Silencing microRNA by interfering nanoparticles in mice

Jie Su¹, Huricha Baigude¹², Joshua McCarroll¹ and Tariq M. Rana¹²,*

¹Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605 and ²Program for RNA Biology, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

Received June 2, 2010; Revised December 3, 2010; Accepted December 8, 2010

ABSTRACT

MicroRNAs (miRNAs) are small endogenous non-coding RNAs that regulate post-transcriptional gene expression and are important in many biological processes. Disease-associated miRNAs have been shown to become potential targets for therapeutic intervention. Functions of miRNAs can be inhibited by using antisense oligonucleotides, called anti-miRs, complimentary to the miRNA sequences. Here, we show that systemic delivery of a chemically stabilized anti-miR-122 complexed with interfering nanoparticles (iNOPs) effectively silences the liver-expressed miR-122 in mice. Intravenous administration of 2 mg kg⁻¹ chemically modified anti-miR-122 complexed with iNOP-7 resulted in 83.2 ± 3.2% specific silencing of miR-122, which was accompanied by regulating gene expression in liver and lowering of plasma cholesterol. The specific silencing of miR-122 was long lasting and did not induce an immune response. Our results demonstrate that iNOPs can successfully deliver anti-miR to specifically target and silence miRNA in clinically acceptable and therapeutically affordable doses.

INTRODUCTION

MicroRNAs (miRNAs) are small endogenous non-coding RNAs that post-transcriptionally regulate gene expression by binding with imperfect complementarity in 3'-untranslated regions (3'-UTR) of their target mRNAs (1–4). miRNAs have been shown to play an important role in development, and cell growth and differentiation (5,6). Recent studies have highlighted the role of miRNAs in various disease states and in regulating host-pathogen interactions (7). For example, miRNAs have been implicated in cardiovascular disease (8), inflammation (9), viral infections (10–13) and cancers (14,15). Hence, disease-associated miRNAs could become potential targets for therapeutic intervention (7).

MiRNA-122 (miR-122) is a liver-specific miRNA, with suggested roles in cholesterol, fatty acid and lipid metabolism (16–18). miR-122 interacts with hepatitis C virus genome facilitating viral replication in host cell (10,19,20). In vivo silencing of miR-122 has been achieved by systemic administration of antagonirs or modified antisense oligonucleotides in mice or non-human primates (16–18,21). These studies were very encouraging in providing proof of the concept for silencing miRNA by its anti-miR sequences in vivo. Recently, therapeutic silencing of miR-122 in primates with chronic hepatitis C virus infection has been reported by using locked nucleic acid (LNA) modified oligonucleotide (SPC3649) complimentary to miR-122 (22). Remarkably, SPC3649 treatment caused long-lasting suppression of HCV viremia in primates with no apparent evidence of side effects or viral resistance indicating that the anti-miR therapeutic strategy could lead to the development of new drugs for viral infections. We recently reported successful silencing of apoB mRNA by iNOP-7 after systemic administration of 1 mg kg⁻¹ siRNA in mice (23). iNOP-7 is a new nanomaterial for systemic delivery of RNAi developed by our laboratory, which is a lysine-containing nanoparticle with the surface functional groups modified with lipid chains (23,24). A clear correlation between siRNA localization, cellular uptake and RNAi activity was observed when siRNA was delivered into cells using siRNA-TAT₄₇–₅₇-peptide, siRNA-TAT₄₇–₅₇-derived oligocarbamate conjugates or nanoparticles (25). For successful RNAi, the localization of siRNA was distinctly cytoplasmic suggesting that siRNA is targeted to these regions for interactions with RISC to induce RNAi (25). Recent advances in understanding the rules for chemically modifying siRNA sequences without compromising their gene-silencing efficiency (26–28), reviewed in (4) have allowed the design and synthesis of therapeutically effective siRNA molecules

*To whom correspondence should be addressed. Tel: +1 858 795 5325; Fax: +1 858 795 5328; Email: trana@sanfordburnham.org

© The Author(s) 2011. Published by Oxford University Press.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
that can silence target genes in vivo ([29–31], reviewed in (32)]. We reasoned that a chemically stabilized anti-miR could be assembled with iNOP-7 to target miRNA sequences and inhibit their functions in vivo. To enhance the stability of anti-miR molecules, we chemically stabilized the oligonucleotides according to modification rules established in our previous studies [(27,28), reviewed in (4)]. In this study, we tested this concept and determined the silencing efficiency of miR-122 using iNOP-7 in mice.

**MATERIALS AND METHODS**

Oligonucleotides

RNAs were purchased from Dharmaco (Lafayette, CO, USA) and consisted of 23-24 nucleotides length with modifications as specified: chemically modified anti-miR-122 (CM): 5'-AC\(^3\)AAC\(^3\)AC\(^3\)C\(^3\)AUUGUCACACUCC A-3'; chemically modified mismatch anti-miR-122 (MM): 5'-UCAC\(^3\)AAC\(^3\)CUC\(^3\)UAAGAAAGAGCUAGA-3'. The superscript letter F represents 2'-O-F modified nucleotides. Chemical modifications were designed based on our previously published rules established in our laboratory [(27,28), reviewed in (4)]. The complexes were prepared by mixing CM or MM anti-miR with iNOP-7 at a ratio of 1:10 (w/w) in HEPES saline or Opti-MEM culture medium (Invitrogen, Carlsbad, CA, USA) and incubating at room temperature for 20 min (see below).

**In vitro silencing miR-122**

Huh-7 cells (kindly provided by John Taylor, Fox Chase Cancer Center) were maintained at 37°C with 5% CO\(_2\) in DMEM with High Glucose culture medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U ml\(^{-1}\) penicillin and 100 \(\mu\)g ml\(^{-1}\) streptomycin. Cells were regularly passaged and plated in 6-well culture plates for 16 h before transfection at 70% confluency. The anti-miRs were transfected in Opti-MEM serum free culture medium for 4 h at 37°C, and then changed to normal medium with 10% FBS. Efficiency of silencing was determined by northern blotting as described below.

**Dual luciferase assay**

The miR-122 luciferase constructs were engineered by inserting the full 23-bp sequence complementary to the mature miR-122 into the 3'-UTR of pGL3-Control (Promega, Madison WI, USA). Huh-7 cells were seeded in 24-well culture plates and transfected with 0.1 \(\mu\)g miR-122 pGL3-control plasmid and 0.015 \(\mu\)g pRL-TK plasmid (Promega, Madison WI, USA) for normalization using iNOP-7. After 4 h of transfection, cells were treated with complete media. Cells were lysed 48 h later, unless otherwise indicated, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison WI, USA). These quantitative assays were conducted in multiple replicates for each concentration.

**In vivo silencing miR-122**

All animal procedures were approved by the Institutional Animal Care and Use Committee (University of Massachusetts Medical School). Six- to eight-week-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) were maintained under a 12 h dark cycle in a pathogen-free animal facility. Mice were administrated with either phosphate buffered saline (PBS) pH 7.4 or iNOP-7 complexes (as indicated) at 2 mg kg\(^{-1}\) body weight in 0.2 ml per injection as bolus injection via the lateral tail vein at 0, 12 and 36 h. Measurements of miRNA or mRNA levels in tissues were performed 24 h after the last injection unless indicated otherwise. Liver and plasma were collected and stored in –80°C until analysis. The in vivo silencing experiments were repeated at least twice and multiple samples were collected from tissues for quantitative analysis.

**Northern blotting**

RNA from cell culture or mouse livers was homogenized in TRIZOL (Invitrogen, Carlsbad, CA, USA) and isolated according to the manufacturer’s instructions. Total RNA was separated on a 14% acrylamide/20% formamide/8 M urea gel, then electroblotted onto Hybond-XL nylon membrane (GE Healthcare, Piscataway, NJ, USA). The probe with \(\gamma\)-\(\alpha\)P-labeled oligonucleotides for miRNA or rRNA was hybridized to the membrane at 42°C. The blots were visualized by scanning in a FLA-5000 scanner (Fujiﬁlm, Stamford, CT, USA).

**Quantitative RT/PCR**

To determine regulations of mRNA levels in mouse tissues after anti-miR treatment, total RNA was extracted with TRIZOL (Invitrogen, Carlsbad, CA, USA) and treated with TURBO DNA-free kit (Applied Biosystems, Foster City, CA, USA) before quantification. At least two liver regions were used to extract total RNA from individual animals in each group of three animals. In preparation for quantitative PCR, total RNA (400 ng) was reverse transcribed by using SuperScript II (Invitrogen, Carlsbad, CA, USA) and random primers according to the manufacturer’s protocol. The expression of mRNA was measured using ABsolute QPCR SYBR green mix (ThermoFisher Scientiﬁc, Epsom, Surrey, UK) normalized to GAPDH according to the manufacturer’s instructions. Quantitative PCR was performed using a Chromo4 Real-Time PCR Detection System (BioRad, Hercules, CA, USA). We used the following primers: **Acacb** forward: GGGGCTCCCCG GATGACAAC, reverse: TCCCCGGAGGATTTCTGGA; **Acas2** forward: GTGGCCATATGC TGCACACCCCTCCTC, reverse: GGACAGCGATCCTC CGGTACATAGT; **Aldoa** forward: CAGGAAAGC AACTGCACCGCGCA C, reverse: GATTTACAGGTTGTCGTCAGTGC; **Apob** forward: TTCCAGGCGATGGGCAACTTTACC, reverse: TACTGAGGGGCTAGTGACAAT; **Fasn** forward: ACGAGTGATGTAATGGGAAG, reverse: GA CCAGGTTGTCCTCAAGT; **Gpdh** forward: ATCA AGAAAGTGTGAGAGCCAAGA, reverse: TGGGAA AGTGAGTGTTGCTGTTGA; **Gys1** forward: CGGCT...
TTGGCTGCTTTATG, reverse: ACCTCCGATCCAGA

ATGTAAATG; HMGCR forward: TCTGGCAGTCAG
TGGGAACTATT, reverse: CCTCGTCCTTCGATCCA

ATTT; P4HA1 forward: CTGTTCTGCCGCTACCAT
GA, reverse: CCCACTCGTCTCCTGCTT; SCD1 forward: TTCCGCCACTCGCCTACA, reverse: CTTTC

CCAGTGCTGAGATCGA; SLC7A1 forward: GCAAC
TTGGACCAGTGCAAA, reverse: ACCCGTCAACCG
CTGTCA.

Measurement of total cholesterol levels in plasma

Plasma cholesterol was measured by Cholesterol E kit according to the manufacturer’s instructions (Wako, Richmond, VA, USA). Three animals in each group and two aliquots of plasma samples were used for measurement of cholesterol.

In vivo interferon induction

To assess for any non-specific immune response to injected iNOP-containing anti-miR, mouse liver tissue RNA was analyzed for expression of the IFN-inducible genes by quantitative RT-PCR. At least two liver regions were used to extract total RNA from individual animals in each group of three animals.

Statistical analysis

Results are given as means ± SD. Statistical analyses were preformed with Student’s t-test, and the null hypothesis was rejected at the 0.05 levels. We also performed one-way ANOVA analysis and the results showed that chemically modified group was significantly different from other two groups ($P < 0.5$).

RESULTS AND DISCUSSION

We first evaluated the silencing potency of iNOP-7 containing chemically modified anti-miR-122 in Huh-7 cells. We transfected these cells by iNOP-7-containing anti-miR-122 and analyzed the miR-122 by northern blotting (Figure 1A). iNOP-7 efficiently delivered anti-miR-122 into the cells and specifically silenced miR-122 in a dose-dependent manner. The endogenous miR-122 was slightly reduced by 10 pM of anti-miR-122 compared with nontransfected control and ~90% of miR-122 was silenced at 1 nM, which is at least 50 times lower than 2'-OMe and/or LNA modified anti-miR inhibitors transfected by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) (33). These results suggest that iNOP-7 can efficiently deliver our chemically modified anti-miR to cytoplasm and the released anti-miR results in lowering the miR-122 levels in cell (17). To determine the specificity of our anti-miR delivery by iNOP, we analyzed miR-22. Since miR-22 is highly expressed both in Huh-7 cell line and mouse liver tissue, therefore, miR-22 quantitation should serve as a control for the specificity of anti-miR-122. Our results showed that the expression level of miR-22 was unaffected by the transfection, indicating that the anti-miR silencing was miRNA specific (Figure 1A and B).

To evaluate the specific anti-miRNA effectiveness, we incorporated the perfectly complementary miR-122 binding sites into the 3'-UTR of a luciferase sensor plasmid as previously reported (34–36). When the reporter was transfected into Huh-7 cells, the endogenously expressed miR-122 strongly repressed the luciferase sensor expression by binding with perfect complementarity and causing cleavage of the mRNA. Introduction of anti-miR-122 by iNOP-7 prevented this miR-122-mediated repression, resulting in increased luciferase expression (Figure 2). The results are inversely consistent with the silencing effect of endogenous miR-122 by iNOP-7 containing anti-miR-122 detected by northern blotting in Figure 1.

We next determined the ability of iNOP-7 to deliver anti-miR-122 to its target and silence miR-122 in vivo. In our previous experiments, we observed efficient silencing of ApoB as reported by Soutschek et al. (30) when we injected mice with 80 mg kg$^{-1}$ chemically modified siRNA conjugated with cholesterol (data not shown). Since 80 mg kg$^{-1}$ doses of RNA are not clinically feasible, our goal in this study was to test the ability of iNOP-7 to inhibit miRNA functions at clinically acceptable doses. Therefore, we did not pursue in vivo experiments using RNA without the iNOP-7 assembly. Mice were injected via tail vein with iNOP-7 complexed to either chemically modified anti-miR-122 or its mismatch
on three consecutive days, and samples of liver and plasma were analyzed. miR-122 was significantly lower in liver tissue from mice treated with 2 mg kg$^{-1}$ iNOP-7 containing chemically modified anti-miR-122 (83.2 ± 6.4%, n = 3 animals) than in livers from control mice (Figure 3). The effects of anti-miR-122 were found to be specific because mice injected with iNOP-7 containing mismatched anti-miR-122 has no effect on miR-122. Furthermore, the expression of miR-22 was unaffected in mice treated with anti-miR-122 and its mismatch, suggesting that silencing was miRNA specific.

We also tested the duration of silencing that could be achieved after the injection of iNOP-7 containing chemically modified anti-miR-122. Mice were injected with 2 mg kg$^{-1}$ iNOP-7 containing chemically modified anti-miR-122 via tail vein, isolating liver tissues at various times after injection, and analyzing liver for miR-122 levels. At day 1, miR-122 level was significantly decreased (over 80% compared to PBS control mice), and the effect lasted at least for 9 days, indicating that silencing of miRNA by iNOP-7 containing chemically modified anti-miR-122 is long lasting effects (Figure S1).

MicroRNA can regulate the mRNA levels of their targets in 3'-UTR (37,38) and pharmacological silencing miRNAs using iNOP-7 containing chemically modified anti-miR-122 might therefore lead to the regulation of many mRNAs. To demonstrate the efficacy of the miRNA inhibition in vivo, the levels of a set of miR-122 target mRNAs identified previously (16,17) were evaluated in quantitative RT-PCR (Figure 4). Four of ten target mRNAs were increased in the anti-miR-122 treated mice, among which SLC7A1 mRNA was most sensitive to miR-122 inhibition (fold increase 5.7 ± 0.7) (Figure 4A). The other six mRNAs were down-regulated after the inhibition of miR-122 and SCD1 was decreased to 4.3 ± 1.7% of control after the inhibition (Figure 4B).

No target mRNA changes were observed in mice treated with mismatched anti-miR-122, demonstrating specific inhibition of miR-122 activity in liver.

To investigate the physiological effects of miR-122 silencing on cholesterol metabolism, we measured total plasma cholesterol levels in mice 24 h after the last injection. Chemically modified anti-miR-122 containing chemically modified anti-miR-122, mismatched chemically modified anti-miR-122, or PBS at 0, 12 and 36 h. Tissues were harvested at 24 h after the last injection to measure miRNA or mRNA levels. Total RNA was isolated from mouse liver 24 h after last injection. Samples were separated in 14% polyacrylamide gel and membrane was probed for miR-122, miR-22 and 5S rRNA. 5S rRNA is shown as a loading control. Quantification of reduced miR-122 levels in mice after treatment with iNOP-7 containing chemically modified anti-miR-122. Northern blots of miR-122 in mice treated with iNOP-7 containing chemically modified anti-miR-122, liver tissue did not alter the expression of these genes in the liver, suggesting iNOP-7 treatment did not induce an immune response in mice (Figure S2). Anti-miR-122 was well...
tolerated after injection of 2 mg kg⁻¹ of chemically modified anti-miR-122 complexed with iNOP-7 on three consecutive days; no alterations in overall health, body weight and food intake were detected. Together, these data indicate that iNOP-7 containing chemically modified anti-miR-122 participates in regulation of the cholesterol biosynthetic pathway and that silencing of a miRNA can be achieved without apparent toxicities.

Taken together, our results show that the newly designed nanoparticles can be used to deliver both duplex siRNA as we reported previously (23) and single-stranded anti-miR oligonucleotides to silence disease-related endogenous genes or miRNA in clinically acceptable and therapeutically affordable doses. When we injected iNOP-7 containing chemically modified anti-miR into animals, miRNA was specifically silenced and the expression of target genes were subsequently altered in liver, total plasma cholesterol was lowered. In addition, iNOP-7 treatment was non-toxic and did not induce an immune response. An important benefit of employing anti-miR oligonucleotides at low doses as presented here is that these strategies would avoid non-specific effects generated by the presence of excess anti-miR during purification and analysis procedures. Furthermore, iNOP-7 can be modified to target specific tissues and to modulate pharmacological properties to develop tissue-specific RNAi-based therapies. Although additional studies will be needed to optimize the dosing regimen and to assess the biodistribution and safety after long-term treatment, our findings highlight the utility of nanoparticles to deliver chemically modified anti-miR to liver tissues to silence endogenous miRNA in mice at clinical feasible doses.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Mike Czech, Greg Hannon, Craig Mello and members of the Rana lab for helpful discussions and critical reading of the manuscript.
