Association of a peptoid ligand with the apical loop of pri-miR-21 inhibits cleavage by Drosha

INTRODUCTION

MicroRNAs are important regulators of cell metabolism and phenotype. These short (~22 nt), noncoding RNAs recognize complementary base sequences in target mRNAs and suppress their translation (Bartel 2004; Chekulaeva and Filipowicz 2009). They are appealing therapeutic targets, because aberrant expression of specific miRNAs is often associated with diseases of dis-regulated translation. For example, a direct role for miRNAs in cancer is well established (Garzon et al. 2009), some miRNAs acting as tumor suppressors, others acting as tumor promoters (oncomirs). An example of an oncomir is miR-21, a miRNA that is dramatically up-regulated in the expression signatures of many tumors (Volinia et al. 2006). It targets tumor suppressor genes, including pro-apoptotic genes such as PDCD4 (programmed cell death 4) (Zhu et al. 2007, 2008; Asangani et al. 2008). By suppressing translation of pro-apoptotic genes, heightened expression of miR-21 helps cancer cells evade apoptosis, a requirement for tumor progression (Chan et al. 2005). Thus, genetic deletion of miR-21 in a mouse model of non–small cell lung cancer protects against tumor formation (Hatley et al. 2010), and suppression of miR-21 in tumor cells by transfection with complementary oligonucleotides results in increased apoptosis (Chan et al. 2005) or increased sensitivity to pro-apoptotic drugs (Moriyama et al. 2009). Consequently, approaches to intervening specifically in the production of miR-21 hold great promise for cancer treatment.

Production of miRNAs is regulated, in part, by modulation of their maturation from primary transcripts (Obernosterer et al. 2006; Thomson et al. 2006). In a typical miRNA primary transcript (pri-miRNA), such as that of miR-21, the mature miRNA sequence resides in the stem of a hairpin structure (Bartel 2004). This hairpin is initially excised from the longer pri-miRNA by the microprocessor, a complex of the endoribonuclease Drosha with the RNA binding protein DGCR8 (Lee et al. 2003; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004). The excised hairpin structure (pre-miRNA) is exported from the nucleus by exportin-5 (Lund et al. 2004) and cleaved by another endoribonuclease, Dicer, to release the mature miRNA (Hutvagner et al. 2001). Individual steps of this maturation process can be inhibited by specific nucleic acid ligands (Moriyama et al. 2009). As an extension of this work, we have identified novel ligands that bind to a 50-bp loop of pri-miR-21 that is essential for microprocessor cleavage and that may provide an additional target for the development of small molecules that modulate the production of this miRNA.
be suppressed or enhanced to alter the level of mature miRNA. For example, the RNA-binding protein Lin-28 mediates diminished processing of Let-7 family pri-miRNAs by association with the precursor hairpins (Newman et al. 2008; Piskounova et al. 2008). Conversely, factors that recruit the microprocessor to pri-miR-21 promote its maturation, increasing the level of mature miRNA (Davis et al. 2008, 2010). Post-transcriptional regulation of miRNA levels suggests interference with maturation of specific miRNAs as a tool to probe miRNA processing and function as well as a possible avenue of therapeutic intervention.

Several efforts to interfere in miRNA maturation have focused on the Dicer step of processing. Oligonucleotide-related molecules, such as PNA (Avitabile et al. 2012), and small molecules (Krishnamurthy et al. 2007; Neubacher et al. 2011; Bose et al. 2012; Maiti et al. 2012; Murata et al. 2013) have been targeted to the Dicer cleavage sites in pre-miRNAs. However, less activity has been directed toward efforts to inhibit the cleavage by Drosha of specific pri-miRNAs. The microprocessor recognizes structural elements of pri-miRNAs to differentiate hairpins containing miRNAs from the many other hairpin structures in the transcriptome (Zeng et al. 2005a,b; Zeng et al. 2005; Zhang and Zeng 2010), and each of these structural elements is a potential target for interfering with cleavage of a miRNA by the microprocessor. Essential features of the hairpin include a terminal (apical) loop, a predominantly double-stranded stem, which includes the miRNA sequence, and single-stranded RNA flanking the stem at its base, approximately one helical turn from the end of the mature miRNA sequence (Zeng and Cullen 2005a,b; Zeng et al. 2005; Zhang and Zeng 2010).

Though the apical loop is at the opposite end of the pri-miRNA hairpin from the Drosha cleavage site, it plays an important role in the recognition and cleavage of pri-miRNAs by the microprocessor. No specific sequence requirement is evident, but mutations that stabilize predicted base-pairing adjacent to the apical loops of several pri-miRNAs diminish cleavage by Drosha, suggesting that conformational flexibility in and around the loop is a requirement for microprocessor recognition (Zeng et al. 2005; Zhang and Zeng 2010). Consistent with that requirement, an aptamer that binds to the terminal loop of pri-miR-18a inhibits cleavage of that pri-miRNA by the microprocessor complex (Lunse et al. 2010).

The apical loops of pri-miRNAs also act as sites for association with trans-acting factors that regulate microprocessor cleavage. For example, the protein hnRNP A1 promotes microprocessor-directed cleavage of pri-miR-18a by inducing relaxation of base-paired structure within the loop region (Michlewski et al. 2008). KSRP is another protein that binds to the terminal loops of a subset of miRNA precursors, including pri-miR-21, promoting their cleavage by Drosha (Trabucchi et al. 2009). On the other hand, Lin28 inhibits pri-let-7 processing by Drosha by binding to the apical loops of let-7 primary transcripts (Newman et al. 2008; Viswanathan et al. 2008). Many pri-miRNAs have highly conserved loops, suggesting the importance of their recognition by auxiliary factors in the regulation of miRNA maturation (Michlewski et al. 2008).

The importance of the apical loop for regulation of pri-miRNA maturation suggests that a small molecule ligand for a pri-miRNA apical loop might modulate processing by Drosha. Such a molecule could be used to probe the dynamics of miRNA processing and could also have therapeutic value. However, no small molecule has previously been shown to inhibit microprocessor cleavage by binding to a pri-miRNA terminal loop. In our earlier efforts to develop ligands that bind to the apical loop of pri-miR-21 and modulate miR-21 maturation, we identified compound 1 (Fig. 1; Chirayil et al. 2009). This peptidomimetic compound was derived from a screen of a combinatorial library of peptid-peptide conjugates in a microarray format. It binds specifically to the pri-miR-21 hairpin loop with low micromolar affinity in the absence of Mg2+. However, it does not bind detectably to the targeted hairpin loop in concentrations of Mg2+ required for microprocessor activity in vitro assays. To identify compounds that bind more tightly to the hairpin loop of pri-miR-21, we have modified our screening protocol and screened microarrays of redesigned peptoid libraries. We identified higher affinity compounds, one of which inhibits cleavage by the microprocessor in vitro.

RESULTS

Library design and screening

Microarrays were created by spotting combinatorial libraries of peptoids (N-substituted oligoglycines) to glass slides. The peptoid scaffold possesses a number of desirable characteristics, including cell permeability (Kwon and Kodadek 2007), protease resistance, and ease of modification, so we sought new compounds comprised entirely of peptoid monomers. The design of the libraries was informed by the structure/activity relationship that we found for compound 1. The arginine of 1 is critical for binding to the target RNA. Therefore, the new libraries included an invariant arginine analog at a central position. In addition, the two glycines to the C-terminal side of the arginine in 1, though originally included only as a linker, contributed significantly to the affinity of the
identified ligand. Therefore, two variable peptoid residues were included to the C-terminal side of the invariant arginine analog.

The library was synthesized as shown in Figure 2 and, while still bead-bound, was divided into two portions. One portion of the library, Sublibrary A, was used in array preparation without further modification. To the other portion, Sublibrary B, an azide derivative of phenylboronic acid was appended to propargyl side chains by copper-catalyzed cyclo-addition wherever they occurred (Fig. 2C). The boronic acid moiety was included to allow for the possibility of forming boronate esters with 2'-hydroxyls on the RNA or conformationally constraining peptoids through intra-molecular esterification with sugar moieties (Chirayil and Luebke 2012).

The peptoid-bearing beads were sorted into microtiter plates, one bead (i.e., one peptoid) per well, and printed to microarrays as previously described (Chirayil et al. 2009). The boronate-containing library was sorted separately and printed to its own set of microarrays. Seven thousand twelve different peptoids were printed to each replicate microarray along with 128 spots of a fluorescein-labeled peptoid to be used as markers for orientation on the array. Thus, a total of 14,024 different compounds were screened. The total possible diversity of the libraries (160,000) makes duplication on the array unlikely.

The arrays were screened to identify ligands specific for an RNA hairpin corresponding to the apical loop of pri-miR-21 (RNA I) (Fig. 3A). MiR-21 hp (RNA I) with a fluorescent label at its 5'-end was applied to a peptoid array, and after washing, spots to which it bound were identified by fluorescence scanning. Features with a ratio of signal to local background (SNR) > 1.2, the threshold for visibly discernable fluorescence on the scanned image, were considered to have signal above background. To be considered positive, an array feature was required to have fluorescence signal above background in each of three replicate arrays treated with labeled RNA I. The requirement for reproducibility on three arrays was imposed to rule out false positives due to washing artifacts (e.g., water spots) or other irregularities on the array (e.g., scratches). Six hundred forty-three features from Sublibrary A and 1,174 features from Sublibrary B were positive for binding RNA I.

To eliminate from consideration compounds with a high nonspecific affinity for RNA hairpins or for the dye molecule and to narrow the search for compounds that recognize distinctive features of the pri-miR-21 hairpin, a dye-labeled control hairpin was tested. This control hairpin was similar to the target hairpin but differed from it in several respects to allow for the possibility of recognizing various features.
of the target. In the control hairpin, the predicted G•U pairs were changed to G•C pairs, the putatively unpaired U was omitted, and the loop sequence was scrambled, maintaining the base composition but changing the sequence order.

For each library, most of the features that were fluorescent above background after treatment with hairpin I were also fluorescent on one or more of three replicate arrays treated with RNA II. Examples of such features can be seen in Figure 3, B and C, identifiable as fluorescent features flanking the feature indicated with an arrow. These features were considered to be nonspecific in their affinity for the labeled RNA, though some might have specificity for the target that was not distinguished at the level of stringency of the binding and washing conditions used. Eight features from library A and three features from library B were positively specifically in response to RNA I.

Mass spectral analysis revealed these features to comprise compounds 2–9 (library A) and 10–12 (library B) (Fig. 4; cf. also Supplemental Data). Compound 12 is notably similar to the previously identified compound 1, each having an N-terminal methlypyridine side chain followed by a hydroxyethyl side chain. The phenylbenzyl, diphenylethyl, and galactose side chains (each found five times) and especially the cyclobutyl side chain (found nine times) are represented within the hit compounds with significantly higher frequency than statistically expected (2–3 times in the 44 variable side chains). Compounds containing boronic acid were not obtained as hits from the screen of Sublibrary B.

Affinities and specificities of ‘hit’ compounds

Binding of ligands to RNA hairpins I and II in solution was assessed using the base analog 2-ap as a fluorescent probe (Bradrick and Marino 2004). For these measurements, the fluorescent base was substituted for the adenines indicated in Figure 3, and the peptoid-dependent change in fluorescence was used to determine the dissociation constant. We previously found that dissociation constants measured for hairpins I and II with this technique correspond to dissociation constants measured by isothermal titration calorimetry and in-line cleavage (Chirayil et al. 2009). A concentration-dependent change in fluorescence of 10% or greater was taken as an indication of peptoid binding. Compound binding typically resulted in a 20%–50% decrease in fluorescence at saturating concentrations. Limited solubility of some of the peptoids and precipitation of the RNA at high peptoid concentrations prevented reaching saturation in some binding curves, limiting the dissociation constant that could be measured to <300 µM. The measured dissociation constants for each peptoid with RNAs I and II are shown in Figure 4.

Each of the hit compounds had measurable binding to RNA hairpin I in solution (K_d < 300 µM), six having dissociation constants below 20 µM. Eight showed specificity for RNA I over RNA II, by factors ranging from 1.7 (11) to >25 (12). Two of the compounds (7, 10) showed little or no preference for RNA I over RNA II, and one compound (8) bound RNA II preferentially. It is important to note that the previously identified compound 1, which binds RNA I with a K_d of 2 µM in the absence of Mg^{2+}, does not bind measurably to RNA I under the conditions for which these dissociation constants were measured, specifically in the presence of 5 mM Mg^{2+} (data not shown). An improved library design and screening with a lower concentration of RNA might account in part for this improvement.

Effect of compound 12 on in-line cleavage of the target and control hairpins

In light of the relatively high affinity and specificity of compound 12, as well as its similarity to our previous lead compound, we tested it further for its binding to the pri-miR-21 hairpin and the control hairpin used for screening. Its effect on Mg^{2+}-induced hydrolytic cleavage was used to characterize its binding. Hydrolytic cleavage of the RNA backbone occurs principally through nucleophilic attack of a 2′-hydroxyl on the adjacent phosphodiester, displacing the 5′-hydroxyl of the following nucleotide. For this displacement to occur, the attacking 2′-hydroxyl must be in-line with the scissile phosphorus–oxygen bond (Soukup and Breaker 1999). Thus, in-line cleavage, which is stimulated by divalent metal ions such as Mg^{2+}, is a useful probe of conformational changes such as those that are frequently induced by ligand binding.

The effect of 12 on in-line cleavage of RNA I is shown in Figure 5, A and B. In the absence of peptoid, Mg^{2+}-induced cleavage occurs predominantly within the 3′ side of the predicted hairpin loop, after U16, C17, and U18, and after U21. With addition of 3–10 µM 12, cleavage after the loop nucleotides increases, accompanied by an increase in cleavage after C19. Cleavage after U11 and U12 also intensifies. At higher concentrations of 12 (300–1000 µM), overall cleavage increases further, with appearance of bands due to cleavage after C8, U9, G10, G13, A14, and A15.

The observed patterns of cleavage suggest two binding modes for 12, one with a dissociation constant ~3–10 µM and a weaker interaction with dissociation constant >300 µM. The higher affinity interaction affects the conformation or flexibility of loop nucleotides, supporting a model of direct and specific interaction of the peptoid with the loop. The lower affinity interaction affects the conformation or flexibility of nucleotides in the putatively base-paired stem adjacent to the loop.

The effect of 12 on in-line cleavage of RNA II is shown in Figure 5, C and D. In the absence of peptoid, weak cleavage is observed within the loop (after U13, A14, C15, and U16). The peptoid induces little change in the cleavage pattern up to (and including) 100 µM. Mg^{2+}-induced cleavage intensifies in the presence of 300 µM peptoid, with strongest cleavage occurring after U13 and U16. Cleavage at these positions is greatly intensified by addition of 1 mM peptoid. This response of the cleavage pattern to addition of peptoid
is consistent with the measurement made using 2-ap as a probe, indicating an interaction of 12 and the control hairpin loop with a dissociation constant >300 µM.

**Microprocessor cleavage of pri-miR-21 is specifically inhibited by compound 12**

We examined the effect of 12 on cleavage of primary miRNAs by the microprocessor complex. A model pri-miR-21 was synthesized that contains the pre-miR-21 hairpin as well as ~100 nt of the sequence flanking it on each side in human pri-miR-21. This RNA was a substrate for the microprocessor (Fig. 6A), producing a 57-nt fragment consistent with pre-miR-21. The flanking segments were further cleaved under the conditions of our experiment to products that include the small fragment indicated on the gel. Addition of compound 12 led to inhibition of cleavage. Inhibition was clearly evident at a concentration of 150 µM 12, and cleavage was
produced chimeric pri-miRs in which the loop sequences were swapped between the stem and flanking sequences of pri-miR-21 and pri-miR-16. A transcript with the loop sequence of pri-miR-21 and the stem and flanking sequences of pri-miR-16 (Fig. 6C) is protected from cleavage in the presence of 250 µM 12. The inhibition of cleavage is dose-dependent, most clearly visible as the dose-dependent increase in the full-length transcript with increasing peptoid. A transcript with the loop sequence of pri-miR-16 and the stem and flanking sequences of pri-miR-21 (Fig. 6D) is also protected from cleavage by 12, following a similar concentration profile as inhibition of pri-miR-21. Though pre-miR-21 is produced from both of these substrates, the cleavage products smaller than pre-miR-21 in this reaction are shorter when the loop sequence is from pri-miR-16.

DISCUSSION

The apical loop of miR-21 is an intriguing target for development of molecules that inhibit microprocessor cleavage. In addition to being important for recognition of pri-miR-21 by the microprocessor, it is a site of recognition by other endogenous factors that regulate interaction of the microprocessor with the primary transcript of miR-21. Furthermore, hairpin loops in general represent attractive targets for recognition by small molecules. In support of this notion, Tran and Disney have recently concluded from a multidimensional screening analysis that hairpin loops represent a preferred RNA motif space for binding small molecules (Tran and Disney 2012). Nevertheless, few sequence-specific small molecule ligands are known for RNA hairpin loops (Thomas and Hergenrother 2008).

Having developed a microarray-based screen for ligands of the apical loop of miR-21, we sought compounds of higher affinity than those we derived from our initial implementation of the screen. The arginine of 1 is critical for the compound’s binding to the RNA hairpin, suggestive of a significant electrostatic contribution to binding affinity. It is not surprising, therefore, that its affinity would diminish substantially with increasing divalent ion concentration, and indeed, no binding is detectable for 1 in the presence of 5 mM MgCl₂. We expected that additional positive binding interactions could be found that would allow binding under more electrostatically demanding conditions. To find such compounds, we modified both the design of the libraries...
FIGURE 6. Electrophoretic analysis of cleavage of radiolabeled pri-miRNA substrates by the microprocessor. The concentration of 12 is indicated above each lane containing microprocessor. Size standards (215 nt, 149 nt, and pre-miR-16, generated as described in Materials and Methods) correspond to the products of cleavage of pre-miR-16 from the pri-miR-16 substrate without degradation of the flanking sequences. Arrows indicate the positions on the gel of the pre-miRNA products. Products smaller than the pre-miRNA are labeled with an asterisk. (A) Comparison of effects of 12 on microprocessor cleavage of pri-miR-16 and pri-miR-21. (B) Effect on microprocessor cleavage of a derivative of 12 in which the guanidinium side chain is replaced with a methyl group. (C) Comparison of the effects of 12 on microprocessor cleavage of pri-miR-16 and pri-miR-21 with the apical loop of pri-miR-21. The loop regions are shown above the gel, with the loop sequences that distinguish the two substrates shaded. (D) Comparison of the effects of 12 on microprocessor cleavage of pri-miR-21 and pri-miR-16 with the apical loop of pri-miR-16. The loop regions are shown above the gel, with the loop sequences that distinguish the two substrates shaded.

we screened and the conditions of screening from those we used previously. In particular, we used a lower concentration of labeled RNA in the screen, 417 nM rather than 5 μM. Reducing the concentration of the target and control RNA used may have allowed for the identification of peptides that would have been eliminated due to binding to the control RNA at higher concentrations. The outcome was a panel of compounds with affinity and specificity for the targeted RNA under the desired conditions.

Noteworthy generalizations are apparent among the 11 lead compounds we obtained from the screen. The cyclobutyl side chain occurs with a substantially higher frequency than expected from its representation in the library. Although only one of 20 side chains in the library, it is nearly one of every five side chains in the identified compounds, present in eight of the 11 hits and six of the eight hits with clear specificity for the pri-miR-21 hairpin. Its position in the hit compounds is nonrandom, being adjacent to the central arginine analog in seven of the eight compounds and five of the six with pri-miR-21 specificity. Phenylbenzyl and diphenylethyl side chains are also overrepresented in the hit compounds, each being present in five of 44 positions. The positions of these groups in the hits are also nonrandom, placing them in the two positions closest to the C terminus (except in 10, which is not specific for the pri-miR-21 hairpin over the control hairpin). The galactose side chain is overrepresented by virtue of its prevalence in 8 and 9, in both of which it comprises the two N-terminal residues. However, only one of these compounds has the desired specificity. The prevalent features of these compounds likely reflect a structure-activity relationship.

The phenylboronic acid side chain that distinguishes Sublibrary B from Sublibrary A is conspicuously absent from the hit compounds. Though other side chains are absent from the hits, including the propargyl side chain among hits from Sublibrary A, it is notable that almost twice as many spots from Sublibrary B bind the control RNA compared to Sublibrary A. The number of these spots is comparable to the number of compounds expected to contain at least one phenylboronic acid. This difference might suggest that the phenylboronic acid contributes nonspecific affinity for RNA under the screening conditions employed, eliminating it from the hit compounds in the counter-screen.

Compounds 12 and 1 share a clear structural similarity. Each incorporates a methylpyridine at its N terminus and a hydroxyethyl group at the second position from its N terminus. These groups contribute to the specific binding of 1 (Chirayil et al. 2009). Though 12 lacks the furfuryl moiety of 1, that group contributes little to the affinity of 1 for the target RNA, and the difference in spacing of the hydroxyethyl and guanidinium groups between 1 and 12 is only three atoms (seven atoms vs. 10 atoms). The recurrence of the structural element comprising the pyridine, hydroxyethyl, and guanidinium moieties in the two screens using different libraries and different screening conditions, in conjunction with the fact that 12 has the highest affinity and specificity of the hit compounds from the screen reported here, implicate this element as a potential pharmacophore for recognition of the hairpin loop of pri-miR-21. The large increase in both affinity and specificity for 12 over 1 also indicates the positive involvement of the two C-terminal residues, consistent with the high frequency of cyclobutyl and phenylbenzyl residues in the hit compounds.

Three of the identified compounds (7, 8, and 10) were not specific for binding the target RNA with preference over the control RNA in solution, though they preferred the target RNA in the context of the microarray. Others who have compared RNA ligand binding specificities in heterogeneous and homogeneous formats have found them to be discrepant (Carlson and Beal 2002; Disney and Seeberger 2004).
This discrepancy apparently reflects the effect of the surface, linker, and linker position on the binding properties of the compounds. Nevertheless, the microarray format is useful for identifying compounds with a desired specificity, most (eight out of 11) hit compounds binding the target RNA preferentially.

Compounds 3, 4, and 12 have the highest affinities and specificities of the identified ligands. We focused our attention on 12 due to its similarity to 1. Changes in the Mg\(^{2+}\)-mediated cleavage of the RNA in the presence of 12 indicate that its binding site is in and proximal to the loop, consistent with the measured changes in fluorescence for 2-ap situated within the loop. At peptoid concentrations up to 100 µM, the changes induced in the Mg\(^{2+}\)-mediated cleavage pattern by 12 are similar to those produced by 1, in particular, intensification of cleavage after U16 and C19. At higher concentrations, a second binding mode is evident, resulting in cleavage along the 5’ side of the upper stem. The alteration in the cleavage pattern within the putatively base-paired region suggests that the binding site for this low affinity interaction includes this portion of the RNA. Disposition of the phosphodiester backbone that allows in-line attack and strand cleavage is not possible within the context of A-form double helical RNA. Therefore, cleavage within this stem region is indicative of destabilization of base-pairing. Despite a weak interaction (K\(_d\) > 300 µM) of the peptoid with the control hairpin loop, there is no indication of peptoid-induced in-line cleavage of the stem region of the control hairpin, suggesting that the interaction of 12 with the region adjacent to the loop of the pri-miR-21 hairpin is somewhat specific to that sequence and structural context.

Compound 12 inhibits microprocessor cleavage with a clear specificity for pri-miR-21 over pri-miR-16. This specificity indicates that the peptoid acts by binding to the RNA rather than general inactivation of microprocessor. The derivative of 12 that lacks the central guanidinium-bearing side chain has diminished affinity for the loop (affinity decreased six- to 12-fold) and, as expected for a peptoid that inhibits cleavage by binding to the RNA, also has a greatly diminished effect on cleavage by the microprocessor. Furthermore, suppression of cleavage of pri-miR-16 bearing the apical loop sequence of miR-21 demonstrates that interaction of 12 with the apical loop of pri-miR-21 is sufficient to inhibit cleavage. Saturation of this effect (i.e., nearly complete suppression) by 250 µM 12 suggests that the higher-affinity binding mode (K\(_d\) = 12 µM) is responsible for suppression of cleavage, because the lower affinity binding mode is not saturated at 300 µM, changes in the Mg\(^{2+}\)-induced cleavage pattern occurring between 300 and 1000 µM 12. In particular, cleavage after C8 and U9 increases, and cleavage after U21 is diminished. Thus, the peptoid concentration required for effective inhibition of microprocessor cleavage is 20-fold higher than the dissociation constant for the responsible binding interaction. This peptoid concentration is in vast excess over the concentration of the RNA substrate (3.3 nM), so it is not reflective of a need to saturate off-target sites. Notably, an aptamer that binds the apical loop of pri-miR-18a with a dissociation constant of 40 nM also requires a concentration more than 20-fold above its K\(_d\) to inhibit microprocessor cleavage (Lunse et al. 2010).

The molecular basis for recognition of the apical loop by the microprocessor and for its inhibition by an associated ligand is unknown. A ligand that simply associates with the apical loop does not necessarily result in inhibition of microprocessor cleavage, since association of factors such as KSRP and hnRNP A1 with apical loops can promote cleavage (Michlewski et al. 2008; Trabucchi et al. 2009). Therefore, suppression of microprocessor cleavage by association with the loop is likely not due to simple competitive inhibition, which might account for the requirement of concentrations above the K\(_{50}\) of the peptoid and the aptamer for inhibitory efficacy. Further investigation of the thermodynamics of inhibition by 12 is warranted and could provide insight into the nature of the microprocessor-RNA interaction as well as the peptoid-RNA interaction.

Although the apical loop sequence of pri-miR-21 is sufficient to allow microprocessor inhibition by 12, it is not necessary: pri-miR-21 in which the apical loop sequence of pri-miR-16 has replaced the native apical loop is also protected from microprocessor cleavage by 12. This inhibition of cleavage indicates the presence of another binding site for the peptoid in pri-miR-21, outside of the loop. The basal segments proximal to the Drosha cleavage site are possible sites for this interaction. Beisel and coworkers have demonstrated that ligand binding to an aptamer integrated into the basal segments of a hairpin substrate inhibits Drosha processing (Beisel et al. 2011). However, we have not ruled out more distal sites within the pri-miR-21 substrate or sites within the stem. Inhibition of cleavage by binding of the peptoid to the site outside of the loop is effective at lower peptoid concentrations than inhibition of cleavage by binding to the targeted loop. This greater effectiveness could be due to a higher affinity for the second site but could also be due to a different mechanism of inhibition, even with a lower affinity. Work is under way to identify other sites of interaction of 12 with pri-miR-21.

This work demonstrates for the first time that a small molecule can interfere with processing of a specific primary miRNA by association with the apical loop. A small molecule that interferes with a specific step in the processing of a specific miRNA, especially one as relevant to disease as miR-21, will be useful as a probe of the dynamics of miRNA maturation, which are still poorly understood. Furthermore, suppression of production of miR-21 is a clear therapeutic goal toward treatment of the many cancers that evade apoptosis by up-regulation of miR-21. While not sufficiently potent to hold promise as a drug itself, the molecule reported here demonstrates for the first time the principle of inhibiting Drosha with a loop-binding small molecule, and later generation compounds with improved efficacy could be generating compounds with improved efficacy could be
of therapeutic utility. We are currently assessing the effects of the compound on processing of miR-21 and other micro-RNAs in a cellular context.

MATERIALS AND METHODS

Synthesis of peptoid libraries

Library synthesis was done by the split/mix method (Lam et al. 1991) as described previously (Chirayil et al. 2009), according to the library design shown in Figure 2. The amines used (obtained from Sigma unless specified otherwise) were 3-amino-1-propyne; 1-amino-2-methylpropane; 1-amino-3-methoxypropane; 3-amino-1-propene, glycine t-butyl ester; 2-aminomethylfluorur; O-t-butyl-2-aminoethanol (CSPS Pharmaceuticals); exo-2-aminonorbornane; 3-((2-aminoethyl)indole; 3,4-(methyleneedioxy)benzylamine; benzylamine; R-(-)-1-phenylethylamine; 2,2-diphenylethylamine; cyclobutylamine; 2,2-dimethyl-1,3-dioxolane-4-methanamine (Acros); 4-(2-aminoethyl)phenol; 4-aminomethylpyridine; 2-(aminomethyl)-5-methylpyrazine (TCI); 4-phenylbenzylamine; and 6-amino-6-deoxy-D-galactopyranose diacetonide (Uno et al. 1999). These amines are shown in their unprotected forms in Figure 2B. The Boc-protected form of the invariant guanidinium-bearing amine was synthesized as described by Nnanabu and Burgess (2006).

Peptoids were assembled on polystyrene macrobeads (2.0 g; 500-560 µm; substitution: 0.56 mmol/g, Rapp Polymere) (Uno et al. 1999). A cysteine (Fmoc-Cys(Ttr)-OH, Novabiochem) was coupled first to provide a thiol for attachment to the maleimide-coated array surface. Following addition of the last peptoid monomer, the library beads were split into two portions. For one portion of the beads, Sublibrary B, azidomethyl phenyl boronic acid (Alvarez and Alvarez 1997) was reacted with propargyl-bearing side-groups by copper-catalyzed 1,3-dipolar cyclo-addition as previously described (Chirayil and Luebke 2012).

To characterize the final distribution of monomers in the libraries, 20 individual library elements, i.e., products from individual beads, were randomly chosen from each peptoid sublibrary and analyzed by MALDI mass spectrometry. The mass spectra indicated a single major component in each of the products, which was confirmed by HPLC for five of the products from each library. Each sampled peptoid was sequenced by MS/MS. Each of the 20 monomers was present at least once in the set of sampled sequences, the most common frequencies being three and five times. The highest frequency of occurrence in this sample is six times. This distribution is consistent with the expected random incorporation of submonomers.

A fluorescein-labeled peptoid for spotting at defined reference positions was synthesized by coupling 5(6)-carboxyfluorescein (Sigma) to the N terminus of a cysteine-bearing peptoid prior to cleavage from solid support. Coupling used four equivalents of HBTU and 10 equivalents of DIPEA in DMF, and after cleavage from support, the labeled peptoid was purified by HPLC. Library beads are sorted into the wells of microtiter plates (one bead/well). Peptoids were cleaved from the beads (95% trifluoroacetic acid, 2.5% trisopropylsilane and 2.5% water), concomitantly removing side chain protective groups. After evaporation of the cleavage cocktail, the cleaved peptoids were dissolved in spotting buffer (50% DMSO, 50% TBS) and printed at a density of 500 spots/cm² onto 1-in × 3-in glass microscope slides coated with polyethyl-

e of ene glycol and maleimide using an ArrayIt robotic printer (Telechem International, Inc.).

General RNA sample preparation

RNA samples were obtained purified from Thermo Scientific with the 2'-ACE protective groups left in place. Protective groups were removed immediately prior to use according to the manufacturer’s protocol. The de-protected RNA was precipitated with ethanol and sodium acetate prior to use.

Array probing with RNA

Five nanomoles of RNA I or II, labeled at its 5’ terminus with Dy547 (Thermo Scientific), were de-protected and precipitated. The precipitate was dissolved in 200 µL of 0.1× PBS and heated briefly to 95°C, then allowed to cool slowly (2 h) to room temperature. The annealed RNA was diluted to 12 mL with 1× PBS, for a final concentration of 417 nM. The peptoid array was immersed in the RNA solution for 1 h at room temperature, then washed four times briefly with PBS before centrifugal drying. The array was scanned on a GenePix 4100A scanner (Axon Instruments) with laser excitation at 532 nm and a 550- to 600-nm emission filter (Cy3 emission maximum at 570 nm). Hit compounds were identified by MS/MS analysis (ABI 4700 Proteomics Analyzer) of the stock solutions of the library members spotted to the positive features.

Affinity measurements

RNA samples with 2-aminopurine (2-ap) substituted for adenine were de-protected and precipitated, then dissolved in 0.1× PBS phosphate buffered saline to a concentration of 18 µM. The resulting solution was heated briefly to 95°C, then allowed to cool slowly (2 h) to 25°C. The RNA was diluted to 6 µM with water. Peptoid solutions were prepared as serial dilutions in 2× assay buffer (10 mM MgCl2, 230 mM NaCl, 5.4 mM KCl, 50 mM Tris-HCl, pH 7.4), and equal volumes of peptoid and RNA solutions were mixed prior to delivery to wells of a standard black 96-well plate. The final RNA concentration in each well was 3 µM. Background fluorescence from the ligand was assessed from wells containing ligand at each final concentration mixed with a sample of RNA lacking 2-ap but treated identically to the 2-ap samples. After delivery of test solutions, each plate was centrifuged (1000 RPM, 1 min) and allowed to incubate at room temperature for 30 min. The plates were centrifuged again prior to measuring fluorescence on a SpectraMax M5 Microplate Reader (Molecular Devices), with excitation at 320 nm, emission measured at 390 nm, and a 325-nm cutoff filter. The dissociation constant was determined by fitting a sigmoidal dose-response curve to the mean fluorescence of six separate measurements after subtraction of background fluorescence due to ligand. Dissociation constants were determined in triplicate, and the mean and standard deviation are reported.

In-line cleavage analysis

The precipitate of de-protected RNA (5’-labeled with Dy547) was dissolved in 0.1× PBS to a final concentration of 15 µM, then heated briefly to 95°C and allowed to cool slowly (over 2 h) to room temperature. The resulting RNA was incubated, at a final concentration
of 3 µM, in 10 mM KCl, 50 mM Tris, pH 8.5. MgCl2 and peptoid were added to specified concentrations as stock solutions. Reaction mixtures were incubated at room temperature in darkness for 7 d, then precipitated with ethanol, sodium acetate, and 5 µg of yeast phenylalanine tRNA. Alkaline hydrolysis was carried out in 10 mM NaHCO3, pH 9.0, at 95°C for 4 min.

Ribonuclease T1 digestion was carried out with 0.007 units of ribonuclease T1 (Ambion) in 20 mM Tris, pH 7.15, 50 mM NaCl, 0.1 mM MgCl2, at room temperature for 20 min. The reaction was stopped by addition of 0.2 volumes of 5 M EDTA and precipitation with ethanol, sodium acetate, and 5 µg of yeast phenylalanine tRNA. The precipitates were heated briefly at 95°C in formamide prior to analysis by electrophoresis on a polyacrylamide gel (20%, 19:1 crosslinking, 8 M urea). The gel was visualized by fluorescence (332-nm excitation) with a Typhoon 9200 scanner (Amersham Biosciences) and analyzed with ImageQuant software.

### Construction of microprocessor substrates

RNA substrates for microprocessor cleavage were prepared by in vitro transcription of DNA templates. The pri-miR-21 and pri-miR-16 substrates were amplified from plasmid pCMV-MIR with pri-miR-21 and pri-miR-16 inserts, respectively (Origene). The pri-miR-21 substrate consisted of 319 nt of human pri-miR-21, with 140 nt flanking pre-miR-21 on its 5’ side and 122 nt flanking it on its 3’ side. The DNA template for this substrate was amplified by PCR using the following primers: 5’-ATAATGACTCCTATATTAGGAGTAAATGACGACGAC-3’ (forward) and 5’-GACTCTGAAGTGCCACACGACGAC-3’ (reverse). The forward primers for each substrate included the promoter sequence for T7 RNA polymerase.

Templates for chimeric substrates were generated by PCR amplification of the portion of the pri-miR-16 template to the 3’ side of the apical loop using primers 5’-CTTGGATGGCATTCAATAC-3’ and 5’-CCAGTATTAACTGTGCTGCTG-3’ (forward). These primers excluded the pri-miR-16 loop sequence before precipitating the products with ethanol and analyzing on an 8% polyacrylamide gel (19:1 crosslinking, 8 M urea). The products were excised from the gel and eluted by crushing the gel slice and soaking in 3 M sodium acetate, pH 5.2 overnight. The product RNA was then precipitated with ethanol prior to use in microprocessor reactions. Size standards were transcribed from templates amplified by PCR and used without purification.

### Microprocessor cleavage reactions

Microprocessor was prepared essentially as previously described (Han et al. 2006). Plasmids pCK-Drosha-FLAG and pCK-V5-DGCR8 were obtained from Professor V. Narry Kim and propagated in Escherichia coli (strain JM109, [Stratagene] for pCK-V5-DGCR8 and 5 µl Iq C2992H [NEB] for pCK-Drosha-FLAG). These plasmids were cotransfected into HEK293T with polyethyleneimine (PEI), using 16 µg pCK-Drosha-FLAG and 4 µg pCK-V5-DGCR8 for each 10-cm plate of cells. To generate a cell lysate for microprocessor-free controls, HEK293T cells were treated with PEI in the absence of plasmid. Cells were harvested 48 h after transfection and lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) containing three Complete Mini Protease Inhibitor Cocktail Tablets (Roche) per 25 mL lysis buffer. One milliliter of lysis buffer was added to 100 mg of cell pellet, and after 10 min of lysis at 4°C, the mixtures were centrifuged (14,000g). The supernatant (500 µL) was applied to an anti-FLAG M2 affinity gel (40 µL, Sigma) and incubated with mixing overnight at 4°C. The gel was washed with TBS, and the microprocessor complex was eluted with 100 µL of 3X FLAG peptide (15 µg/100 µL in TBS). The eluted protein was quantitated by Bradford assay (Pierce BCA assay kit, Thermo Scientific).

Microprocessor reactions contained radiolabeled substrate (3.3 nM), MgCl2 (6.4 mM), recombinant ribonuclease inhibitor (1.4 units/µL, Promega), NaCl (0.5 mM), Tris-HCl (0.2 mM, pH 7.4), and peptoid 12 at specified concentrations. Reaction mixtures were preincubated for 15 min prior to addition of microprocessor. Microprocessor or eluate from microprocessor-free cells was added to a final concentration of 0.4 µg/µL protein. Reactions were carried out at 37°C for 30 min, then extracted with phenol and chloroform before precipitating the products with ethanol and analyzing on an 8% polyacrylamide gel (19:1 crosslinking, 8 M urea). The gel was visualized by phosphor storage analysis (Typhoon 9200 scanner, Amersham Biosciences).

### SUPPLEMENTAL MATERIAL

Supplemental material is available for this article and shows representative microarray scans and binding curves with RNAs I and II for compounds 2–12.
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