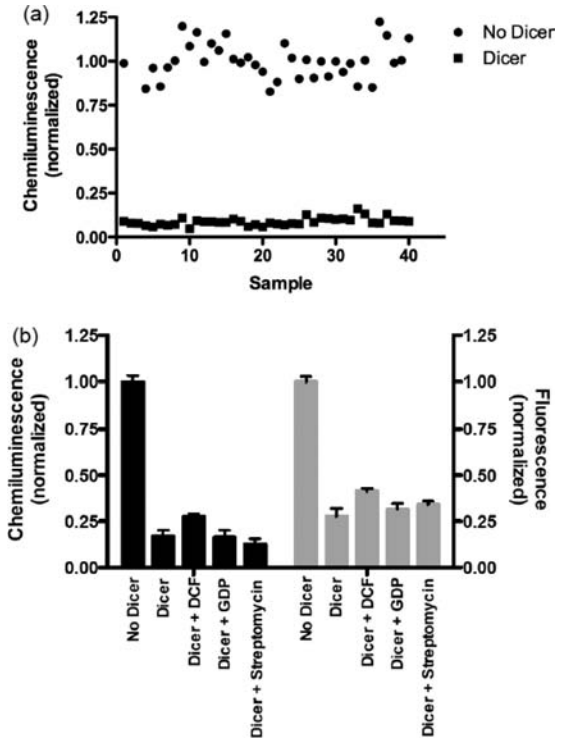


Figure 4. Characterization of HTS suitability. (a) Z′ factor. (b) Effect of common false hits and streptomycin on cat-ELCCA. [DCF], [GDP] = 10 μM; [streptomycin] = 2 μM.

the assay, either with chemiluminescence or fluorescence readout. We anticipate that fluorescent molecules or quenchers in a screening library will only be observed as hits if they actually bind to the pre-miRNA or Dicer, thus removing these classes of compounds as interferents. This is in contrast to other known FRET-based assays for Dicer cleavage.[17−19] In a recent report, compound interference by such molecules was cited as a major limitation and the group only screened 2816 compounds.[19]

To test a potential pre-miR-21 binder, we assayed streptomycin, which was reported to selectively disrupt Dicer processing of pre-miR-21.[31] Unfortunately, in our hands, this aminoglycoside had no inhibitory properties in cat-ELCCA (Figure 4b) or in solution (see Figure S4 of the Supporting Information). At higher concentrations (≥50 μM), we observed some inhibition; however, this is the same concentration range reported for streptomycin binding to TAR RNA.[32] Thus, the compound is not specific for pre-miR-21, and inhibition likely results from nonspecific electrostatic interactions. Current efforts are underway to apply cat-ELCCA in two-dimensional HTS to identify small molecule probes that are specific for select pre-miRNAs.

In summary, we have described a new platform screening technology for the identification of pre-miRNA-selective small molecule probes. Major advantages of cat-ELCCA include its high-throughput potential, resistance to compound interference, and ease of implementation. These attributes will finally enable the development of robust screening assays that will not cost considerable time and money in false hit validation. Due to the modularity of our approach, we envision ready adaptation to other RNA or even DNA molecules, regardless of secondary structure. The merging of chemistry and biology for assay design and development is a unique approach for solving challenging problems, such as the discovery of RNA-specific ligands. As we have already demonstrated the potential of cat-ELCCA for monitoring and inhibiting post-translational modifications (e.g., fatty acid acylation)[24,25] and now RNA, we believe that cat-ELCCA has the potential for translation to other difficult biological targets, including protein−protein and RNA−protein interactions. Future efforts will be devoted toward these goals. It is our hope that the advantages of cat-ELCCA over other single-turnover methods, like FRET and fluorescence polarization, will catalyze its positioning as the premiere fluorescence/chemiluminescence-based biochemical assay for probe and drug discovery in the near future.

■ ASSOCIATED CONTENT

Supporting Information

General methods and materials, assay protocols, and supplemental figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: algarner@umich.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

D.A.L. and J.M.S. are grateful for fellowships from the Graduate Assistance in Areas of National Need (GAANN) program. This work was supported through a generous start-up package from the University of Michigan College of Pharmacy. We thank Mr. Jim Windak (Department of Chemistry) for assisting with mass spectrometry analyses.

■ ABBREVIATIONS

miRNA, microRNA; HTS, high-throughput screening; cat-ELCCA, catalytic enzyme-linked click chemistry assay; HRP, horseradish peroxidase

■ REFERENCES

(1) Friedman, R. C., Kai-How Farh, K., Burge, C. B., and Bartel, D. P. (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res. 19*, 92−105.

(2) Li, Z., and Rana, T. M. (2014) Therapeutic targeting of microRNAs: current status and future challenges. *Nat. Rev. Drug Discovery 13*, 622−638.

(3) Thomas, J. R., and Hergenrother, P. J. (2008) Targeting RNA with small molecules. *Chem. Rev. 108*, 1171−1224.

(4) Guan, L., and Disney, M. D. (2012) Recent advances in developing small molecules targeting RNA. *ACS Chem. Biol. 7*, 73−86.

(5) Chirayil, S., Chirayil, R., and Luebke, K. J. (2009) Discovering ligands for a microRNA precursor with peptoid microarrays. *Nucleic Acids Res. 37*, 5486−5497.

(6) Diaz, J. P., Chirayil, R., Chirayil, S., Tom, M., Head, K. J., and Luebke, K. J. (2014) Association of a peptoid ligand with the apical loop of pri-miR-21 inhibits cleavage by Drosha. *RNA 20*, 528−539.

(7) Velagapudi, S. P., Gallo, S. M., and Disney, M. D. (2014) Sequence-based design of bioactive small molecules that target precursor microRNAs. *Nat. Chem. Biol. 10*, 291−297.

(8) Velagapudi, S. P., and Disney, M. D. (2014) Two-dimensional combinatorial screening enables the bottom-up design of a microRNA-10b inhibitor. *Chem. Commun. 50*, 3027−3029.

(9) Velagapudi, S. P., Seedhouse, S. J., French, J., and Disney, M. D. (2011) Defining the RNA internal loops preferred by benzimidazole

derivatives via 2D combinatorial screening and computational analysis. *J. Am. Chem. Soc. 133*, 10111−10118.
(10) Tran, T., and Disney, M. D. (2012) Identifying the preferred RNA motifs and chemotypes that interact by probing millions of combinations. *Nat. Commun. 3*, 1125.
(11) Childs-Disney, J. L., Wu, M., Pushechnikov, A., Aminova, O., and Disney, M. D. (2007) A small molecule microarray platform to select RNA internal loop-ligand interactions. *ACS Chem. Biol. 2*, 745−754.
(12) Velagapudi, S. P., Seedhouse, S. J., and Disney, M. D. (2010) Structure-activity relationships through sequencing (StARTS) defines optimal and suboptimal RNA motif targets for small molecules. *Angew. Chem., Int. Ed. 49*, 3816−3818.
(13) Gumireddy, K., Young, D. D., Xiong, X., Hogenesch, J. B., Huang, Q., and Deiters, A. (2008) Small-molecule inhibitors of microRNA miR-21 function. *Angew. Chem., Int. Ed. 47*, 7482−7484.
(14) Young, D. D., Connelly, C. M., Grohmann, C., and Deiters, A. (2010) Small molecule modifiers of microRNA miR-122 function for the treatment of hepatitis C virus infection and hepatocellular carcinoma. *J. Am. Chem. Soc. 132*, 7976−7981.
(15) Bryan, M. C., and Wong, C.-H. (2004) Aminoglycoside array for the high-throughput analysis of small molecule-RNA interactions. *Tetrahedron Lett. 45*, 3639−3642.
(16) Disney, M. D., and Seeberger, P. H. (2004) Aminoglycoside microarray to explore interactions of antibiotics with RNAs and proteins. *Chem.—Eur. J. 10*, 3308−3314.
(17) Davies, B. P., and Arenz, C. (2006) A homogeneous assay for microRNA maturation. *Angew. Chem., Int. Ed. 45*, 5550−5552.
(18) Davies, B. P., and Arenz, C. (2007) A fluorescence probe for assaying microRNA maturation. *Bioorg. Med. Chem. 16*, 49−55.
(19) Podolska, K., Sedlak, D., Bartunek, P., and Svoboda, P. (2014) Fluorescence-based high-throughput screening of Dicer cleavage activity. *J. Biomol. Screen. 19*, 417−426.
(20) Janzen, W. P. (2014) Screening technologies for small molecule discovery: the state of the art. *Chem. Biol. 21*, 1162−1170.
(21) Imbert, P.-E., Unterreiner, V., Siebert, D., Gubler, H., Parker, C., and Gabriel, D. (2007) Recommendations for the reduction of compound artifacts in time-resolved fluorescence resonance energy transfer assays. *Assay Drug Dev. Technol. 5*, 363−372.
(22) Comley, J. (2003) Assay interference a limiting factor in HTS? *Drug Discover World 4*, 91−98.
(23) Tawfik, D. S., Green, B. S., Chap, R., Sela, M., and Eshhar, Z. (1993) catELISA: a facile general route to catalytic antibodies. *Proc. Natl. Acad. Sci., U. S. A. 90*, 373−377.
(24) Garner, A. L., and Janda, K. D. (2010) cat-ELCCA: a robust method to monitor the fatty acid acyltransferase activity of ghrelin O-acyltransferase (GOAT). *Angew. Chem., Int. Ed. 49*, 9630−9634.
(25) Garner, A. L., and Janda, K. D. (2011) A small molecule antagonist of ghrelin O-acyltransferase (GOAT). *Chem. Commun. 47*, 7512−7514.
(26) Ha, M., and Kim, V. N. (2014) Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol. 15*, 509−524.
(27) MacRae, I. J., Zhou, K., and Doudna, J. A. (2007) Structural determinants of RNA recognition and cleavage by Dicer. *Nat. Struct. Mol. Biol. 14*, 934−940.
(28) Feng, Y., zhang, X., Graves, P., and Zeng, Y. (2012) A comprehensive analysis of precursor microRNA cleavage by human Dicer. *RNA 18*, 2083−2092.
(29) Chakravarthy, S., Sternberg, S. H., Kellenberger, C. A., and Doudna, J. A. (2010) Substrate-specific kinetics of Dicer-catalyzed RNA processing. *J. Mol. Biol. 404*, 392−402.
(30) Zhang, J.-H., Chung, T. D. Y., and Oldenburg, K. R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen. 4*, 67−73.
(31) Bose, D., Jayaraj, G., Suryawanshi, H., Agarwala, P., Pore, S. K., Banerjee, R., and Maiti, S. (2012) The tuberculosis drug streptomycin as a potential cancer therapeutic: inhibition of miR-21 function by directly targeting its precursor. *Angew. Chem., Int. Ed. 51*, 1019−1023.
(32) Mei, H.-Y., Galan, A. A., Halim, N. S., Mack, D. P., Moreland, D. W., Sanders, K. B., Truong, H. N., and Czarnik, A. W. (1995) Inhibition of an HIV-1 Tat-derived peptide bindings to TAR RNA by aminoglycoside antibiotics. *Bioorg. Med. Chem. Lett. 5*, 2755−2760.