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Original Article

Efficacy of microRNA silencing by lipid-conjugated double-stranded antisense oligonucleotides

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Abstract

MicroRNAs (miRNAs) are important therapeutic targets for intractable diseases and antisense oligonucleotides that silence microRNA (antagomirs) have been developed for clinical applications. Although conjugation of ligands to antagomirs is a promising means of delivering them to target tissues and cells, the efficacy of these constructs is yet to be optimized. In this study, we designed a novel antagomir construct that comprise an antagomir strand and its complementary RNA strand. We were then able to indirectly conjugate ligands to this double-stranded antagomir via the complementary RNA strand. We then used single- or double-stranded antagomirs to examine effects of ligand type, conjugation site, or chemical modifications of the antagomir strand on miRNA silencing *in vitro*. We found that indirect conjugation of cholesterol ligand to a double-stranded antagomir produced a construct with comparable miRNA-silencing efficacy as that of a single-stranded antagomir directly conjugated with the ligand. Our findings support application of this technology for the therapeutic regulation of miRNA.

Key words: microRNA, delivery ligands, antagomir

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Introduction

MicroRNAs (miRNAs) are 21–23 nucleotides non-coding RNAs which regulate mRNA post-transcriptionally[1]. miRNAs play important roles in various aspects of life phenomenon and response to pathogens[2] and various human diseases are caused by altered expression and profiling of miRNAs[3, 4]. Therefore, regulation of each miRNA expression is expected as a novel therapeutic strategy for intractable diseases[4, 5].

Two approaches have been used to modulate miRNA from a therapeutic perspective[4]. One is the restoration of miRNA activity by synthetic RNA duplexes, the so-called 'miRNA mimics'. The other approach is the inhibition of miRNA by antisense oligonucleotides (ASO) known as antagomir[6]. Antagomir is a chemically modified ASO with improved biostability. The chemistries used most often are phosphorothioate bond (PS)[7] in internucleotide linkage and modification at ribose moiety, such as 2′-*O*-methylated ribose moieties (OM)[8] or locked nucleic acid (LNA)[9-12]. Although development of antagomir has been progressed, efficacy of antagomir without carriers which deliver antagomir to target tissues remains limited[13].

One promising approach to address this limited efficacy of antagomirs is a conjugation of delivery-ligand for target organs or cells. The first ligand reported to improve the potency of antagomirs *in vivo* was cholesterol which was conjugated to fully OM-modified antagomir[8]. Recent studies suggested that siRNAs conjugated with cholesterol are incorporated into lipoproteins and are delivered to liver, gut, kidney and steroidogenic organs[14]. In addition, N-acetylgalactosamine (GalNAc) is a liver-targeted ASO ligand that binds to asialoglycoprotein receptor with high affinity[15-17]. Regulus Therapeutics Inc., a biopharmaceutical company, has developed a

GalNAc-conjugated antagomir targeting miR-122, RG-101[18]. Although several ligands for antagomirs have been developed, improvement of their *in vivo* potency and dose reduction are highly desirable to counter the high cost for synthesizing chemically modified oligonucleotides and to avoid adverse effects

Recently, we reported a new class of mRNA targeting ASO, DNA–RNA heteroduplex oligonucleotide (HDO), which comprise an ASO-strand and its complementary RNA (cRNA) strand[19]. When HDO was conjugated with a lipid-ligand such as alpha-tocopherol (Toc), it improved the silencing effect by 10- to 20-fold in liver. However, application of this HDO technology to the construction of antagomirs is difficult because antagomirs are different from ASOs targeting mRNA with respect to their chemical modifications and mechanisms of silencing target RNAs. The chemical modifications of ASO targeting mRNA are mainly of gapmer-type including at least four consecutive DNAs and its silencing mechanism is mediated by RNase H. RNase H recognizes DNA–RNA heteroduplex which composes ASO and the target mRNA and then cleaves the mRNA, resulting in degradation of the mRNA. In our recent study, we found that the cRNA strand bound to gapmer type of ASO was totally cleaved by Rnase H, indicating that RNase H was able to cleave not only target mRNAs but also the cRNA of HDO[19]. In contrast, chemical modifications of antagomir, such as fully OM-modified or mixmer type, does not induce RNase H mediated degradation[20-22]. Therefore RNase H would not cleave the cRNA strand of HDO using those antagomirs, indicating that silencing effect of the antagomir may be blocked by the cRNA.

In the present study, we designed a variety of double-stranded antagomirs which include various types of antagomirs or ligands. We then investigated efficacy of those antagomirs when we transfected those into cells to elucidate which types of double-stranded antagomir is promising for application to *in vivo* evaluation.

Materials and Methods

Design and synthesis of antisense oligonucleotides

Antagomirs targeting miR-122 were based on previous reports[8,10] and were synthesized by Gene Design (Osaka, Japan) with the following sequences; OM-modified antagomir, 5′-A*C*AAAACACCAUUGUCACACU*C*C*A-3′; DNA/LNA mixmer type of antagomir, 5′-c*c*a*t*t*g*t*c*a*c*a*c*t*c*c-3′, where uppercase underlined letters represent OM, lower case represents DNA, lower case underlined represents LNA and * indicates phosphorothioate internucleotide linkage. In addition, two cRNAs were synthesized by Hokkaido System Science (Sapporo, Japan) with following sequences; cRNA for OM-modified antagomir, 5′- U*G*G*AGUGUGACAAUGGUGUU*U*G*U-3′; cRNA for DNA/LNA mixmer type of antagomir, 5′-G*G*A*GUGUGACAA*U*G*G-3′, where uppercase represents RNA. To generate double-stranded antagomir, equimolar amounts of antagomir and cRNA strands were heated in PBS (Sigma-Aldrich) at 95°C for 5 minutes and cooled to room temperature over 1 hour.

Cell transfection

Huh-7 cells (human hepatocellular carcinoma cell line) in 24-well plates were transfected with increasing concentrations of antagomirs in Opti-MEM containing 10 μL/mL Lipofectamine RNAiMAX reagent (Life Technology). After 6hours, the transfection medium was replaced with complete medium consisting of DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and the cells were incubated for a further 18 hours and then used in the subsequent experiments. All protocols met ethics and safety guidelines for experimentation and were approved by Tokyo Medical and Dental University (2016-030C2).

Quantitative RT-PCR.

Total RNA was isolated from the cells by the MagNA Pure 96 system (MagNA Pure 96 Cellular RNA Large Volume Kit, Roche Diagnostics). cDNAs were synthesized by TaqMan miRNA assays (Applied Biosystems) for miRNA experiments. qRT-PCR analysis normalized to small RNA U6 for miRNA experiments was performed with a TaqMan primer (Applied Biosystems) in a LightCycler 480 System with a LightCycler 480 Probes Master kit (Roche).

Statistical analysis

Comparisons between single- and double stranded antagomir groups were performed using paired t test. $P < 0.05$ was considered to indicate statistical significance; Prism version 6.05 (GraphPad Software) was used to perform all statistical analyses.

Results

Design of a double-stranded, fully OM-modified, ligand-conjugated antagomir

In this study, we selected miR-122 as the target miR because antagomirs which silenced miR-122 is the most advanced antagomir in development of antagomir

therapy[18,23]. Since miR-122 is specific and most abundant in hepatocyte[24], we transfected antagomirs into Huh-7 which is a human hepatocyte cell line. To elucidate whether the double-stranded antagomir technology could be used to silence miRNA, we designed a double-stranded antagomir comprising an antagomir-strand and its cRNA-strand conjugated with a lipid ligand (Fig. 1). The antagomir-strand is a 23-mer fully OM-modified antisense oligonucleotide with a complementary sequence to that of mature miR-122 and carries five PS-backbones. Previous studies reported that the same type of antagomir which was conjugated with cholesterol ligand (Chol) at the 3′ end of the antagomir can efficiently silence target miR-122[8,25]. Both 3′- and 5′- wing portions of the cRNA-strand include three PS modifications. We conjugated two types of lipid ligands, Chol or alpha-tocopherol (Toc), as delivery ligand targeting liver with single-stranded angatomir or cRNA-strand of double-stranded antagomir. The Chol ligand could be attached to the end of either 3′- or 5′-end of oligonucleotides, whereas Toc could only be attached to the end of 5′-end due to methodological issues in the synthesis.

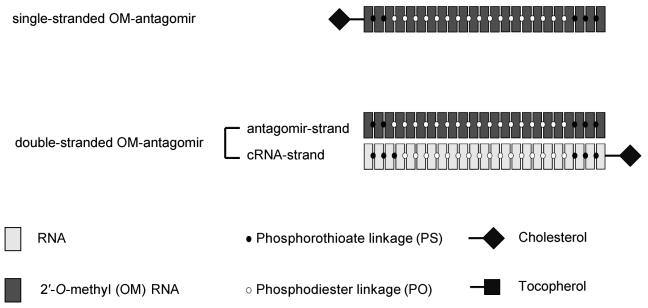

Figure 1. Design of the single- or double-stranded fully OM-modified antagomir

Schematic illustrations of the parent single-stranded antagomir which is fully 2′-O-methyl (OM) RNA and the double-stranded antagomir. A lipid ligand is conjugated directly with the single-stranded antagomir or conjugated indirectly with the double-stranded antagomir.

miRNA-silencing by Chol-conjugated single- or double-stranded OM-modified antagomir

We first attached the Chol to the 3′ end of the parent single-stranded OM-modified antagomir and the cRNA strand of double-stranded antagomir targeting miR-122 and evaluated its silencing effect in Huh-7 cells. Because fully OM-modified antagomir are not e recognized by RNase H[20-22], we assumed that the double-stranded antagomir would not be unwound and the antagomir strand would not be released. Therefore we were concerned that the double-stranded antagomir would show no miRNA silencing effect due to blocking by the cRNA strand. However, treatment with the double-stranded antagomir silenced the target miRNA in a dose-dependent manner as measured by miR-122-specific RT-PCR and no significant differences were found between miRNA silencing effect of the two antagomirs (Fig. 2).

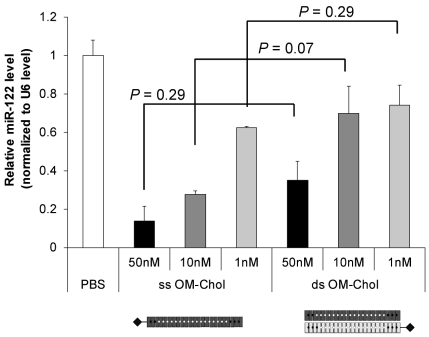

Figure 2. miRNA silencing by a single or double-stranded fully OM-modified antagomir with Chol

Quantitative RT-PCR (qRT-PCR) analysis of relative miR-122 expression in Huh-7 cells treated with phosphate buffered saline (PBS), the single-stranded (ss) antagomir or double-stranded (ds) antagomir conjugated with cholesterol (Chol). Data are presented as means ± standard errors of the mean (SEM.); n = 3 well per group.

Effect of Chol -ligand position (3′- or 5′) on miRNA silencing by a double-st randed antagomir

To examine whether the position of the ligand affected the efficacy of miRNA inhibition by double-stranded antagomir, we compared the potency of double-stranded antagomirs where Chol conjugation was made in either the 3′- or 5′-end in the cRNA strands. The double-stranded antagomir with Chol at the 3′-end showed greater miR-122 silencing significantly with 1 nM transfection than that by the double-stranded antagomir at the 5′-end did (Fig. 3).

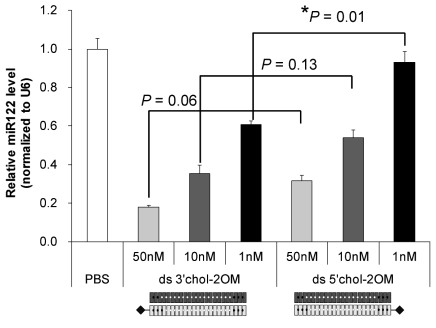

Figure 3. Effect of Chol-ligand position (3′- or 5′ end) on miRNA silencing by the double-stranded OM-modified antagomirs

qRT-PCR analysis of miR-122 level in Huh-7 cells treated with the double-stranded antagomirs which were conjugated with Chol at the 3′- or 5′-end of the cRNA strand. Data are presented as means ±SEM; n = 3 well per group; *$P < 0.05$.

Effect of Toc-conjugation to OM-modified antagomirs on miRNA-silencing

To investigate the effect of a different type of lipid ligand on the miRNA silencing with the antagomirs, we attached Toc to either 5′-end of the parent single-stranded OM-modified antagomir or the cRNA strand of double-stranded antagomir. The miR-122 silencing in Huh-7 cells treated with the double-stranded antagomir with Toc was comparable with the single-stranded antagomir with Toc (Fig. 4).

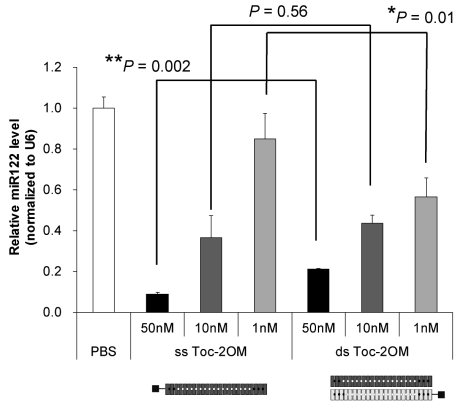

Figure 4. Efficacy of miRNA silencing by the single or double-stranded fully OM-modified antagomir with Toc

qRT-PCR analysis of relative miR-122 expression in Huh-7 cells treated with PBS, the ss-antagomir or ds-antagomir conjugated with tocopherol (Toc). Data are presented as means ± SEM; n = 3 well per group; *$P < 0.05$, **$P < 0.01$.

Effect of Chol- or Toc- conjugation to single- or double-stranded DNA/LNA-modified antagomirs on miRNA-silencing

To evaluate potency of a double-stranded antagomir with an antagomir strand that was not fully OM-modified, we designed double-stranded antagomir conjugated with lipid ligands, which comprise DNA/LNA mixmer type of antagomir which includes no more than three consecutive DNAs targeting miR-122 (Fig. 5). This DNA/LNA mixmer type of antagomir has advanced to phase II study for the treatment of chronic HCV infection[26]. The double-stranded DNA/LNA mixmer antagomir conjugated with Chol was less potent significantly by 10 nM transfection than the parent single-stranded DNA/LNA mixmer with Chol (Fig. 6). However, there were no significant differences between the single- or double-stranded Toc-conjugated DNA/LNA antagomirs.

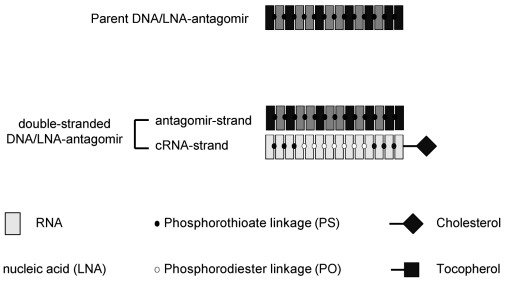

Figure 5. Design of the single- or double-stranded DNA/LNA mixmer antagomir

Schematic illustrations of the DNA/LNA mixmer type single- and double- stranded antagomir. Lipid ligand were conjugated directly to the single-stranded antagomir or conjugated indirectly with the double-stranded antagomir.

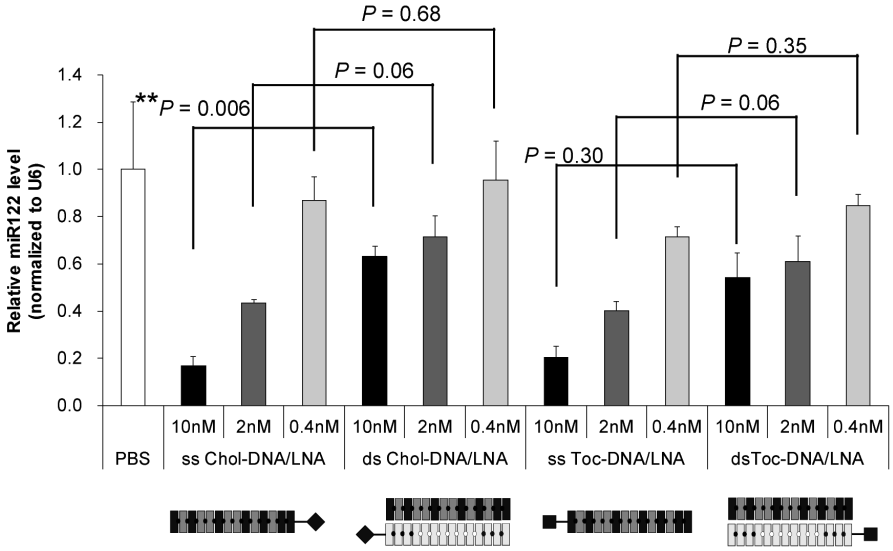

Figure 6. Potency of single- or double-stranded DNA/LNA mixmer antagomirs with Chol or Toc

qRT-PCR analysis of miR-122 level in Huh-7 cells treated with PBS, or Toc or Chol-conjugated ss or ds DNA/LNA mixmer antagomiR. Data are presented as means ±SEM; n = 3 well per group; *$P < 0.05$, **$P < 0.01$.

Discussion

Here we describe a new type of antagomir, a double-stranded antagomir, in which molecular design are composed of the antagomirs and complementary RNAs strand conjugated with lipid ligands. Although such a hybridization by cRNA to antagomir is usually expected to block the miRNA silencing, this double-stranded antagomir, to our surprise, showed inhibition of miRNA. In addition, the double-stranded antagomir with which cholesterol was conjugated at 3′-end of the cRNA inhibited miRNA more efficiently than that at 5′-end (Fig. 3), indicating that the conjugation site of Chol affected efficacy.

In previous studies, antagomirs, including fully OM-modified and DNA/LNA mixmer types, did not induce RNA degradation via RNase H[20-22], suggesting that double-stranded antagomirs are not recognized by RNase H and the antagomir strand cannot be unwound and released. Therefore, we first thought that we would use a double-stranded antagomir as a negative control. However, the double-stranded antagomir did silence miRNA although its silencing effect was no better than the parent single-stranded antagomir. This finding indicated that there may be other as-yet-unidentified RNases that are responsible for cleavage of the cRNA strand or there is unwinding mechanism of double-stranded antagomir which is independent from cleavage.

The position of cholesterol conjugation (3′- vs 5′-end) was found to affect potency of double-stranded antagomir. Previous study reported that 3′-conjugation of cholesterol to ASO targeting mRNA increased potency of ASO in mouse liver and hepatic accumulation in comparison with 5′-conjugation[27]. In the present study, 3′-conjugation of cholesterol to the cRNA strand of double-stranded antagomir may enhance uptake to hepatocyte, resulting in higher potency than 5′-conjugation.

A limitation of the present study concerns the transfection of antagomir by cationic liposome reagent. Recent studies show that types and affinities of carrier molecules in blood determinates the cellular uptake and intracellular trafficking of ASOs[28-30], indicating that cellular uptake of antagomir via cationic liposome in our *in vitro* system may be different from that *in vivo*. Moreover, the conjugation of Toc to double-stranded ASO targeting mRNA increase delivery efficiency to the liver in our previous report[19]. This improved delivery might be caused by increased retention in blood because of two speculated mechanisms as follow. One is that the double-stranded structure could be more resistant to nuclease in blood than the single-stranded structure. The other is that double-stranded structure which has a greater molecular size than the single-stranded structure could escape from renal excretion. These led us to expect combining double-stranded antagomir with a delivery ligand for target tissue or cells to enable higher efficiency *in vivo*. Thus, further investigations of double-stranded antagomir *in vivo* are required.

In conclusion, we developed a novel oligonucleotide construct, double-stranded antagomir, and demonstrated that the type of lipid ligands and its conjugation-site are the important determinants of potency.

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