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Caged oligonucleotides for bidirectional photomodulation of *let-7* miRNA in zebrafish embryos

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Abstract

Many biological functions of microRNA (miRNA) have been identified in the past decade. However, a single miRNA can regulate multiple gene targets, thus it has been a challenge to elucidate the specific functions of each miRNA in different locations and times. New chemical tools make it possible to modulate miRNA activity with higher spatiotemporal resolution. Here, we describe light-activated (caged) constructs for switching *let-7* miRNA “on” or “off” with 365 nm light in developing zebrafish embryos.

1. Introduction

MicroRNA (miRNA) is a large class of non-coding RNA that interferes with post-transcriptional gene expression through binding to the 3' UTR of mRNA. Since the discovery of the first miRNA, *lin-4*, in 1993, it has been found that miRNAs are abundant short RNAs that have important roles in normal developmental and cellular processes, as well as in human disease.^{1, 2}

Unlike short interfering RNA (siRNA), a single miRNA can interfere with multiple gene targets, as binding does not require perfect complementarity except in the 5' seed region.^{3, 4} This makes elucidation of miRNA function particularly challenging as a single miRNA can have multiple roles that vary with cellular location and timing.⁵ Loss-of-function miRNA studies are useful in determining miRNA pathways,⁶ but fall short in identifying multiple miRNA functions with spatial and temporal resolution.

Loss-of-function studies of miRNA are most commonly performed using anti-miRNA oligonucleotides, also known as antagomirs.⁷ These oligonucleotides are perfectly complementary to the biologically active sequence of the miRNA and sterically block function by outcompeting the target mRNA. Various oligonucleotide backbone and ribose modifications have been previously studied to improve the efficacy of antagomirs, such as phosphorothioation, 2'-F RNA, 2'-OMe RNA, locked nucleic acid (LNA), as well as morpholinos which are commonly used in antisense applications.⁴ Reverse complementary 2'-OMe RNA can block miRNA function in *C. elegans*, based on nuclease resistance and increased binding affinity as shown by melting temperature (T_m) studies.^{6, 8}

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The complexity of gene expression patterns established in metazoan early development has motivated the creation of biochemical tools for controlling genes with higher spatial and temporal resolution.^{9–12} Light-activated (i.e., “caged”) approaches show considerable promise as near-UV/Vis/near-IR light of desired wavelength can be applied dose-dependently and in a spatially and temporally controlled manner. In recent years, there have been advances in caging siRNA, allowing for spatial and temporal control of the RNA interference (RNAi) pathway. In 2005 the Friedman lab first reported caged siRNA by labeling the phosphate backbone of a siRNA duplex with 1-(4,5-dimethoxy-2-nitrophenyl)ethyl moieties, which blocked the siRNA-RISC interaction before photolysis.¹³ More recent work by the Friedman lab blocked siRNA activity with a larger photocleavable cyclo-dodecyl (dimethoxy-2-nitrophenyl)ethyl moiety.¹⁴ The addition of steric bulk to the terminal phosphates prevents Dicer and nucleases that could remove the caging group, from interacting with the siRNA.

While these and other^{15, 16} advances have been made towards spatiotemporal control of the RNAi pathway, there has been much less reported for the biochemical modulation of miRNA. Recently, Zheng et. al. reported a novel photoactivatable antagomir for controlling miRNA in *C. elegans*.¹⁷ In this work, a caged hairpin-like structure was created by attaching a complementary blocking strand to the antagomir via a coumarin moiety. After irradiation, dissociation of the antagomir became more favorable, allowing binding to a miRNA target.¹⁷ Additionally, Connelly et al. reported a method of photomodulating miRNA in cells by attaching caging groups on specific bases of the *miR-122* and *miR-21* antagomirs.¹⁸ Here, we expand upon the caged hairpin antagomir. Moreover, we provide the first example of a caged miRNA, which makes it possible to “turn on” exogenously supplied miRNA via photoactivation.

Let-7, the miRNA of interest in this study, was discovered in 2000 in *C. elegans* by Reinhart et al. and was shown to play an important role in developmental timing.¹⁹ Interestingly, this particular miRNA is evolutionarily conserved across humans, flies, mice, and zebrafish.²⁰ *Let-7* miRNA was chosen for the current investigation due to the developmental arrest caused by overexpression in *Danio rerio* (zebrafish) embryos, resulting in a readily observable phenotype.⁵ This phenotype is characterized by reduced head and eye formation, shortened tail, and limited yolk sack extension.⁵ *Let-7* miRNA is not endogenously expressed in zebrafish embryos until 48 hours post fertilization (hpf), but an early *let-7* expression phenotype can be induced by injecting the *let-7* miRNA at the one-cell stage. This phenotype is clearly visible at 24 hpf.⁵ Representative examples of zebrafish embryos showing the *let-7* early induction phenotype are shown in Figure 1. This provides a miRNA-vertebrate model system to photoregulate miRNA in both directions--from “off to on” and “on to off”.

We developed novel oligonucleotide constructs for regulating miRNA in zebrafish embryos: two caged hairpin antagomirs (CHANT1, CHANT2) for blocking miRNA function with 365 nm light, as well as a caged circular miRNA (CIRC*let7*) for introducing exogenous miRNA into a system with 365 nm light. The design of CHANT1 was guided by our lab’s experience in developing caged hairpins with a single photocleavable linker for photoactivating antisense oligonucleotides in zebrafish embryos.²¹ This is similar to the caged hairpin antagomir recently published by Zheng et al.¹⁷ In CHANT2, we expand upon this design by incorporating a longer blocking sense strand and second photocleavable linker. Finally, the circular miRNA expands on our previous circular design involving the 10–23 DNase, ²² and also draws inspiration from several notable circular caged oligonucleotide constructs that have recently been published.^{23–25}

2. Results and Discussion

2.1. CHANT1

A caged hairpin antagomir (CHANT1) was designed and synthesized to “turn off” *let-7* miRNA with a low dose of 365 nm irradiation. This antagomir consisted of a 22-mer oligonucleotide synthesized with all 2'-OMe RNA bases (Figure 2A). The function of this antagomir was inhibited by a shorter 12-mer 2'-OMe RNA blocking strand. The antagomir and blocking strand were covalently attached by a single nitrobenzyl photocleavable linker, PL (shown in red). Prior to photolysis, PL stabilized the hairpin hybrid form, thereby blocking the function of the antagomir. Upon near-UV irradiation, PL was cleaved, resulting in a destabilized hybrid. The photoactivated 22-mer 2'-OMe RNA antagomir can readily hybridize to the complementary miRNA and sterically block its function.

In designing CHANT1, a balance was struck between antagomir blocking efficiency and restoration of antagomir function post-irradiation. In principle, all antagomir nucleotides can be sterically blocked with a 22-mer blocking sequence; however, this would disfavor dissociation post-irradiation. For this particular construct, a 12-mer blocking strand was chosen to block the antagomir at the 5' seed region, as this 6–8 nucleotide sequence has been shown to be important in miRNA-mRNA target recognition.^{26, 27} Additionally, terminating the blocking sequence with a guanine or cytosine base should result in a more stable hybrid due to the increase in hydrogen bonding. For this reason, the placement and length of the blocking strand for this particular sequence was chosen to be a 12-mer as shown in Figure 2A. CHANT1 was synthesized by solid-phase synthesis, isolated by HPLC purification (Figure S1), and confirmed by MALDI-TOF MS to have the correct mass (Figure S2A). Melting temperature analysis has proved to be a useful tool to determine nearly the optimal length of the blocking strand. In this case, melting temperature analysis showed a CHANT1 melting temperature of 85 °C, indicating favorable duplex stability. The exact melting temperature cannot be determined because a full transition cannot be observed in water at such a high T_m . The T_m dropped to 64 °C post-photolysis, decreasing the melting temperature (T_m) by at least 21 °C (Figure S3A). This T_m indicates significant photomodulation of duplex stability and is comparable to many caged hairpins synthesized in our lab.^{28–30} However, the post-photolysis T_m of 64 °C suggests that a significant amount of antagomir remains bound to the blocking strand (and therefore inactive), even after uncaging.

To confirm the efficacy of the exogenous *let-7* miRNA *in vivo*, a 10 μ M solution was injected into zebrafish embryos at the one-cell stage ($n = 35$). Injection resulted in the *let-7* miRNA early induction phenotype in 92% of embryos (Figure 1). *In vivo* studies with CHANT1 were subsequently performed by co-injecting a 10 μ M solution of *let-7* miRNA, together with varying concentrations (40 – 100 μ M) of CHANT1 into embryos at the one cell stage. CHANT1 at 50 μ M exhibited high activity with minimal toxicity. Thus, a 10 μ M solution of *let-7* miRNA and 50 μ M CHANT1 was subsequently injected into all zebrafish embryos at the one-cell stage. Half of the injected embryos were allowed to incubate at 28 °C in the dark, while the other half were irradiated at 1 hpf. A near-UV transilluminator was used to irradiate the embryos for 10 min in E3 zebrafish media. The embryos were incubated for 24 h at 28 °C, and then imaged to determine phenotypic response. Of the embryos that were irradiated with 365 nm light ($n = 21$), the majority (86%) developed normally, indicating that the caged antagomir was photoactivated and able to block *let-7* miRNA function (Figure 3). In the non-irradiated embryo group ($n = 15$), the majority (80%) developed with the *let-7* miRNA early induction phenotype, as expected. However, a significant minority (20%) of the embryos developed normally (Figure 5), indicating

incomplete caging of CHANT1. Although this caged hairpin exhibited a very high T_m , only 12 of the 22 antagomir nucleotides were blocked from *let-7* miRNA binding.

2.2. CHANT2

A second caged hairpin antagomir, CHANT2 (Figure 2B), was developed with the goal of minimizing background miRNA binding pre-photolysis, while maximizing miRNA binding post-photolysis. To achieve these aims, we reasoned that the caged hairpin must form a longer, stable duplex structure prior to irradiation, and become less thermodynamically stable afterwards. In particular, the high melting temperature of CHANT1 post-photolysis indicated a route to further optimization. By adding an additional photocleavable moiety in the blocking strand, it was possible to incorporate more bases within the blocking strand, without sacrificing antagomir activity after irradiation.

The same 22-mer antagomir complementary to *let-7* miRNA was covalently attached via a PL to a blocking strand comprised of a 9-mer and 10-mer linked by an additional PL (Figure 2B). This PL blocking strand design was guided by our previously published RNA “bandage” which used two shorter sequences linked by a PL to achieve mRNA blocking pre-photolysis and dissociation post-photolysis.³¹ Consistent with successful RNA bandage designs,³¹ a one nucleotide gap was introduced at the position of the PL. Finally, because terminating the blocking sequence with a guanine or cytosine base should result in a more stable duplex, the blocking strand was extended by one base on the 3' end to a 10-mer to facilitate G/C base-pair termination.

CHANT2 was synthesized on solid support, purified by HPLC (Figure S1) and the mass was confirmed by MALDI-TOF MS (Figure S2). The T_m for CHANT2 was measured to be -38°C : $T_m(-\text{UV}) = 71^{\circ}\text{C}$, $T_m(+\text{UV}) = 33^{\circ}\text{C}$ (Figure S3B). Significantly, $T_m(+\text{UV})$ was much closer to rt than observed for CHANT1, which should improve the miRNA-binding activity of the uncaged antagomir. Moreover, relative to CHANT1 with a single 12-mer blocking strand, CHANT2 exhibited a significantly greater difference in thermal stability pre- vs. post-photolysis, $T_m = 17^{\circ}\text{C}$. Interestingly, $T_m(-\text{UV})$ was significantly lower for CHANT2 than CHANT1, which indicates that the 9-mer-PL-10-mer blocking strand did not behave as a contiguous 19-mer. This is likely due to the intervening PL, and also the one nucleotide gap included in the 9-mer-PL-10-mer design. This is further corroborated by the broad transition observed in the CHANT2 melting temperature curve. Upon photolysis, the two short oligonucleotides of different lengths will dissociate at somewhat different temperatures, causing the melting curve to broaden.

Based on T_m data alone, one might expect the CHANT1 hairpin structure to be more stable *in vivo* than CHANT2 prior to photolysis, due to a higher melting temperature. However, thermal stability alone is not a reliable predictor of mRNA blocking efficiency and the different hairpin structures must be taken into account. As both T_m 's are significantly above incubation temperature, it is expected that CHANT1 and CHANT2 will remain annealed. A more important factor becomes the antagomir bases that remain accessible when the caged hairpin is fully annealed. For *in vivo* studies, particularly when the target miRNA is co-injected, the second PL, in conjunction with the longer blocking strand, is critical for improving photomodulation efficiency.

Preliminary *in vivo* studies were performed by co-injecting a 10 μM solution of *let-7* miRNA with varying concentrations (30 – 100 μM) of CHANT2 into embryos at the 1-cell stage. CHANT2 at 50 μM exhibited high activity with minimal toxicity. Thus, a 10 μM solution of *let-7* miRNA and 50 μM CHANT2 was subsequently injected into all zebrafish embryos at the one-cell stage. Half of the injected embryos were incubated at 28°C in the dark, while the other half were irradiated at 1 hpf for 10 min with the near-UV

transilluminator. The embryos were incubated for 24 h in the dark at 28 °C and then imaged to determine phenotypic response (Figure 4). The embryos that were irradiated ($n = 40$) developed normally which clearly showed that the uncaged antagomir was active. Photoactivated CHANT2 rescued the normal wildtype phenotype for nearly 90% of zebrafish embryos, vs. 80% for CHANT1 embryos (Figure 5). A marked improvement was also seen in antagomir caging in the non-irradiated embryos ($n = 45$), with just 2% background activity for CHANT2 compared to 20% for CHANT1 (Figure 5). This 2% background can be attributed to injection error, which in our experience can range from 0 – 10% of embryos. This construct was also tested at lower concentrations (30 and 37 μM) and it was demonstrated that *in vivo* efficiency varied with CHANT2 concentration. Post-photolysis antagomir activity increases from 64% to 76% and 87% with increasing CHANT2 concentration (30 μM , 37 μM , 50 μM), indicating that *in vivo* activity is strongly dependent on caged antagomir concentration. As the T_m indicates, at 33 °C, this construct should be half dissociated. As the embryos are incubated at 28 °C, which is below the post-photolysis T_m , less than half of the antagomir will be dissociated at this temperature, resulting in the need for higher concentrations of caged antagomir. The observation of maximum antagomir activity at 50 μM is well below the concentration (100 μM) where significant toxicity was observed.

The significant decrease in CHANT2 background activity as compared to CHANT1 highlights that the 9-mer-PL-10-mer blocking strand caged the antagomir more effectively than the original 12-mer, despite the lower thermal stability of CHANT2 ($T_m = 72$ °C) relative to CHANT1 ($T_m = 85$ °C) (Figure S3A–B). The data indicate that CHANT2 is nearly optimized for photomodulating the *let-7* antagomir in the current experimental paradigm, where miRNA is co-injected in zebrafish embryos.

With creation of CHANT1 and CHANT2 and the two previously published caged antagomirs,^{18, 32} a new class of tools for downregulating miRNA with light is emerging. Connelly et. al. presented an elegant method of caging antagomirs miR-122 and miR-21 with minimal background activity and showed efficient antagomir restoration post-photolysis; however, this involved the use of custom caged bases. Zheng et al. presented the first example of *in vivo* miRNA photomodulation by using a caged hairpin antagomir. To achieve very efficient caging and uncaging, it was necessary to design and test a large number of caged antagomirs. Our CHANT2 design minimizes the need for optimization, as the second PL allows a much larger portion of the antagomir to be blocked without sacrificing uncaging efficiency. This will facilitate the targeting of multiple miRNAs without optimization necessary for each sequence. Additionally, the commercial availability of the 2'-OMe RNA phosphoramidites and PL used in CHANT2 make it readily accessible to biological researchers.

2.3. Caged Circular miRNA, CIRC*let7*

In addition to the caged hairpin antagomirs, which provide a method to “turn off” miRNA with light, caged circular *let-7* miRNA (CIRC*let7*) was designed to “turn on” exogenous miRNA with light. The circular design provides a way to introduce miRNA into a biological system and subsequently activate at the desired place and time by irradiating with 365 nm light.

Similar to the caged antagomirs, this caged miRNA uses fully 2'-O-methylated bases to limit degradation that is problematic with unmodified RNA. A complementary 22-mer 2'-OMe RNA miRNA and blocking strand were synthesized containing two PLs, a free amine on the 3' end, and a disulfide on the 5' end as shown in Scheme 1. The 5' and 3' ends were linked together in a “circularization reaction” by first reacting the succinimide of a commercially available heterobifunctional linker with the 3'-amine. The 5'-disulfide was then reduced with

TCEP, allowing subsequent reaction with the linker maleimide and resulting in a circularized oligonucleotide. Both the linear and circularized oligonucleotides were purified by HPLC (Figure S4). Upon photolysis, the circular miRNA was linearized as shown in Scheme 1. The phosphate group revealed on the 5' end of the biologically active miRNA allows for processing by the multiprotein RNA-induced silencing complex (RISC). Our lab's previous circular design for caged DNazymes used CircLigase for circularization, which limited the reaction to the picomole scale.³³ By using the heterobifunctional linker, more oligonucleotide can be circularized in a single reaction. Circularization yield, calculated from gel band intensity, was determined to be 45% when performed on a 1 nmol scale (Figure S5A). Additionally, a method was developed to purify the circular construct by HPLC without having to do lengthy gel extractions to remove linear starting material. Purity was confirmed by a well-resolved product peak on HPLC which was further analyzed using gel electrophoresis (Figures S4, S5).

To test this circular miRNA *in vivo*, a 20 μ M solution of CIRC*let7* was injected into zebrafish embryos at the one-cell stage. Half of the injected population was incubated in the dark at 28 °C (n = 18). The other half was irradiated with 365 nm light for 10 min at 1 hpf before incubation (n = 21). The embryos were analyzed and imaged at 24 hpf for phenotypic response. As shown in Figure 6, embryos injected with the caged miRNA developed normally, while embryos injected and irradiated developed with the *let-7* miRNA early induction phenotype. These results were seen in 100% of embryos injected, indicating that there was no background activity prior to photolysis and uncaging completely restored miRNA activity. Interestingly, the phenotype induced by the 2'-OMe RNA circular design varied slightly from the phenotype induced by the *let-7* miRNA. A 2'-OMe RNA control miRNA was injected into zebrafish embryos (Figure S6) to confirm that this was not a result of the circular construct, but rather a result of an analog used to induce the miRNA phenotype. As shown in Figure 1, the phenotype induced with *let-7* miRNA was dominated by a thickened yolk. This particular feature was not seen with the 2'-OMe RNA *let-7* early induction phenotype, although other characteristic features were present such as a significant decrease in tail formation, and decreased head and eye formation (Figure S6).

3. Conclusion

miRNAs are an abundant class of small gene-regulating RNAs, and our understanding of their diverse biological functions is still incomplete. New chemical tools, such as those we have introduced here, will make it possible to probe how each miRNA regulates a multitude of genes in different locations and at different times *in vivo*.

We have designed a set of caged antagomir and caged miRNA constructs with the ability to regulate miRNA from “on to off” as well as “off to on” with light. These tools were shown to control exogenously supplied miRNA and have the potential to control endogenous miRNA as well. These caged constructs are effective in zebrafish embryos and could readily be optimized for use in other model organisms, such as *Xenopus*³⁴ and *C. elegans*.^{6, 8} By using 2'-OMe RNA oligonucleotides, we greatly simplified the synthesis of these constructs, as these analogs can be prepared very successfully on solid support using commercially available phosphoramidites.

We described two caged antagomir designs, CHANT1 and CHANT2. With the addition of the second photocleavable linker in CHANT2, more antagomir bases were sterically blocked prior to irradiation without sacrificing antagomir activity post-photolysis. With the addition of a second PL, the blocking strand could be lengthened to diminish background activity seen in the first design. Previously, we described several examples of caged antisense

hairpins with a similar design to CHANT1 using only one PL.^{21, 30} Based on this current study, it appears beneficial to incorporate a second PL in many caged hairpin constructs.

To introduce an exogenous miRNA and provide a method for turning a miRNA from “off to on”, a circular caged miRNA was designed. CIRC*let7* is the first example of a caged miRNA, and was demonstrated in a zebrafish model system. As many developmental programs stem from the downregulation of mRNA via miRNA, it is also useful to have a tool to introduce either a *de novo* miRNA, or to probe the effects of miRNA upregulation in different systems. The tools described herein will provide new ways to probe miRNA function, which until now has been difficult to resolve with loss-of-function experiments. A direct follow-up to this work could be the use of light-activatable probes to determine more precisely the genes on which *let-7* miRNA acts in different locations and times in early embryo development, as it is known that there are multiple targets.⁵ While these constructs have been developed specifically for *let-7* miRNA in zebrafish, these methods can very easily be applied to a wide range of miRNAs in many biological systems.

4. Experimental

4.1. Materials

2'-OMe RNA phosphoramidites for solid-phase oligonucleotide synthesis and all modified phosphoramidites, including CPG solid supports, were purchased from Glen Research (Sterling, VA). Product numbers for photocleavable spacer phosphoramidite, amino modifier CPG, and thiol modifier phosphoramidite are 10-4913-90, 20-2957-01, 10-1926-90, respectively. N- ϵ -maleimidocaproyl-oxysulfosuccinimide ester (Sulfo-EMCS) linker was purchased from Thermo Scientific (Pierce Protein Biology Products). NAP-5/10 desalting columns were purchased from GE Healthcare. *Let-7* miRNA (5'-UGAGGUAGUAGGUUGUAUAGUU-3') was ordered from Integrated DNA Technologies (IDT).

4.2. Synthesis, purification, and characterization of CHANT1 and CHANT2

CHANT1 and CHANT2 were synthesized on a 1 μ mol scale using published methods for oligonucleotide synthesis on solid support using an ABI 394 DNA synthesizer.^{35, 36} CHANT1 and CHANT2 were cleaved from solid support using concentrated ammonium hydroxide for 16 h, and HPLC purified (Figure S1). A reverse-phase C18 semi-preparatory column was used on an Agilent 1100 HPLC system. A gradient of 10% - 80% acetonitrile in 0.05 M triethylammonium acetate (TEAA) was used for purification of all oligonucleotides containing a dimethoxytrityl (DMT) protecting group. The DMT protecting group was removed using 80% glacial acetic acid for 20 min at rt. A NAP-5 column was used for buffer exchange after purification and deprotection. After HPLC purification, approximately 100 nmol of pure material was attained.

4.3. Synthesis and purification of CIRC*let7*

CIRC*let7* was synthesized on a 1 μ mol scale using published methods for oligonucleotide synthesis on solid support using an ABI 394 DNA synthesizer.³⁷ A Fmoc-protected amino solid support was used on the 3' end and a disulfide phosphoramidite was incorporated at the 5' end. The construct was cleaved from solid support using concentrated ammonium hydroxide for 16 h, and HPLC purified (Figure S4). A reverse-phase C18 column was used on an Agilent 1100 HPLC system. A gradient of 10% - 80% acetonitrile in 0.05 M triethylammonium acetate (TEAA) was used for purification of all oligonucleotides containing a dimethoxytrityl (DMT) protecting group. The DMT protecting group was removed using 80% glacial acetic acid for 20 min at rt. A NAP-5 column was used for

buffer exchange and desalting after purification and deprotection. After HPLC purification, approximately 30 nmol of pure material was attained.

The 3' amine was reacted with an excess of sulfo-EMCS linker. A 0.4 mg/mL linker solution was prepared in 0.1 M PBS buffer, pH 7.2. The reaction was allowed to proceed for 30 min at 37 °C and quenched by removing excess sulfo-EMCS with a NAP-5 column. The 5' disulfide was then reduced with TCEP at 37 °C, and the circularization reaction with 5' thiol was allowed to proceed for 24 h. Circularization was confirmed by visualizing bands by gel electrophoresis (Figure S5A). Circularization yield was determined by band quantification using ImageQuant TL 7.0 Image Analysis Software with the rubber band background subtraction method. Circular product was purified from linear starting material using anion exchange HPLC (Figure S5B). A Dionex DNAPac PA100 anion exchange column was used with a gradient (Figure S4B) of NaClO₄ in the presence of Tris-Cl pH 8.0 to separate linear starting material from circularized product (Figure S4B).

4.4. Characterization

Melting temperature analysis was performed on a Beckman Coulter DU800 spectrophotometer with a Peltier temperature controller and T_m analysis software (Figure S3). A solution of 1 μ M oligonucleotide in 50 mM NaCl in deionized H₂O was used for all melting temperature experiments. Samples were irradiated using a Spectroline TR-365R transilluminator delivering 9 mW/cm² centered at 365 nm for 10 min. Melting temperatures were analyzed by varying the temperature between 15 °C and 90 °C at a rate of 1 °C per min, in both forward and reverse directions.

Gel electrophoresis was performed using a 20% polyacrylamide gel containing 7 M urea. Gels were run at 300 V for 45 min and stained with ethidium bromide. Gels were imaged with a GE Typhoon FLA 7000 imager (Figure S5).

Masses were confirmed by the Wistar Proteomics Facility using an Applied Biosystems Voyager 6030 with 3-hydroxypicolinic acid (3-HPA) as matrix (Figure S2).

4.5. *In vivo* studies

Zebrafish embryos were obtained from CDB Zebrafish Core Facility at the University of Pennsylvania. Both Tu and TLF wildtype embryos were used for these studies. Zebrafish embryo injection solutions were prepared to contain a final concentration of 0.1 M KCl and 0.25% phenol red dye. All injections were performed at the one-cell stage and injected into the cell compartment only. A Harvard Apparatus PLI-100 pico-injector was used to inject controlled volumes. Injection volume was calibrated to dispense 8 – 10 nL per embryo. Zebrafish embryos were incubated at 28 °C in E3 zebrafish medium.³⁸ All embryos were incubated in the dark, except for irradiated samples which were exposed to near-UV light for the stated time and returned to the dark incubator. Embryo micrographs were collected at 24 hpf with an Olympus FV1000 laser scanning confocal microscope using transmitted light imaging. A 10x air objective was used for single embryo imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *let-7* early induction phenotype

Zebrafish embryos injected at the 1-cell stage with 10 μ M *let-7* miRNA show the *let-7* early induction phenotype when imaged at 24 and 50 hpf (right images), compared to un-injected wildtype (Tu $\times$ Tu) embryos (left images). The early induction *let-7* miRNA phenotype is characterized by decreased head formation, shortened tail, and yolk that fails to extend along the tail.

Figure 2. Structures of caged hairpin antagomirs CHANT1 and CHANT2

A) CHANT1: A 22-mer miRNA antagomir targeting *let-7* miRNA was covalently attached to a complementary 12-mer blocking sequence via a nitrobenzyl photocleavable linker, PL (in red). Upon photolysis, the 12-mer more readily dissociated, restoring antagomir function.

B) CHANT2: A 22-mer miRNA antagomir targeting *let-7* miRNA was covalently attached via PL (in red) to a 5-9-mer-PL-10-mer blocking sequence. Upon PL photolysis, antagomir function was restored. All sequences consisted of 2'-OMe RNA bases.

Figure 3. CHANT1 *in vivo* experiments

A) Embryos injected at the 1-cell stage with 10 μM *let-7* miRNA showed *let-7* early induction phenotype. B) Embryos injected with 10 μM *let-7* miRNA + 50 μM CHANT1 showed similar *let-7* early induction phenotype prior to 365 nm irradiation. C) Embryos injected with 10 μM *let-7* miRNA + 50 μM CHANT1 developed normally after 365 nm irradiation for 10 min at 1 hpf. All embryos were imaged at 24 hpf.

Figure 4. CHANT2 *in vivo* experiments

A) Embryos injected at the 1-cell stage with 10 μ M *let-7* miRNA showed *let-7* early induction phenotype. B) Embryos injected with 10 μ M *let-7* miRNA + 50 μ M CHANT2 antagomir showed *let-7* early induction phenotype prior to 365 nm irradiation. C) Embryos injected with 10 μ M *let-7* miRNA + 50 μ M CHANT2 developed normally after 365 nm irradiation for 10 min at 1 hpf. All embryos were imaged at 24 hpf.

Figure 5.
CHANT1 vs. CHANT2 *in vivo* efficiency.

Figure 6. CIRClet7 in vivo experiments

A) Zebrafish embryos injected with 20 μ M CIRClet7 and incubated in the dark developed normally. B) Embryos irradiated with 365 nm light developed with the early induction *let-7* miRNA phenotype. All embryos were imaged at 24 hpf.

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