Review

MicroRNA Silencing in Primates: Towards Development of Novel Therapeutics

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Abstract

MicroRNAs (miRNA) comprise an abundant class of small noncoding RNAs that act as important posttranscriptional regulators of gene expression. Accumulating evidence showing that aberrantly expressed miRNAs play important roles in human cancers underscores them as potential targets for therapeutic intervention. Recent reports on efficient miRNA silencing in rodents and nonhuman primates using high-affinity targeting by chemically modified antisense oligonucleotides highlight the utility of such compounds in the development of miRNA-based cancer therapeutics. [Cancer Res 2009;69(2):393–5]

Background

MicroRNAs (miRNAs) are a class of small noncoding RNAs that bind to partially complementary sites in the 3′ untranslated regions (UTR) of target mRNAs and modulate gene expression by facilitating translational repression or mRNA degradation (1). *In silico* predictions estimate that up to one third of the human transcriptome may be regulated by miRNAs (2) and, indeed, mounting evidence suggests that miRNAs play important roles in the control of many biological processes (3). In addition, miRNAs have been implicated in viral infections, cardiovascular disease, as well as in the onset and progression of cancers (4–8). Thus, disease-associated miRNAs could represent a novel group of viable targets for therapeutic interventions.

MicroRNA-122 (miR-122) is a highly abundant, liver-specific miRNA implicated in liver homeostasis and cholesterol metabolism (9–12). The potential of this miRNA as a therapeutic target for treatment of hypercholesterolaemia is supported by the fact that silencing of miR-122 *in vivo* leads to lowering of plasma cholesterol (10–12). Moreover, the cholesterol lowering effect of miR-122 antagonists serves as a biomarker for measuring their pharmacologic effect. miR-122 is expressed in the hepatocellular carcinoma (HCC) cell line Huh7 but is absent from the hepatoblastoma cell line HepG2. This discrepancy led Jopling and colleauges (5) to explore whether the difference in miR-122 expression could explain why Huh7 cells are permissive for hepatitis C virus (HCV) replication, whereas HepG2 cells are not. The HCV genome contains two closely spaced sites in the 5′ noncoding region predicted to interact with the miR-122 seed (5, 13). In a series of experiments, Jopling and colleagues (5, 13) have shown that both miR-122 target sites are directly required for HCV replication and, indeed, transfection of Huh7 cells with a 2′-O-methyl antisense oligonucleotide complementary to miR-122 resulted in an 80% decrease in viral replicon RNA.

HCV is a leading cause of liver disease with nearly 200 million HCV infected individuals worldwide. Moreover, a high proportion of HCV infections become chronic and lead to HCC, which is one of the most common cancers in the world. Due to suboptimal efficacy and safety profile of current HCV therapy, there is a high unmet medical need for novel HCV-targeted therapeutics. The recent findings that miR-122 is required for HCV replication underscores its potential as a therapeutic target for treatment of HCV infection, which necessitates the development of effective and safe approaches for miR-122 targeting *in vivo*.

The binding of miRNAs to their target sites is governed by Watson-Crick base pairing. Thus, an obvious inhibitor of miRNA function is an oligonucleotide, which is complementary to the miRNA and basepairs with the miRNA in competition with cellular target mRNAs. Locked Nucleic Acids (LNA) are bicyclic high-affinity RNA analogues in which the furanose ring in the sugar-phosphate backbone is chemically locked in an RNA mimicking N-type (C3′-endo) conformation by the introduction of a 2′-O,4′-C methylene bridge (14). Several studies have shown that LNA-modified oligonucleotides exhibit unprecedented thermal stability when hybridized with their RNA target molecules (14). Consequently, an increase in melting temperature of +2°C to 10°C per monomer against complementary RNA compared with unmodified duplexes has been reported. LNA substitutions are known to generally improve mismatch discrimination compared with unmodified reference oligonucleotides (15). In addition, LNA oligonucleotides display high stability, low toxicity, and exhibit good aqueous solubility and potent antisense activity *in vivo* (14). Besides LNA, other chemically modified oligonucleotides have also proven efficient in functional inhibition of miRNAs. These include morpholinos (16), 2′-O-methyl- (12), and 2′-O-methoxyethyl–modified (2′-MOE) oligonucleotides (11).

MicroRNA Silencing in Nonhuman Primates

We have recently shown efficient miR-122 silencing *in vivo* using phosphorothiolated 15 and 16 nucleotide LNA/DNA mixmer oligonucleotides (LNA-antimiRs) complementary to the 5′ end of miR-122 (10, 17). Administration of unconjugated, saline-formulated LNA-antimiR to mice either i.p. or i.v. resulted in efficient uptake of the compound in the liver, which coincided with a dose-dependent depletion of mature miR-122 and the detection of a shifted band in Northern blots. This suggests that mature miR-122 is sequestered in a highly stable heteroduplex with LNA-antimiR, which seems to be different from the degradation mechanism described previously for miR-122 using either cholesterol-conjugated 2′-O-methyl oligonucleotides (antagomirs; ref. 12), or 2′-MOE–modified oligonucleotides (11). These studies reported effective miR-122 knockdown in mice using total i.v. doses of 120 to 240 mg/kg of antagomir-122 or twice weekly i.p. doses of 25 to 150 mg/kg for 2′-MOE–modified oligonucleotide, leading

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to a 30% to 40% decrease in total plasma cholesterol (11, 12). Using single i.p. injections of a high-affinity LNA-antimiR (melting temperature, 80°C) at doses ranging from 1 to 200 mg/kg, we observed a dose-dependent lowering of plasma cholesterol in normal mice with a ED_{50} of 10 mg/kg, whereas treatment of high-fat diet–induced obese mice with 5 mg/kg LNA-antimiR twice weekly for 6 weeks led to sustained lowering of plasma cholesterol by 30% (10). Microarray expression profiling studies have shown that among genes exhibiting increased expression in miR-122 antagonized animals, there is a significant overrepresentation of transcripts with miR-122 seed matches in the 3′ UTR (10, 12, 17). This suggests that the detected liver mRNA changes in the treated mice are primarily due to silencing of miR-122.

To investigate whether the high-affinity LNA-antimiR could mediate miR-122 antagonism in nonhuman primates, we carried out an efficacy study in African green monkeys. Systemic administration of PBS-formulated LNA-antimiR at doses ranging from 3 × 1 to 3 × 10 mg/kg with 3 i.v. infusions over 5 days resulted in dose-dependent and long-lasting decrease of total plasma cholesterol in African green monkeys (10). The high-dose group showed a maximum of 40% and the middle-dose group a maximum of 20% lowering of plasma cholesterol, respectively (10). In accordance with our findings in mice, we observed dose-dependent sequestration of mature miR-122 and concomitant appearance of a shifted LNA-antimiR/miR-122 heteroduplex in Northern blots, which coincided with accumulation of the LNA-antimiR compound in the cytoplasm of hepatocytes in treated animals (10). Silencing of miR-122 in monkeys was reversible where the levels of cholesterol gradually returned to normal over a 3-month period after LNA-antimiR treatment. Northern blot analysis and *in situ* detection performed on monkey liver biopsies taken 96 days after initiation of the study showed that the LNA-antimiR compound had been cleared from the liver. The LNA-antimiR oligonucleotides were well-tolerated in both mice and primates as we observed no acute or subchronic toxicities in the treated animals. Notably, we did not detect any hepatotoxicity or renal toxicity in the LNA-antimiR–treated primates as shown by unaltered levels of the plasma transaminases alanine aminotransferase and aspartate aminotransferase, bilirubin and creatinine phosphokinase, or creatinine in the high-dose LNA-antimiR group compared with vehicle-treated controls (10). Moreover, we observed no changes in blood coagulation profiles of LNA-antimiR–treated monkeys and no abnormalities in histopathologic investigations of the primate liver biopsies (10). Considered together, our data show that efficient antagonism of miR-122 can be achieved in rodents and nonhuman primates by simple delivery of unconjugated LNA-antimiR oligonucleotides.

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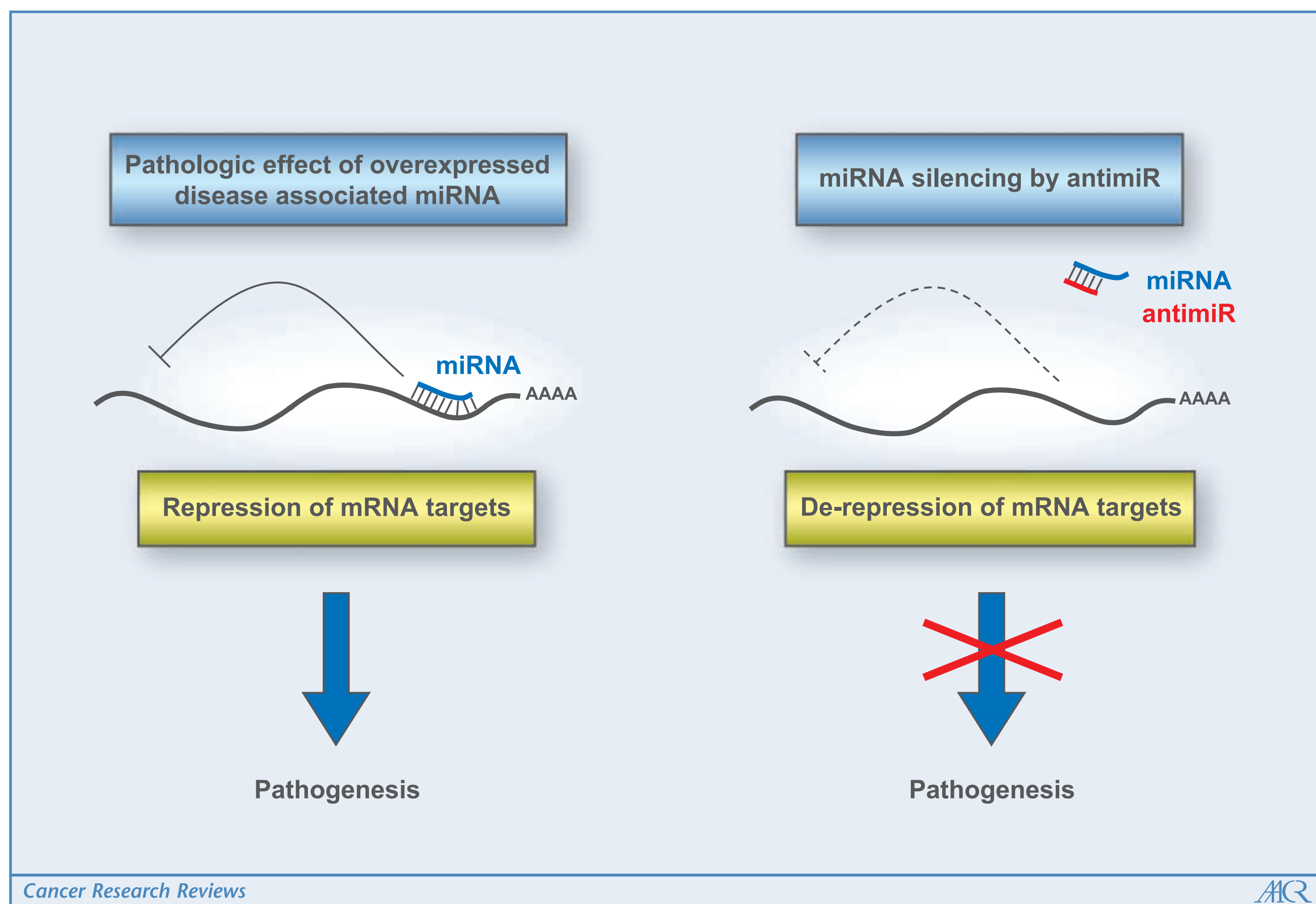

Figure 1. MiRNAs have been implicated in the onset and progression of human cancers where their misexpression leads to cancer pathogenesis. Antagonizing miRNAs using chemically modified complementary antimiR oligonucleotides holds great promise for *in vivo* targeting of disease-implicated miRNAs and for the development of novel therapeutic approaches targeting cancer-associated microRNAs.

Implications for MicroRNA-Based Cancer Therapeutics

MicroRNA expression is perturbed in many human cancers, and both tumor suppressor and oncogenic miRNAs have been identified (6). For example, miR-15a and miR-16-1, which are deleted or down-regulated in B-cell chronic lymphocytic leukemia, and let-7 family members that are down-regulated in lung cancer are likely to act as tumor suppressors, whereas the miR-34 family has been implicated in the p53 tumor suppressor network (6, 18).

The oncogenic activities of the miR-17-92 cluster, amplified in human B-cell lymphomas, and miR-155, which is overexpressed in Burkitt's and Hodgkin's lymphomas, have been confirmed in transgenic mice where their overexpression leads to accelerated tumor progression in a B-cell lymphoma model and spontaneous B-cell malignancy, respectively (4, 19). Furthermore, miR-221 and miR-222 are overexpressed in, e.g., glioblastomas and prostate cancer and seem to function as oncogenes by controlling cell cycle progression via posttranscriptional regulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ via two target sites for miR-221 and miR-222 in the 3′ UTR (20).

Several studies have shown that miR-21 is overexpressed in glioblastomas and in breast, lung, colon, prostate, and stomach cancers (21), suggesting that miR-21 acts as an oncogene. Recently, miR-21 was shown to repress many target genes associated with glioma cell apoptosis, migration, and invasiveness, including genes, which suppress malignancy and inhibit matrix metalloproteinases (MMP). These data imply that miR-21 plays a role in glioma malignancy by repressing MMP inhibitors, which, in turn, leads to activation of MMPs, thereby accelerating glioma cell invasiveness (21).

Efficient *in vivo* delivery of therapeutic oligonucleotides is a critical factor for the development of successful microRNA-based treatment modalities. Many peripheral tissues can be effectively targeted by systemically delivered antisense oligonucleotides containing phosphorothioate backbone modifications, which provide good pharmacokinetic properties and tissue uptake along with protection against nucleases (22). Consistent with these findings, unconjugated, phosphorothiolated 2′-MOE– and LNA-modified antisense oligonucleotides have been used to antagonize miR-122 function *in vivo*, whereas the use of cholesterol-conjugated 2′-O-methyl antisense oligonucleotides represents another approach to silence miRNA function (10–12). A number of alternative strategies for siRNA delivery are being tested, and these could also be applied to miRNA-based therapy. For example, ligands for specific cell surface receptors capable of being internalized can be conjugated to oligonucleotides, thereby facilitating both cellular uptake and cell type–specific delivery (23). The use of supramolecular nano-carriers, such as liposomes, and polymeric nanoparticles represents another strategy for delivering antisense oligonucleotides (for review, see ref. 23). Although success has thus far been limited, the potential benefit from using suitable carriers or conjugates *in vivo* justify that these strategies should be pursued in the future.

The importance of miRNAs in tumor development underscores them as potential targets for cancer therapeutics. On the other hand, recent reports on antagonizing miRNAs using chemically modified oligonucleotides hold great promise for *in vivo* targeting of disease-implicated miRNAs (Fig. 1). Among the nucleotide modifications, LNA possesses the highest affinity for complementary target RNA. Our findings that silencing of miR-122 in primates can result in stable and long-term functional inhibition suggest that miRNA antagonists composed of LNA may be valuable tools for studying the role of miRNAs in mouse models of cancer. In addition, the high metabolic stability of LNA-antimiRs, due in part to increased nuclease resistance, their small size, and lack of acute and subchronic toxicities in nonhuman primates imply that LNA-antimiRs may be well-suited for the development of novel therapeutic approaches targeting cancer-associated miRNAs.

Disclosure of Potential Conflicts of Interest

A. Petri, M. Lindow, and S. Kauppinen are employees of Santaris Pharma, a clinical stage biopharmaceutical company that develops RNA-based therapeutics.

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